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Agonist-induced Desensitization of Muscarinic Acetylcholine Receptor in Rat Brain

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Abstract □ Intact brain cell aggregates were dissociated from adult rat brains without cerebellum using a sieving technique. This preparation was used to elucidate the binding characteristics of agonist to muscarinic acetylcholine receptors (mAchR) in brain. Incubation of cells with carbamylcholine (carbachol) was shown agonist-induced receptor down-regulation depending on the concentration of agonist, not depending on the incubation time. This effect of carbachol was due to a reduction in the maximal binding capacity (B_{max}) to the mAchR without decreasing the affinity of the remaining receptors in incubation at 37 °C but was not apparent in incubation at 15 °C. In addition, it was abolished when the receptors were blocked by atropine. The decline in (^{3}H)N-methylscopolamine((^{3}H)NMS) binding induced by agonist was reflected as a significant reduction in the receptor density with no change in receptor affinity, suggesting that 'true' receptor down-regulation takes place. Moreover, when the receptors were labeled with the lipophilic antagonist (^{3}H)quinuclidinyl benzilate ((^{3}H)QNB) insted of the hydrophilic ligand (^{3}H)NMS, the magnitude of the observed receptor down-regulation was significantly lower in case of the former than the latter. **This suggests that exposure of intact brain cells to muscarinic agonists might induce a slight** degree of accumulation of receptors in intracellular sites before the receptors are actually degraded.

Keywords □ Intact brain cell aggregates, sieving technique, muscarinic acetylcholine receptors (mAchR), carbamylcholine (carbachol) down-regulation, maximal binding capacity (B_{max}), (^{3}H)N-methylscopolamine((^{3}H)NMS, (^{3}H)Quinuclidinyl benzilate((^{3}H)QNB).

The density of muscarinic acetylcholine receptors in the central nervous system is regulated by the concentration of muscarinic agonists in the vicinity of the receptor.

The level of muscarinic acetylcholine receptors in the brain is regulated by chronic receptor activation. For example, long-term treatment with muscarinic agonists[1,2] or anticholinesterase agents[3,4] results in a decrease in muscarinic receptor concentration of several brain areas.

Interaction of muscarinic acetylcholine receptor agonists with their specific receptors triggers a specific sequence of electrophysiological and biochemical events that leads, ultimately, to certain changes in cellular function[5]. The concentration of muscarinic acetylcholine receptors on the cell surface and the receptor sensitivity are both a dynamic function of the concentration of the agonist in contact with the receptors.

Studies of the possible mechanisms underlying muscarinic receptor regulation in the central nervous system require the use of intact cells, and clonal cell lines of nervous origin have been used extensively for this purpose[6-10]. Recently we have established the usefulness of this preparation in investigating the binding characteristics of muscarinic receptors[11].

In the present work, we have studied the details of this agonist-induced regulation of muscarinic receptors in this system.

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EXPERIMENTAL METHODS

Preparation of intact brain cell aggregates

Adult male or female Sprague-Dawley rats were killed by decapitation and brains were immediately dissected on ice to remove the cerebellum. Tissue was dissociated at 4 °C using a modification of the sieving technique of Kanba and Richelson (1983)[12]. Brains were minced into a paste using a razor blade, then placed in a nylon mesh bag (210 μm pore diameter, Nitex 210, Tetko, Elmsford, NY), sub-

merged in a modified Puck's D_1 solution (medium I) of the following composition (mM): NaCl 138, KCl 5.4, Na_2HPO_4 0.17, KH_2PO_4 0.22, glucose 5.5 and sucrose 58.4 (pH 7.35, 340 mOsm). Tissue was dissociated by gently stroking the bag from the outside with a glass rod. The resulting suspension was filtered by gravity flow through a tighter nylon mesh bag (130 μm pore diameter, Nitex 130) and the resulting tissue was washed twice by centrifugation (400Xg for 3 min at 4 °C) in a physiological buffer solution (medium II) of the following composition (mM): NaCl 110, KCl 5.3, $CaCl_2$ 1.8, $MgCl_2$ 1, glucose 25, sucrose 70 and HEPES ((4-2-hydroxyethyl)-1-piperazineethanesulfonic acid) 20 (pH 7.4, 340 mOsm). Viability tests performed by the trypan blue exclusion method usually yielded viability values of 80-90%.

Muscarinic acetylcholine receptor down-regulation study

For muscarinic receptor down-regulation experiments, cells were suspended in Dulbecco's modified Eagle's medium (Grand Island Biological Co., Grand Island, NY), supplemented with 10% bovine calf serum and buffered at pH 7.35 with 20 mM HEPES (medium III). Cells were preincubated in defferent time periods (from 1/2 hr to 12 hrs) at 37 °C or 15 °C (unless otherwise stated) with or without carbamylcholine (Sigma Chemical Co., St. Louis, MO) in a final volume of 2 m*l* with occasional shaking. At the end of the incubation period, the cell suspension was centrifuged at 400Xg for 3 min at 4 °C, followed by washing twice with 20 m*l* of ice-cold medium II. Washed cells were suspended in cold medium II and used for the receptor binding experiments.

Muscarinic acetylcholine receptor binding assay

To elucidate the effect of preincubation with carbamylcholine on the binding properties of muscarinic receptors, cells were incubated with or without carbamylcholine for 3 hrs at 37 °C, and then washed with medium II as described above. These preincubated cells were incubated in quadruplicate with 0.2 nM (^{3}H)N-methylscopolamine ((^{3}H)NMS), (84.8 Ci/mmol, New England Nuclear, Boston, MA) in a final volume of 1 m*l* of medium II. Incubations were carried out at 15 °C (to prevent recovery of down-regulation, if any) for 90 min, where equilibrium was attained (Lee and El-Fakahany, 1985a)[11] And these cells were also incubated in triplicate with increasing concentrations (0.01-1.0 nM) of (^{3}H)NMS or (^{3}H)quinuclidinyl benzilate ((^{3}H)QNB) (56 Ci/mmol, Amersham Corp., Arlington Heights, IL) in absence and in presence of 2 μM atropine for 90 min at 15 °C. The incubation mixture was filtered under vacuum through Whatman glass fiber GF/B filters using a Cell Harvester (Brandel, Gaithersburg, MD). The filters were washed four times with 5 m*l* of ice-cold 0.9% NaCl solution. Individual filters were placed in scintillation vials to which was added 4 m*l* of a toluene-based scintillation fluid, and radioactivity was measured at least 6 hrs later in a Beckman LS-6800 liquid scintillation counter at about 50% counting efficiency. Non-specific binding was obtained in the presence of 2 μM atropine, and was subtracted from total binding in the absence of atropine. Specific binding was analyzed using Scatchard plots (Scatchard, 1949)[13]. Proteins were determined by a modification of the method of Lowry *et al.* (1949)[14], and statistical analysis was performed using Student's t-test.

RESULTS

Preincubation of dissociated intact rat brain cells with the muscarinic acetylcholine receptor agonist carbamylcholine (1 mM, 37 °C), followed by washing the agonist, resulted in a considerable decrease in the specific binding of (^{3}H)NMS measured at 15 °C. This decrease in muscarinic receptor binding was dependent on the duration of exposure to the agonist. Thus, cells pre-incubated with carbamylcholine showed binding values of

Table I. Time Course of carbamylcholine-induced decrement (1 mM, 37 °C) in specific (^{3}H)NMS binding

Incubation Time (hr)	n	(^{3}H)NMS binding (% control)	(Δ%)
1/2	8	91.6 ± 3.6	−8.4
1	8	82.0 ± 4.5**	−18.0
2	11	80.7 ± 3.8***	−19.3
3	13	70.9 ± 5.8***	−29.1
4	10	71.6 ± 4.8***	−28.4
6	10	71.5 ± 4.1***	−28.5
8	8	79.6 ± 3.5***	−20.4
10	8	81.2 ± 2.6***	−18.8
12	9	82.3 ± 3.9**	−17.7

Mean ± S.E.M., n: number of independent experiments, Each control value determined in quadruplicate was between 437.0 ± 22.2 and 421.3 ± 22.8 fmol/mg protein.
** $P<0.01$, *** $P<0.001$

91.6, 82, 80.7, 70.9, 71.6, 71.5, 79.6, 81.2 and 82.3% of control after 0.5, 1, 2, 3, 4, 6, 8, 10 and 12 hours of incubation, respectively (Table I).

The ability of carbamylcholine to cause down-regulation of specific (^{3}H)NMS binding sites upon preincubating rat brain cell aggregates with agonist for 3 hrs at 37 °C was also dependent on the concentration of carbamylcholine in the incubation medium, and reached a ceiling 94.1, 93, 86.2, 83.1, 82.6, 80.0, 76.7, 73.6, 64.2, 61.8 and 61.4% of control binding value after incubation of the cells with 1μM, 2μM, 4μM, 10μM, 20μM, 40μM, 0.1 mM, 0.2 mM, 0.4 mM, 1 mM and 2 mM for 3 hrs at 37 °C, respectively (Table II).

In addition, when brain cells were challenged with 1 mM carbamylcholine for 3 hrs at 37 °C then washed and incubated with increasing concentrations of (^{3}H)NMS at 15 °C, it was found that pre-exposure to the agonist significantly reduced the maximum binding capacity of the receptors without a significant change in the equilibrium dissociation constant of the lignad-receptor complex (Table III).

No further decline in specific (^{3}H)NMS binding took place by increasing the time of incubation with the agonist from 3 hrs to 6 hrs, however, upon more prolonged incubation with carbamylcholine to 12 hrs, muscarinic acetylcholine receptor binding showed a tendency to maintain plateau (Table I).

The effect of preincubating rat brain cell aggregates with 1 mM carbamylcholine for 3 hrs on subsequent receptor binding was dependent on the incuation temperature during the desensitization step. Although exposure of the tissue to carbamylcholine at 37 °C resulted in (^{3}H)NMS specific binding of 70.9 ± 5.8% of control (mean ± S.E.M.), no significant reduction in muscarinic receptor binding was observed when parallel incubations with the agonist were carried out at 15 °C (101.6 ± 8.3% of control binding) (Fig. 1).

The effect of preincubating rat brain cell aggregates with agonist on the binding properties of muscarinic acetylcholine receptors was investigated. Cells were preincubated with or without 1 mM carbamylcholine for 3 hrs at 37 °C, then the agonist

Table II. Concentration dependence of carbamylcholine-induced (3 hrs, 37 °C) decrement in specific (^{3}H)NMS binding

Conc. of Carbamylcholine (μM)	n	(^{3}H)NMS binding (% control)	(Δ%)
1	5	94.1 ± 3.5	−5.9
2	5	93.0 ± 4.2	−7.0
4	6	86.2 ± 5.1*	−13.8
10	7	83.1 ± 4.9*	−16.9
20	5	82.6 ± 4.2*	−17.4
40	5	80.0 ± 3.8**	−20.0
100	6	76.7 ± 4.7**	−23.3
200	5	73.6 ± 5.6**	−26.4
400	5	64.2 ± 4.9**	−35.8
1000	7	61.8 ± 4.1***	−38.2
2000	5	61.4 ± 5.2**	−38.6

Mean ± S.E.M., n: number of experiments., Each determed in quadruplicated at 0.2 nM (^{3}H)NMS control value averged 435.9 ± 19.6 fmol/mg protein.
* $P<0.05$, ** $P<0.01$, *** $P<0.001$

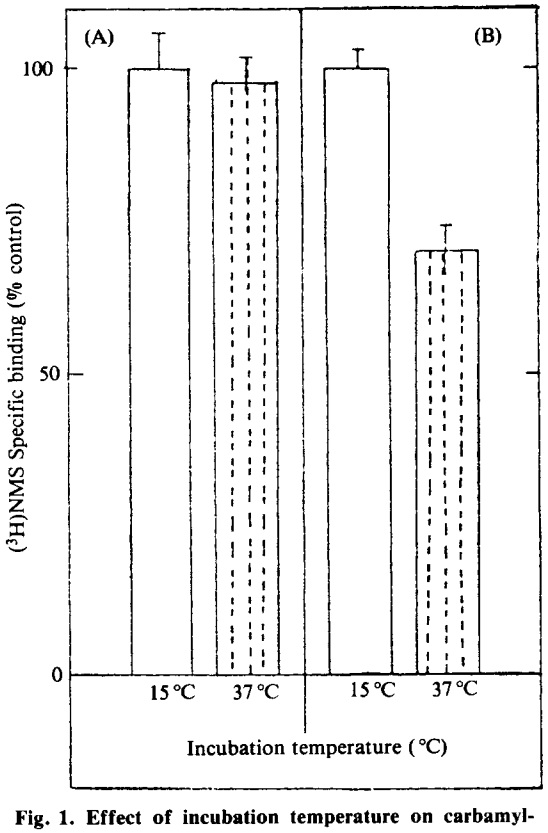

Fig. 1. Effect of incubation temperature on carbamylcholine-induced reduction in specific (^{3}H)NMS binding in intact rat brain cells.
Cells were preincubated in parallel with (B) or without (A) 1 mM carbamylcholine for 3 hr at either 15 or 37 °C, then washed and incubated with 0.2 nM (^{3}H)NMS. The data are presented as the mean ± S.E.M. of 6 to 13 independent experiments each determined in quadruplicate, and are expressed as a percentage of the corresponding control groups preincubated without the agonist.

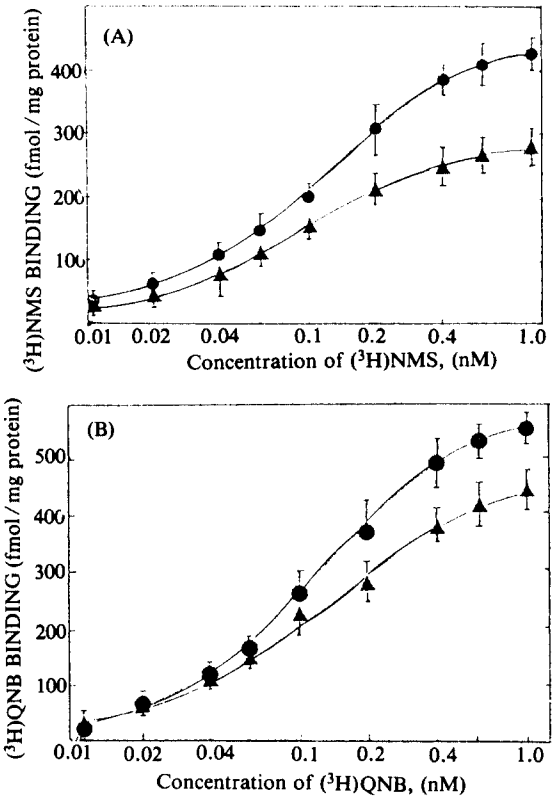

Fig. 2. Saturation isotherms of (^{3}H)NMS (A) and (^{3}H)QNB (B) binding in control and desensitized rat brain cell aggregates.

Cells were preincubated with (▲) and without (●) 1 mM carbamylcholine for 3 hrs at 37 °C, then prepared for the binding assay. Cells were incubated in triplicate with increasing concentration of (^{3}H)NMS or (^{3}H)QNB and specific bindings were determined. The data are presented as the mean of S.E.M. of 8 to 13 independent experiments for control and desensitized cells, respectively.

was washed extensively. When cells were then incubated with increasing concentrations of (^{3}H) NMS for 90 min at 15 °C in absence and in presence of 2 μM atropine, specific (^{3}H)NMS binding was significantly decreased in desensitized cells at all ligand concentrations (Table III & Fig. 2). Scatchard analyses of the saturation isotherms pooled from several independent experiments indicated a significant reduction in the maximal muscarinic receptor density (B_{max}) upon desensitization. When individual B_{max} values were averaged from the independent experiments, this resulted in significantly different ($p < 0.05$) receptor densities of 436 ± 30 (mean ± S.E.M.) and 278 ± 26 fmol/mg protein in control and desensitized cells, respectively (Table III). However, the affinity of the receptors for (^{3}H) NMS did not change significantly since the equilibrium dissociation constant (K_d) of (^{3}H)NMS was 0.156 ± 0.02 and 0.136 ± 0.01 nM in cells preincubated without or with 1 mM carbamylcholine for 3 hrs at 37 °C, respectively (Table III). On the other hand, when the same experiments were repeated using the lipophilic muscarinic receptor antagonist (^{3}H)QNB as a ligand, there was much less reduction in receptor binding upon desensitization, as compared to the corresponding control. In this case, control and desensitized cells showed B_{max} values of 552 ± 45 and 432 ± 46 fmol/mg protein, respectively, which were not significantly different (Table III & Fig. 2), suggesting that muscarinic receptor desensitization in these cells is accompanied by a greater reduction in (^{3}H)NMS binding than in (^{3}H)QNB binding, relative to their own controls. Again, there was no significant change in the affinity of the receptor for (^{3}H)QNB upon desensitization, since the respective K_d values for control and desensitized cells were 0.349 ± 0.04 and 0.307 ± 0.06 nM (Table III).

Table III. Effect of carbamylcholine (1 mM, 3 hr, 37 °C) on the maximum binding capacity (B_{max}) and equilibrium dissociation constant (K_d) of (^{3}H)NMS and (^{3}H)QNB

Treatment	Ligand	n	B_{max} (fmol/mg protein)	K_d (nM)
Control	(^{3}H)NMS	6	436 ± 30	0.156 ± 0.02
	(^{3}H)QNB	5	552 ± 45	0.349 ± 0.04
Desensitized	(^{3}H)NMS	13	278 ± 26*	0.136 ± 0.01
	(^{3}H)QNB	8	432 ± 46	0.307 ± 0.06

Values are reported as the mean ± S.E.M., n: represents the number of independent experiments. * $P < 0.05$

DISCUSSION

Cell and tissue culture techniques have been applied to elucidate the mechanisms underlying agonist-induced down-regulation of muscarinic cholinergic receptors and desensitization of their function.

Several *in vitro* models have also been used to study desensitization of muscarinic agonist-induced effects. These include receptor-mediated contraction of smooth muscle[5,6], the decrease in cardiac muscle contractility[7], the increase in cyclic GMP formation[8,9], and the decrease in cyclic AMP syn-

thesis[20,21] caused by activation of muscarinic receptors.

Recent studies from our laboratory have indicated that binding of (^{3}H)NMS to muscarinic acetylcholine receptors in dissociated rat brain cells is linear with tissue concentration, saturable, and homogeneous, and also other intact cell systems have been used in several laboratories to study muscarinic receptor regulation.

Our present results indicate that muscarinic acetylcholine receptors in intact rat brain cells are regulated by prolonged incubation with the muscarinic receptor, agonist carbamylcholine. This agonist-induced receptor down-regulation is temperature-and concentration-dependent. Similar findings have been reported in other *in vitro* models of muscarinic receptor regulation, for example, Maloteaux *et al.*[22] have found fast disappearance of surface muscarinic receptors labeled by (^{3}H)NMS upon exposure of intact neuroblastoma-glioma hybrid cells to agonists. Short-term incubation of mouse neuroblastoma cells (clone NIE-115) with carbamylcholine leads to complete loss of the ability of muscarinic receptor agonists to induce cyclic GMP formation, with no accompanied loss of (^{3}H) quinuclidinyl benzilate ((^{3}H)QNB) binding. Upon more prolonged incubations with the agonist, however, a significant decline in (^{3}H)QNB binding is observed.

An increase of muscarinic acetylcholine agonist concentration in the receptor biophase results in certain adaptive changes culminating in loss of cellular responsiveness to the agonist, a phenomenon known as subsensitivity, tachyphylaxis or desensitization[22]. Furthermore, if the concentration of the muscarinic receptor agonist in the receptor vicinity remains significantly higher than the physiological concentration for an extended period, cell membrane-bound receptors start to decrease in number (down-regulation), which could also be accompanied by a change in receptor affinity. We have recently reported that when muscarinic receptors in rat brain homogenates are labeled with these two ligands, (^{3}H)NMS is still able to bind only to a fraction of the sites labeled by (^{3}H)QNB[23]. These data have been interpreted in terms of the ability of (^{3}H) QNB to label a hydrophobic domain on the muscarinic receptor which is not easily accessible to hydrophilic ligands such as (^{3}H)NMS[23].

Regulation of muscarinic receptor binding sites by carbamylcholine in brain cells does not occur if incubation with the agonist is performed at 15 °C, which demonstrates the sensitivity of the process to temperature. This is similar to the temperature dependence of desensitization of muscarinic receptor-mediated cyclic GMP formation and down-regulation of these receptors in mouse neuroblastoma (clone NIE-115) cells. The effect of temperature on muscarinic receptor down-regulation in intact rat brain cells might be due to an energy requirement similar to that reported in guinea-pig vas deferens[24], or due to the necessity of a certain degree of membrane fluidity[25]. It might also be due to the involvement of certain muscarinic receptor-mediated biochemical responses, since the latter also demonstrate strong dependence on temperature[25].

It has been reported recently that exposure of other intact cell systems to muscarinic receptor agonists induces a much faster loss in (^{3}H)NMS binding than in (^{3}H)QNB binding[26,27]. These observations have been attributed to the much more hydrophilic nature of (^{3}H)NMS compared to that of (^{3}H) QNB[28]. This difference in physicochemical properties between the two ligands makes (^{3}H)NMS able to identify muscarinic acetylcholine receptors only if they exist on the cell surface, while (^{3}H) QNB, being a lipophilic ligand, can label the receptors even if they are internalized or sequestered inside the cell membrane. These properties have been applied in our laboratory and others to study the differential regulation of cell surface muscarinic receptors as opposed to the total receptor population[22,26,28]. In the present work, although preincubation of rat brain cells with 1 mM carbamylcholine for 3 hrs at 37 °C results in 36% reduction in the density of the receptors labeled by (^{3}H) NMS, similar treatments decrease maximal (^{3}H) QNB binding by only 22%. These data suggest that desensitization of muscarinic acetylcholine receptors in these cells results in some degree of receptor internalization of sequestration into the cell membrane. Alternatively, desensitizing conditions might transform the receptors into the cell membrane. Alternatively, desensitizing conditions might transform the receptors into a conformation to which (^{3}H) QNB binds more efficiently than (^{3}H) NMS. However, the relative reduction in (^{3}H) NMS and (^{3}H)QNB binding in desensitized cells as compared with their own controls is not as large as the difference found in mouse neuroblastoma NIE-115 cells[29]. It might be concluded that under our experimental conditions using intact rat brain cells, there is not as much accumulation of intramembranal of intracellular muscarinic receptors preceding receptor degradation as there is in clonal cell lines. In other words, the rate of receptor internalization might be only somewhat greater than the rate of receptor breakdown. This is also supported by

our present findings that in cells desensitized with 1 mM carbamylcholine for 3 hrs the rate of recovery of (^{3}H)NMS binding, if any, appears to be extremely slow, suggesting that *de novo* receptor synthesis is required for the reappearance of the lost surface receptors.

It is noteworthy that in control cell preparation, (^{3}H) QNB labels a higher receptor density than (^{3}H) NMS. Although this might be taken as evidence for the existence of an internalized receptor population in control cells that is accesible only to (^{3}H)QNB but not to (^{3}H)NMS, findings from our laboratory in broken cell preparations make this possibility rather unlikely.

A general contention which can be gathered from the above mentioned reports is that (^{3}H)NMS is a more appropriate ligand to use in studying regulation of surface-bound muscarinic receptors, which are probably the only receptor population available to hydrophilic agonists. In dissociated brain cells, we have also found a slower agonist-mediated disappearance of muscarinic receptors when receptor binding was assessed using (^{3}H)QNB instead of (^{3}H)NMS.

In summary, our present data demonstrate that muscarinic acetylcholine receptors in intact rat brain cells are subject to regulation upon exposure to receptor agonists, in a fashion which is somewhat different from that observed in cloned cell lines in culture. This agonist-induced receptor down-regulation is dependent on the agonist concentration, in addition to the temperature and not the time of incubation. Furthermore, our data suggest that the receptors might undergo a process of internalization followed rapidly by receptor degradation. These cells provide a less expensive and a more physiologically relevant model to study muscarinic receptor regulation in the central nervous system than clonal cell lines.

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