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Short-term disappearance of muscarinic cell surface receptors in carbachol-induced desensitization

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A rapid carbachol-induced disappearance of muscarinic cell surface receptors was shown using [^{3}H]methyl scopolamine as ligand on intact 108CC15 hybrid cells or rat cerebellar cells. This phenomenon is temperature-dependent, correlated to agonist stimulation and reversible. In these short time periods ($\leqslant$30 min), no change was observed in the total receptor amount measured on membrane preparations. This disappearance of cell surface receptors could represent the first event in cell desensitization which could be followed by receptor recycling in physiological conditions or by receptor degradation if the stimulation by agonists persists, as in long-term regulation.

Muscarinic receptor *[^{3}H]Methylscopolamine* *[^{3}H]QNB* *Desensitization* *Down-regulation* *Cell culture*

1. INTRODUCTION

Desensitization or refractoriness is observed in numerous systems and consists in diminished cellular response subsequent to agonist treatment [1]. This phenomenon, which corresponds to a loss of physiological receptor-mediated response, appears rapidly in a few minutes; it is called acute or short-term desensitization. In contrast, long-term or chronic desensitization, also called down-regulation, develops more slowly and is accompanied by a true loss of receptors but not correlated to decrease in pharmacological activity [1–3]. Down-regulation of muscarinic receptors was reported in neuroblastoma cells [4,5]; long-term incubation of these cells with muscarinic agonists caused a dose- and time-dependent loss of muscarinic receptors when measured on membrane fractions using labelled ligands.

Recently, muscarinic receptor down-regulation was also reported in primary cultures of rat cerebellum [6]. This long-term regulation, because several hours of exposure to agonists were needed [7], presumably involves receptor internalization followed by a degradation process.

A short-term desensitization was also reported in neuroblastoma cells where the exposure to agonists led to a rapid desensitization of the receptor-mediated increases in cGMP levels (cf. [2]). Although the relationships between both types of regulation is not clear, they certainly represent a way to protect the cells against an excess of agonists.

We now report the rapid disappearance of muscarinic receptors from the cell membrane surface of two neuronal cells: neuroblastoma × glioma hybrid cells and cerebellar granule cells. This was measured with the convenient ligand, [^{3}H]methylscopolamine.

2. MATERIALS AND METHODS

Neuroblastoma × glioma hybrid cells (108CC15) were obtained from Dr B. Hamprecht (Wurzbürg). Cells were grown in Petri dishes in a Dulbecco's modified Eagle's medium supplemented with

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15 mM glucose, 10% fetal calf serum, 0.1 mM hypoxanthine, 1 μM aminopterin and 16 μM thymidine (DMEM–HAT).

Cerebellar granule cells were prepared as in [8] and cells were incubated in Petriperm® dishes as described in [9]. This allowed us to obtain virtually pure rat granule cells after 7 days in vitro. All cultures were incubated in a humidified incubator in an atmosphere of air with 10% CO_2 and held at 37°C.

For binding assays, performed on intact cells, the monolayers of cells were gently washed and incubated with 0.5 nM [^{3}H]methylscopolamine and unlabelled drugs as displacer in the following medium: Dulbecco's modified Eagle's medium with 15 mM glucose and 20 mM Hepes, at pH 7.4. Incubation was stopped by washing the cells with chilled buffer. Radioactivity was counted with Insta Gel (Packard) as scintillation liquid in a Packard 460 CD counter.

Membrane fractions from 108CC15 or cerebellar granule cells were prepared as in [10] and incubated for 1 h with 0.5 or 1 nM [^{3}H]QNB at 37°C. Specific binding was defined as the difference between binding with or without 3×10^{-7} M atropine. Protein content was estimated as in [11].

DMEM and fetal calf serum were from Gibco/Biocult Laboratories. Petriperm® dishes were obtained from W.C. Heraeus GmbH (Hanau). *N*-Methyl-[^{3}H]scopolamine (spec. act. 84.8 Ci/mmol) was purchased from NEN (Boston) and [^{3}H]QNB (spec. act. 38.0 Ci/mmol) was from Amersham.

3. RESULTS

Study of muscarinic receptor regulation in whole cells implies an additional ligand property to those needed for binding studies on membrane fractions: it should only label the cell surface receptors without diffusing and be trapped into the cell. In this way, [^{3}H]methylscopolamine seemed to be a ligand of choice when compared to [^{3}H]QNB or [^{3}H]dexetimide (not shown).

Fig.1 shows that the saturation of specific binding was obtained on intact 108CC15 cells in the nM range which is quite comparable to results obtained on membrane fraction of the same cells: the K_d-values on whole cells and membranes were

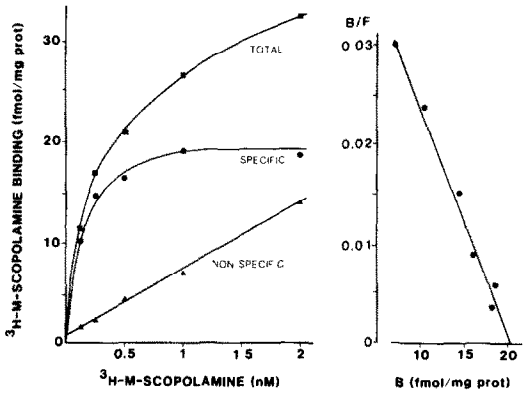

Fig.1. Methyl-[^{3}H]scopolamine-binding in intact 108CC15 cells, measured with increasing concentrations of labelled ligand: (■—■) total binding; (●—●) specific binding; (▲—▲) non-specific binding measured in the presence of 3×10^{-7} M atropine. On the right, Scatchard analysis of the specific binding.

0.145 and 0.143 nM, respectively, whereas the B_{max}-value on intact cells was 20 fmol/mg protein. Note the low non-specific binding. Table 1 shows the IC_{50}-values obtained with methyl-[^{3}H]scopolamine (0.5 nM) or [^{3}H]QNB (0.5 nM) on whole cells or membrane of both 108CC15 and cerebellar cells. There was a marked stereospecificity between dexetimide and levetimide, about 10000-times. IC_{50}-values obtained in 108CC15, granule cells or membranes were correlated with those which were observed in membrane preparation of rat brain [10,12]. Full specific association was obtained within 15 min at 37°C and 30 min at 0°C.

The rate of agonist-induced disappearance of the muscarinic receptors was studied as follows: cells were incubated at 37°C up to 60 min, with the cholinergic agonist carbachol (5×10^{-4} M on cerebellar granule cells or on 108CC15 cells) in the presence of eserine (5×10^{-6} M). At this concentration, eserine did not interfere with specific binding. Afterwards, the medium was removed and cells were incubated for 10 min with chilled buffer without carbachol and maintained at 4°C during the binding assay. This further incubation was performed with 0.5 nM methyl-[^{3}H]scopolamine in cold buffer with or without atropine during 1 h at 4°C. Cells were then washed 3 times with the buffer. There was no change in the cell viability during the course of the experiment.

Table 1

IC_{50}-values for various drugs in methyl-[^{3}H]scopolamine and [^{3}H]QNB-binding measured in intact 108CC15 cells or in membrane fractions of 108CC15 and cerebellar cells

Drug	IC_{50}-values (M)			
	Methyl-[^{3}H]scopolamine			[^{3}H]QNB
	108CC15 cells	108CC15 membrane	Granule cells membrane	108CC15 membrane
Dexetimide	2.0×10^{-9}	1.7×10^{-9}	2.1×10^{-9}	0.9×10^{-9}
Atropine	4.0×10^{-9}	3.2×10^{-9}	5.0×10^{-9}	4.5×10^{-9}
Isopropamide	4.0×10^{-9}	4.4×10^{-9}	–	4.2×10^{-9}
Oxotremorine	4.4×10^{-6}	–	–	7.9×10^{-7}
Carbachol	2.9×10^{-5}	3.1×10^{-6}	3.0×10^{-6}	2.6×10^{-6}
Levetimide	$>10^{-5}$	$>10^{-5}$	$>10^{-5}$	1.8×10^{-5}

IC_{50}-values are mean values of binding measured in triplicate with 0.5 nM of methyl-[^{3}H]scopolamine or [^{3}H]QNB

Fig.2 illustrates the rapid disappearance of cell surface muscarinic receptors on 108CC15 and cerebellar granule cells. Half-life of disappearance in specific binding was 4.8 min on cerebellar neurones and 9.5 min in 108CC15 cells. When the binding was performed in membrane fraction with 1 nM [^{3}H]QNB, the number of muscarinic receptors did not significantly change during the first hour after exposure to carbachol. In contrast, when cells were incubated for 16 h with carbachol, there was a dramatic decrease of [^{3}H]QNB-specific

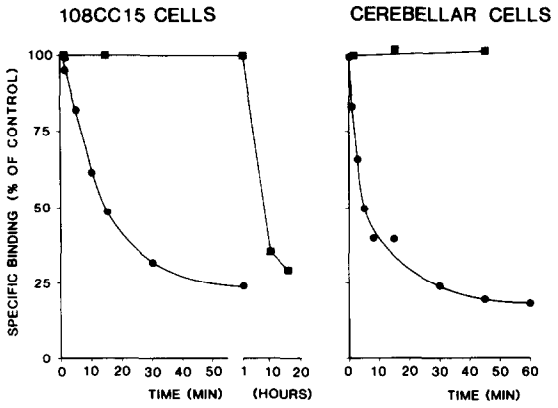

Fig.2. Time course of carbachol-induced effect on muscarinic receptors in intact cells (●—●) and membrane fractions (■—■) in 108CC15 and cerebellar cells. Specific binding was measured in intact cells with 0.5 nM methyl-[^{3}H]scopolamine and in membrane fractions with 1 nM [^{3}H]QNB. Carbachol concentration was 5×10^{-4} M in 108CC15 cells and in cerebellar cells.

binding sites. As a rule, [^{3}H]QNB was preferred to methyl-[^{3}H]scopolamine for measuring muscarinic receptors in membrane fraction because it gave more reproducible results.

The Scatchard analysis of the specific binding obtained with increasing concentrations of methyl-[^{3}H]scopolamine (0.062–2 nM) showed a reduction in B_{max}-values of muscarinic receptors in whole cells after short-term incubation with carbachol and in membranes after 16 h of exposure to carbachol but without change in the K_d-value.

When rat brain membranes were incubated with or without carbachol for 1 h at 37°C, then washed and centrifuged (18000 rev./min for 10 min) at 0°C before performing the binding assay, there was no difference in specific binding, suggesting that cell integrity is necessary to observe the rapid disappearance phase. If the cells (108CC15 or granular cells) were incubated with carbachol for 15, 30 or 60 min at 4°C instead of 37°C, no reduction in the specific binding could be seen. When 108CC15 cells were incubated for 16 h at 4°C with carbachol (5×10^{-4} M), the down-regulation did not appear. The lysosomotropic agent methylamine (3×10^{-2} M) almost completely inhibited the carbachol-induced down-regulation but not the rapid disappearance phase.

Fig.3 shows the reversibility of the agonist-induced disappearance when carbachol was added in the cell culture for short times (15 min–1 h) and then further incubated at 37°C up to 16 h without carbachol, there was no significant change in the

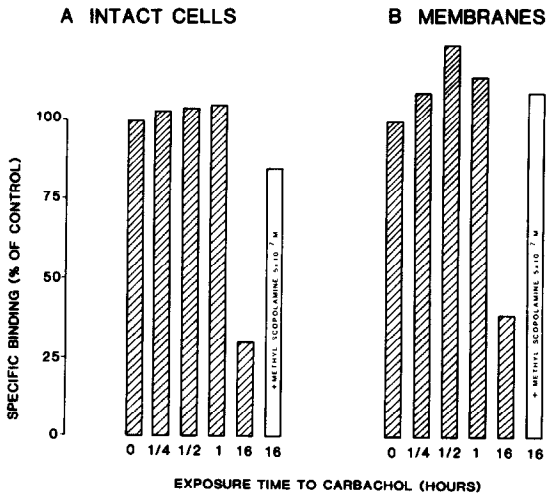

Fig.3. Reversibility of short-term carbachol-induced disappearance of muscarinic receptors. 108CC15 cells were incubated at 37°C for 15, 30 and 60 min with carbachol and then further incubated up to 16 h without agonist. Long-term incubation with carbachol was performed with carbachol for 16 h in the presence and absence of 5×10^{-7} M methylscopolamine. Specific binding was measured in triplicate with 0.5 nM methyl-[^{3}H]scopolamine in intact cells (A) or 0.5 nM [^{3}H]QNB in membrane fractions (B).

number of muscarinic receptors in intact cells or membrane. When the contact with carbachol was prolonged for 16 h, there was a marked decrease of muscarinic receptors in intact cells or membrane

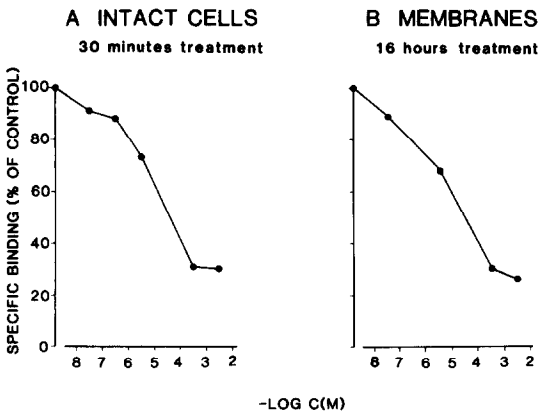

Fig.4. Effect of different concentrations of carbachol on muscarinic receptors measured in intact 108CC15 cells (A) 30 min after carbachol or in membrane fractions, 16 h after carbachol (B). Binding conditions as in fig.3.

fractions; this effect was blocked by 5×10^{-7} M methylscopolamine.

Fig.4 shows the disappearance of muscarinic receptors when 108CC15 cells were incubated for 30 min (intact cells) or 16 h (membrane) with different concentrations of carbachol. The effect was already detectable at 5×10^{-8} M and became maximal at 10^{-3} M.

4. DISCUSSION

A rapid and reversible carbachol-induced disappearance of surface muscarinic receptors was observed in two neuronal cells when the binding assay was carried out in intact cells using methyl-[^{3}H]scopolamine as ligand. This phenomenon, which could not be demonstrated with other ^{3}H-labelled ligands, appeared already within a few minutes after exposure to carbachol and reached a maximum after 30 min. At that time there was no change in the total number of muscarinic receptors when measured in membrane fractions. This suggests that the receptors were still present presumably within the neuronal cells. This rapid disappearance of muscarinic receptors was reversible and dependent on agonist concentration and temperature. That this rapid disappearance was not measurable using [^{3}H]dexetimide and [^{3}H]QNB (not shown) could be due to high non-specific binding and to the fact that they also bind receptor sites located within the cell in addition to the cell surface receptors.

When the neuronal cells were treated with carbachol for a long period of time (several hours) the number of total muscarinic receptors measured in membrane fractions markedly decreased, suggesting that a degradation phenomenon follows the rapid disappearance phase. In contrast, the total number of muscarinic receptors did not decrease after short-term exposure to carbachol, suggesting that they do not undergo this degradation process. Since the receptors appear again at the membrane surface after having disappeared, one may assume that they are recycled after having undergone an internalization process.

This work gives more support to the idea that the refractoriness period of a receptor due to the presence of an agonist should be caused by a rapid agonist-induced disappearance of receptor from the cell surface. Such a phenomenon was reported

for β-adrenergic receptors measured with a ^{3}H-labelled ligand which did not penetrate into the cell [13]. Therefore, the short-term and long-term desensitization could be only two stages of the same phenomenon. However, the fate of the receptor could be 2-fold whether the receptor is recycled after short-term interaction with the agonist or is subsequently degraded if the exposure to the agonist is prolonged.

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