# Agonist-induced down-regulation of m1 muscarinic receptors and reduction of their mRNA level in a transfected cell line

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Agonist-induced reduction in both the number of m1 muscarinic receptors and the mRNA coding for the receptor protein was investigated in Chinese hamster ovary (CHO) cells which were transfected with the m1 muscarinic receptor gene. Receptor concentration was measured by the specific binding of the muscarinic ligand, [$^3$H]quinuclidinyl benzilate ([$^3$H]QNB), and Northern blot hybridization analysis was used to evaluate the levels of receptor mRNA. Incubation of cells with 1 mM of the muscarinic agonist, carbamylcholine (CBC), for 24 h decreased receptor density and mRNA levels in cells by 65% and 73%, respectively. These results indicate that agonist-induced down-regulation of m1 muscarinic receptors might be due to, at least in part, a decrease in receptor synthesis resulting from a reduction in the steady-state level of their mRNA.

Muscarinic receptor; Receptor down-regulation; mRNA regulation; Northern blot analysis; Chinese hamster ovary cell

## 1. INTRODUCTION

Desensitization and/or down-regulation of G protein-coupled receptors is a common phenomenon observed in a variety of cell types upon prolonged stimulation by receptor agonists [1]. Muscarinic receptors, which are members of this superfamily, have been shown to be regulated in a similar fashion [2-6]. However, the molecular basis for agonist-induced reduction in the number and the sensitivity of muscarinic receptors remains unclear. Although it has been suggested that muscarinic receptor down-regulation is due to an increase in the rate of receptor protein degradation [7], there have been no attempts to assess alterations in receptor biosynthesis. An important question to be addressed in this regard is whether prolonged muscarinic receptor activation alters the concentration of mRNA coding for the receptor, similar to what has been shown recently in the case of $\beta$-adrenergic receptors [8]. There are currently five known subtypes of muscarinic receptors [9]. The m1 muscarinic receptor subtype is of a particular importance, since it is abundant in brain regions which are closely associated with memory and cognition [9]. Since memory deficits are treated on a long-term basis with either directly acting muscarinic agonists or acetylcholinesterase inhibitors [9], it is therefore important to investigate the mechanisms underlying regulation of m1 receptors. Unfortunately, however, there are no known established cell lines which express this subtype of muscarinic receptors. Therefore, we have employed Chinese hamster ovary (CHO) cells which were stably transfected with the m1 muscarinic receptor gene [10] for this purpose. Here, we report for the first time that agonist-induced down-regulation of m1 muscarinic receptors is associated with a similar decrease in the steady-state level of the specific mRNA encoding this receptor protein.

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*Abbreviations:* CHO, Chinese hamster ovary; [$^3$H]QNB, [$^3$H]quinuclidinyl benzilate; CBC, carbamylcholine

## 2. MATERIALS AND METHODS

### 2.1. *Cell culture*

Chinese hamster ovary (CHO) cells stably transfected with the rat m1 muscarinic receptor gene (*Sma*I 1.73 kb restriction fragment of genomic clone c71 [10] containing the entire coding region of 1.38 kb) were provided by Drs J.C. Venter and C.M. Fraser at the National Institutes of Health [11]. Cells were grown in tissue culture flask (75 cm$^2$/250 ml) in 20 ml of DMEM 199 supplemented with 10% (v/v) bovine calf serum and 0.005% (w/v) geneticin. Cells were grown at 37°C in a humidified atmosphere consisting of $CO_2$/air (1:9). In all experiments, cells were used after 6 days from subculture.

### 2.2. *Preparation of mRNA and Northern blot analysis*

Total cellular RNA was extracted by the guanidinium thiocyanate method [12]. Poly(A)$^+$ RNA used in Northern blot analysis was isolated by oligo (dT)-cellulose [13]. Samples of poly(A)$^+$ RNA (10-20 $\mu$g) were denatured with 50% formamide and electrophoresed through 1.0% agarose/0.66 M formaldehyde gels [13], then transferred to nitrocellulose membranes (Optibind, Schleicher & Schuell, Keene, NH). Blots were hybridized using a 0.39 kb *Pst*I/*Hin*dIII restriction fragment of the rat m1 muscarinic receptor cDNA cor-

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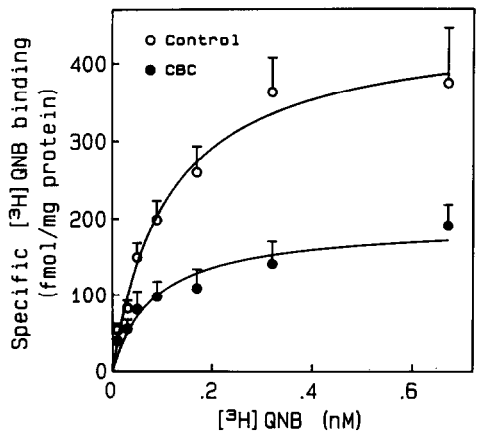


Fig. 1. Saturation isotherm of specific [$^3$H]QNB binding in control and desensitized m1 CHO cells. Cells were incubated in monolayer with or without (control) 1 mM CBC for 24 h at 37°C, then washed and harvested. Intact cells (0.21 ± 0.03 mg protein for control and 0.18 ± 0.03 mg protein for CBC group) were incubated with increasing concentrations of [$^3$H]QNB for 60 min at 37°C followed by filtration as described in section 2. Nonspecific binding was determined in the presence of 10 $\mu$M atropine and subtracted from all values. Data are shown as the mean ± SE from three independent experiments performed in triplicate.

responding to bases number 672–1044 of the sequence [14] (kindly provided by Dr T.I. Bonner, National Institutes of Health). The probe was labeled with [$\alpha$-$^{32}$P]dCTP using a random priming labeling kit (Pharmacia, Piscataway, NJ). Hybridization was performed with $1\text{-}3 \times 10^{-6}$ cpm/ml of the probe at 42°C for 18 h in 3 × SSC (1 × SSC = 0.15 M NaCl/0.015 M Na-citrate, pH 7.0) containing 40% formamide, 10% dextran, 1 × Denhardt's solution and 20 $\mu$g/ml denatured salmon sperm DNA. Blots were washed successively in 1 × SSC/0.1% SDS (2 × 20 min at room temperature and 20 min at 55°C), 0.1 × SSC/0.1% SDS (20 min at 55°C and 15 min at 60°C) and

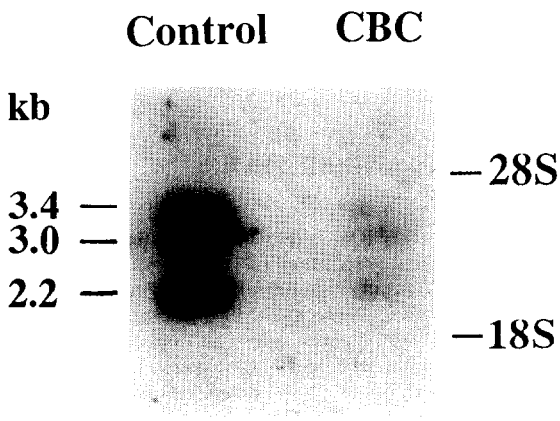


Fig. 2. Northern blot analysis of mRNA in control and desensitized m1 CHO cells. Hybridization with $^{32}$P-labeled m1 muscarinic receptor probe in control cells and in cells which were incubated with 1 mM CBC for 24 h at 37°C (CBC group). In this particular experiment, densitometric scanning of the summation of the three bands yielded a ratio of 5.7 when control cells were compared to densensitized cells. Hybridization of the same blot after removal of the m1 probe with a $^{32}$P-labeled $\beta$-actin probe resulted in the detection of a single band and indicated that the amount of loaded mRNA in the control group was 1.4 times larger than that of the CBC group. 18S and 28S mark the position of the corresponding ribosomal RNA species.

3 × SSC/0.1% SDS (10 min at 65°C), and exposed to X-ray film with intensifying screens for 24–34 h at −70°C. The m1 receptor probe was then removed and the blots were hybridized under the same conditions with a 0.77 kb $^{32}$P-labeled chicken $\beta$-actin cDNA probe (Oncor, Gaithersburg, MD; $1\text{-}3 \times 10^{-6}$ cpm/ml) followed by washing to a final stringency of 0.1 × SSC/0.1% SDS (2 × 20 min at 65°C) and exposure to X-ray film for 12–20 h. Autoradiograms were scanned by a densitometer (Hoefer, San Francisco, CA) and the hybridization signals of the m1 receptor probe were expressed as a ratio of the corresponding signals of the $\beta$-actin probe to account for differences in loading or transfer. Molecular weights were determined using RNA molecular weight markers (BRL, Gaithersburg, MD).

### 2.3. *Muscarinic receptor binding assay*

Suspended intact cells were incubated with [$^3$H]quinuclidinyl benzilate ([$^3$H]QNB) (32.9 Ci/mmol, NEN Research Products, Boston, MA) in a concentration range of 0.01–0.7 nM in a final volume of 1 ml of oxygenated Krebs-Henseleit buffer. Nonspecific binding was measured in the presence of 10 $\mu$M atropine. After 1 h at 37°C samples were filtered under vacuum through Whatman glass fiber GF/B filters, followed by washing 3 times with ice-cold 0.9% NaCl solution. Radioactivity was measured after 6 h of the addition of scintillation fluid by liquid scintillation spectrometry. Saturation isotherms of specific binding were analyzed by Scatchard plots [15] using linear least-squares regression and protein was assayed according to the method of Lowry et al. [16] using bovine serum albumin as a standard.

## 3. RESULTS AND DISCUSSION

Binding studies using the specific muscarinic receptor ligand [$^3$H]QNB in intact CHO cells transfected with the m1 muscarinic receptor gene showed saturable and high affinity binding ($B_{max} = 427.1 \pm 55.1$ fmol/mg protein, $K_d = 41.0 \pm 5.7$ pM) (Fig. 1). Incubation of cells in monolayer with a receptor-saturating concentration (1 mM) of carbamylcholine (CBC) for 24 h at 37°C resulted in a significant ($P < 0.05$) decline in $B_{max}$ of [$^3$H]QNB to 147.4 ± 29.4 fmol/mg protein, without a significant change in the $K_d$ (30.6 ± 8.6 pM) (Fig. 1). The absence of changes in $K_d$ suggests that the observed reduction in binding is not due to the presence of residual CBC in the binding assay. Thus, agonist treatment reduced receptor concentration by 66 ± 6% (mean ± S.E.M.). These data are similar to those obtained in other cell lines [3–6] and primary tissues [17]. The decrease in receptor density by 1 mM CBC was time dependent, with a half-maximal effect of 15 h. It was also dependent on the concentration of CBC ($EC_{50} \approx 1$ $\mu$M) when cells were desensitized for 24 h (data not shown). The presence of 10 $\mu$M atropine during incubation of cells with 1 mM CBC for 24 h completely abolished CBC-induced receptor down-regulation (135% of control; J. Hu, S.-Z. Wang and E.E. El-Fakahany, manuscript in preparation). Taken together, these data suggest that this effect of CBC is associated specifically with its activation of muscarinic receptors.

Northern blot hybridization using a $^{32}$P-labeled specific cDNA probe for m1 muscarinic receptors detected three distinct mRNA species with molecular weights of 2.2 (corresponding to the mol. wt. of the transfected fragment), 3.0 and 3.4 kb (Fig. 2), while

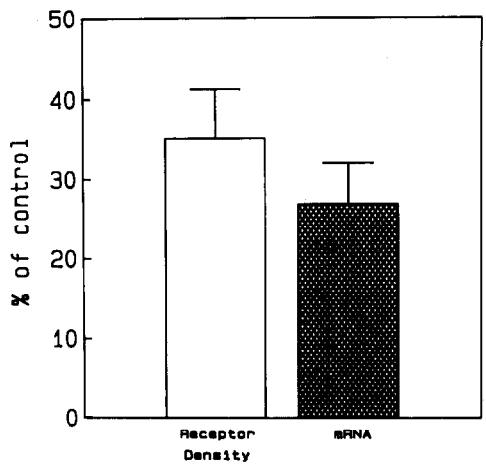


Fig. 3. Comparison between CBC-induced receptor down-regulation and decrease in m1 mRNA. The data shown were derived from the experiments presented in Figs 1 and 2, where control is the corresponding value in cells which were not treated with CBC. Receptor density is presented as the $B_{max}$ of [$^3$H]QNB binding. mRNA levels were calculated by densitometric scanning of the three bands which hybridized with the m1 probe (see Fig. 2) after correction for the intensity of hybridization of the $\beta$-actin probe for each sample. The data shown are the means ± SE for three independent experiments.

hybridization with a $\beta$-actin probe demonstrated the presence of a single molecular weight mRNA (not shown). The reasons for the appearance of these multiple bands are not clear, and it is not possible to provide a conclusive explanation for this phenomenon at present. However, all three bands are most likely related to a specific hybridization signal, particularly since the probe was homogeneous (data not shown) and the hybridization conditions were relatively stringent. Furthermore, screening of an EMBL database indicated that there is no more than 52% homology with any of 24 000 non-muscarinic receptor cloned sequences (also see below). Thus, the observed multiple bands might reflect different degrees of processing of the m1 receptor mRNA resulting from the stable transfection of the foreign muscarinic receptor gene into the genome, perhaps in multiple copies per genome.

Incubation of cells in monolayer with 1 mM CBC for 24 h at 37°C resulted in reducing the level of mRNA encoding m1 muscarinic receptors by 73 ± 5% (Figs 2 and 3). This reduction was observed for all the three hybridizable mRNA species to a similar extent, supporting the notion that they all correspond to an m1 receptor transcript. Possible differences in the amount of loaded mRNA between control and treated cells or differences in the efficiency of mRNA transfer could not account for the observed decrease in mRNA hybridization, since all data were corrected for the hybridization of a $\beta$-actin probe to the same blots. Similar results have been published recently where incubation with isoproterenol resulted in a decrease in mRNA encoding $\beta$-adrenergic receptors in Chinese hamster fibroblasts [18], DDT$_1$ MF-2 hamster vas deferens cells [8] and S49 mouse lymphoma cells [19]. In the presence of 10 $\mu$M atropine, the magnitude of the reduction of m1 mRNA concentration by CBC was attenuated from 73% to 27% ($n = 2$, data not shown), suggesting that the effects of CBC are mediated by activation of muscarinic receptors. It is noteworthy that in the present study there was an excellent correspondence in the magnitude of agonist-induced receptor down-regulation and the observed decrease in m1 receptor mRNA after desensitization with 1 mM CBC for 24 h (Fig. 3). Preliminary experiments showed that this correlation between the decrease in mRNA and receptor down-regulation holds at shorter time points. Thus, there was a decrease in the concentration of m1 receptor mRNA of 15% and 34% after incubation of cells with 1 mM CBC for 6 and 12 h, respectively, which is in reasonable agreement with the observed 18% and 58% decrease in receptor density at the same time points ($n = 2$, data not shown). Moreover, the two parameters were reduced by approximately 50% at 1 $\mu$M CBC for 24 h (47% and 55% reduction in mRNA and $B_{max}$, respectively, $n = 2$, data not shown). These data suggest that the lowered steady-state level of muscarinic receptors following prolonged treatment with agonist is not solely the result of an increase in the rate of receptor degradation as has been suggested previously [7], but could also result from a decrease in receptor protein synthesis as a consequence of a decrease in the level of its mRNA. These data also suggest that the transcription regulatory elements which influence the concentration of mRNA might reside on or near the receptor coding region. The m1 receptor gene construct used for transfecting CHO cells contains 0.14 and 0.21 kb of untranslated sequences at the 5′ and 3′ end, respectively. Careful analysis of the upstream untranslated sequence should provide valuable information regarding the existence and localization of such regulatory elements. Future studies should aim at determining whether the observed decrease in the steady-state level of m1 muscarinic receptor mRNA is due to a decrease in the rate of transcription, a decrease in the stability of mRNA or a combination of both. The possible role of muscarinic receptor-induced second messenger signals in the observed effects should also be investigated.

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