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Long-term carbachol treatment-induced down-regulation of muscarinic M_2-receptors but not m_2 receptor mRNA in a human lung cell line

E.-B. Haddad, J. Rousell, J.C.W. Mak & P.J. Barnes

Department of Thoracic Medicine, National Heart & Lung Institute, Dovehouse Street, London SW3 6LY

1 The molecular mechanisms involved in the regulation of muscarinic receptor gene expression are poorly understood. We have investigated the effect of homologous stimulation on the regulation of M_2 muscarinic receptor protein and gene in human embryonic lung fibroblasts (HEL 299 cells).

2 Saturation studies performed with the non-selective hydrophilic ([^{3}H]-N-methyl-scopolamine, [^{3}H]-NMS) and lipophilic ([^{3}H]-quinuclidinyl benzilate, [^{3}H]-QNB) muscarinic antagonists revealed a single class of high affinity binding sites.

3 Carbachol (1 mM) induced a rapid down-regulation of [^{3}H]-NMS binding sites. Within 12 h, the process had approached steady state with 40 to 60% loss of receptors at 12 and 24 h.

4 The loss of [^{3}H]-QNB binding sites (40% reduction at 24 h) occurred at a slower rate than did loss of [^{3}H]-NMS binding sites as a result of receptor sequestration.

5 Carbachol treatment was accompanied by a functional desensitization of the receptor after 24 h of agonist treatment. In untreated cells, forskolin induced a large increase in cyclic AMP accumulation which was inhibited significantly by carbachol. The inhibitory effect of carbachol on forskolin-induced cyclic AMP accumulation was lost following 24 h carbachol stimulation.

6 The steady state level of muscarinic m_2 mRNA measured by Northern blot analysis was not affected by carbachol treatment over the time course investigated and half-life studies with actinomycin D suggest that carbachol had no effect on the stability of m_2 mRNA.

7 The rate of transcription of m_2 muscarinic receptor gene as measured by nuclear RNA run-on assay was unaltered by carbachol stimulation.

8 These results suggest that homologous sequestration, desensitization, and down-regulation of M_2 muscarinic receptors in HEL 299 cells does not involve transcriptional or post-transcriptional modifications of m_2 muscarinic receptor mRNAs.

Keywords: Homologous regulation; M_2 muscarinic receptors; cyclic AMP; Northern blot; Nuclear RNA run-on assay; gene transcription

Introduction

Accumulating evidence suggests the classification of muscarinic receptors into four major subtypes (M_1–M_4) on the basis of their antagonist affinities (Caulfield, 1993). The molecular cloning of five muscarinic acetylcholine receptor genes (m_1–m_5) has provided the molecular basis of muscarinic receptor subtypes (Hulme *et al.*, 1990). Through the stable expression of these cloned muscarinic receptors a large body of data regarding the structure, function and pharmacology of muscarinic receptor subtypes has accumulated (Hulme *et al.*, 1990; Wess, 1993). However, much less is known about the factors that regulate the expression of muscarinic receptors, in part because the non-coding promoter and enhancer regions that directly control transcription of the individual muscarinic receptor genes have not yet been sequenced.

Chronic agonist stimulation of muscarinic receptors induces desensitization and down-regulation (Maloteaux & Hermans, 1994), although the mechanism by which these processes occur is not well understood. Several studies have highlighted the crucial role of phosphorylation by specific cellular kinases in the receptor desensitization process (El-Fakahany & Cioffi, 1990; Hosey, 1992, Haga *et al.*, 1993).

While several studies have investigated the regulation of muscarinic receptors at the protein level, few have focused on gene expression. In this study, we investigate whether the homologous down-regulation of muscarinic m_2 receptors is mediated through changes in muscarinic m_2 receptor gene transcription in HEL 299 cells.

[1] Author for correspondence.

Methods

Cell culture

All tissue culture reagents except for Hanks Balanced Salt Solution (HBSS) and Dubelcco's modified Eagle's medium (DMEM, Gibco BRL, Paisley, UK) were obtained from Sigma, (Poole, UK). HEL 299 cells were obtained from the American Type Culture Collection (ATCC code CCL 137; Rockville, MD, U.S.A.) and maintained in DMEM supplemented with 10% foetal calf serum, 2 mM L-glutamine, 100 iu ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 2.5 μg l^{-1} amphotericin B in 95% air and 5% CO_2 at 37°C in a humidifier incubator. All experiments were performed on cells at passage 9 as it has been reported muscarinic receptor levels decrease with increasing passage number in NIE-115 murine neuroblastoma cells (McKinney *et al.*, 1984). The medium was replaced every 3–4 days and on reaching confluence, cells were subcultured by detaching the monolayer with 0.05% trypsin/1 mM EDTA. Treatments were carried out such that cells could be harvested simultaneously at preconfluence.

Radioligand binding studies

All the membrane preparation procedures were performed at 4°C. Cells were washed twice with ice-cold Tris buffer (25 mM, pH 7.4), harvested by cell scraping and homogenized with an Ultra-Turax homogenizer. Membranes were pelleted by centrifugation at 40,000 *g* for 20 min and resuspended in an appropriate volume of Tris buffer. The protein concentration was measured according to Lowry *et al.* (1951).

[^{3}H]-N-methyl-scopolamine ([^{3}H]-NMS; 79.5 Ci mmol^{-1}; New England Nuclear, Stevenage, UK) and [phenyl ^{3}H]-quinuclidinyl benzilate ([^{3}H]-QNB; 49 Ci nmol^{-1}; Amersham plc, Little Chalfont, UK) saturation curves were carried out using a concentration-range varying from 0.04 to 4 nM. Non-specific binding was measured in the presence of 10 μM atropine. Incubations were performed at 30°C for 2 ([^{3}H]-NMS) or 3 h ([^{3}H]-QNB) and terminated by rapid vacuum filtration over 0.2% polyethyleneimine pretreated Whatman GF/C glass fibre filters using a Brandel cell harvester. The filters were washed three times with 4 ml of ice-cold Tris buffer and placed in vials with 4 ml of scintillation cocktail (Filtron X, National Diagnostics, Manville, NJ, U.S.A.) and counted on a Packard beta counter (Packard 2200 CA model). Binding data were analysed with the computerised non-linear regression programme LIGAND (Munson & Rodbard, 1980).

Cyclic AMP measurements

Following stimulation, cells were washed with HBSS and the adenosine 3′: 5′-cyclic monophosphate (cyclic AMP-phosphodiesterade inhibitor zardaverine (10 μM) was added to fresh media for 20 min at 37°C. From each group of treatments basal levels of cyclic AMP were measured, as well as accumulation following forskolin exposure (10 μM) for 10 min in the presence and absence of carbachol (100 μM). Cells were harvested by addition of 1 ml of boiling water directly to each well. Cells were then boiled for a further 2 min before centrifugation at full speed in a microcentrifuge at 4°C for 10 min. The supernatant was collected and stored at −20°C before being assayed for cyclic AMP accumulation by radio immunoassay. Protein assays were performed using a Bio-Rad protein assay (Hemel Hempstead, UK), according to the manufacturer's instructions.

Northern blot analysis

Cells were washed twice with HBSS and harvested by detachment of the monolayer with trypsin/EDTA. Total RNAs were isolated by phenol/chloroform extraction and isopropanol precipitation. Purity was assessed by A_{260}/A_{280} spectrophotometric measurements. Poly $(A)^+$ RNA was prepared using PolyTract mRNA system kit (Promega, Southampton, UK) according to the manufacturer's instructions. Samples of mRNA were size fractionated on a 1% agarose/formaldehyde gel containing 20 mM morpholinosulphonic acid (MOPS), 5 mM sodium acetate and 1 mM EDTA (pH 7.0) and blotted onto Hybond-N filters (Amersham, U.K.) by capillary action using 20× SSC (Standard Saline Citrate, 1× SSC, 0.15 mM NaCl and 0.015 M sodium citrate at pH 7.0).

Cloned human muscarinic receptor cDNAs (Hm_1–Hm_4) were used as probes. These cDNAs corresponded to the third intracellular loop and consisted of a HindIII/EcoRI fragment (271 bp) of Hm_1 cDNA, an EcoRI/PstI fragment (550 bp) of Hm_2 cDNA, a PstI/PvuII fragment (250 bp) of Hm_3 cDNA and a KpnI/HindIII fragment (315 bp) of Hm_4 cDNA.

Prehybridizations and hybridizations were carried out at 42°C with the probes labelled to approximately 1.5×10^6 c.p.m. ml^{-1} in a buffer containing 50% formamide, 50 mM Tris-HCl (pH 7.5), 5× Denhardts solution, 0.1% sodium dodecyl sulphate (SDS), 5 mM EDTA and 250 μg ml^{-1} denatured salmon sperm DNA. Following hybridization the blots were washed to a stringency of 0.1× SSC, 0.1% SDS at 65°C before exposure to Kodak X-OMAT-S film. To account for differences in loading or transfer of the RNA, the blots were hybridized with a 1272 bp PstI fragment from rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA. The intensity of the signals was then quantified by laser densitometry (Quantity One Software, pdi, New York, U.S.A.).

Measurement of nuclear RNA run-on gene transcription in isolated nuclei

Nuclei were prepared as described by Greenberg & Ziff (1984). Isolated nuclei were resuspended in Tris-HCl (10 mM, pH 7.4), $MgCl_2$ (5 mM), glycerol (50%), sorbitol (0.5 M), Ficoll (2.5%), spermidine (0.008%) and DTT(1 mM) and stored at −70°C until use. Nuclei (5×10^7) were incubated for 30 min at 27°C with 300 μCi [^{32}P]-UTP, ATP (0.625 mM), CTP and GTP (0.31 mM), Tris-HCl (40 mM), NH_4Cl (150 mM), $MgCl_2$ (7.5 mM) and RNasin (120 u). DNA digestion was carried out with a 15 min incubation at 27°C with RQ-1 DNase (75 u) and RNasin (40 u) before protein digestion for 3 h at 37°C with Proteinase K (1 mg ml^{-1}) in buffer containing Tris-HCl (pH 7.4, 10 mM), EDTA (15 mM), SDS (3%) and heparin (3 mg ml^{-1}). RNA extraction was then carried out with a phenol, phenol/chloroform (1:1) and a chloroform wash and then precipitated three times with 100% ethanol in the presence of 1.33 M ammonium acetate. The radiolabelled RNAs were dissolved in 100 μl TE buffer (10 mM Tris-HCl pH 7.4, 1 mM EDTA) and added to 2 ml hybridization solution (50% formamide, 5× SSC, 0.1% SDS, 1 mM EDTA, 10 mM Tris-HCl pH 7.5, 5× Denhardt's solution, 50 μg ml^{-1} yeast tRNA, 100 μg ml^{-1} salmon sperm DNA, 0.02 μg poly A and 0.02 μg poly G RNA). Following 4 h prehybridization in the above buffer, hybridization was carried out at 42°C for 72 h to 10 μg of the immobilised plasmid pGEM3Z as a control or to plasmids containing inserts of rat GAPDH cDNA and human m_2-muscarinic receptor cDNA as described previously under Northern analysis. The filters were washed first in buffer A (300 mM NaCl, 10 mM Tris-HCl pH 7.4, 2 mM EDTA, 0.1% SDS, 1 μg ml^{-1} RNase A and 10 u ml^{-1} RNase T1) at 37°C for 30 min then in buffer B (10 mM NaCl, 10 mM Tris-HCl pH 7.4, 2 mM EDTA and 0.4% SDS) to a stringency of 55°C for 30 min and autoradiographed.

Results

Effect of carbachol on muscarinic receptor density and function

All receptor binding studies were performed on cells at approximately 70% confluence as a significant loss in the number of receptor binding sites was observed when the cells were allowed to reach a confluent state (data not shown). Saturation studies performed with the non-selective hydrophilic ([^{3}H]-NMS) and the lipophilic ([^{3}H]-QNB) muscarinic antagonists revealed a single class of binding sites with equilibrium dissociation constants of 0.21 ± 0.09 nM and 22.94 ± 3.15 pM for [^{3}H]-NMS and [^{3}H]-QNB, respectively. At concentrations near their K_d values, the level of specific binding was around 80 to 97% of total binding. The ratio of the B_{max} values of [^{3}H]-QNB to [^{3}H]-NMS in control membranes was 1.2 (1.1–1.4). Carbachol (1 mM) induced a time-dependent down-regulation of M_2 muscarinic receptors in HEL 299 cells (Figure 1). The rate of muscarinic receptor loss, measured with [^{3}H]-NMS was greatest during the first 2 h of down-regulation. Within 12 h, the process had approached steady state with 40 to 60% loss of receptors at 12 and 24 h. The loss of [^{3}H]-NMS binding sites occurred at a faster rate than did loss of [^{3}H]-QNB binding sites (Figure 1). This preferential decrease in [^{3}H]-NMS binding sites at early time points may mirror receptor sequestration induced by carbachol.

Functional desensitization was assessed by measurement of cyclic AMP accumulation following carbachol stimulation. In untreated cells, forskolin induced a large increase in cyclic AMP accumulation which was inhibited significantly by carbachol. The inhibitory effect of carbachol on forskolin-induced cyclic AMP accumulation was lost following 24 h carbachol treatment (Figure 2) or 4 h pertussis toxin (500 ng ml^{-1}) treatment (data not shown). The basal level of cyclic AMP accumulation was not affected by such treatment (Figure 2).

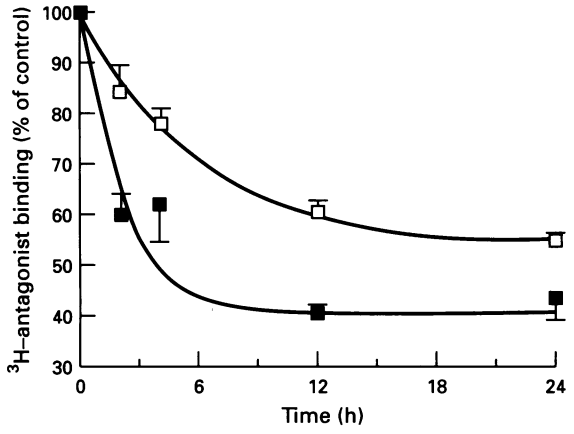


Figure 1 Time-course of carbachol effect on [^{3}H]-NMS (■) and [^{3}H]-QNB (□) binding in HEL 299 cells. Cells were treated with 1 mM carbachol at 37°C for the time indicated. After this incubation period, cells were washed three times with HBSS and the ^{3}H-antagonists binding was carried out as indicated in Methods. [^{3}H]-NMS and [^{3}H]-QNB receptor densities in control cells are 386 ± 18 and 428 ± 24 fmol mg^{-1} protein, respectively. Data, expressed as percentage binding in untreated cells, are the mean ± s.e.mean of three to six independent experiments performed on separate membrane preparations.

Effect of carbachol on the gene expression of m_2 muscarinic receptors

To address the question of whether the down-regulation of muscarinic M_2 receptors in HEL 299 cells is mediated through changes in the steady-state levels of m_2 receptor mRNA, the level of cellular m_2 mRNA was measured by Northern blot analyses following carbachol treatment. Northern blot analysis on isolated messenger RNAs revealed the presence of a single transcript around 6.1 kb corresponding to the m_2 muscarinic receptor with no evidence for the presence of m_1-, m_3-, or m_4-receptor mRNAs. Figure 3 shows that over the time course investigated, carbachol (1 mM) had no significant effect on the level of m_2 mRNA expression. Under the same conditions, atropine (1 μM) induced an up-regulation of m_2 mRNAs after 24 h atropine-treatment (Figure 4). A similar result was observed at the protein level where a 2.4 fold increase in [^{3}H]-NMS binding sites was obtained after 24 h of atropine stimulation (Control, B_{max} 340 ± 42 fmol mg^{-1} protein; atropine, 739 ± 63 fmol mg^{-1} protein. The effect of carbachol on the m_2 mRNA stability was also investigated. The m_2 mRNA half-life ($t_{1/2} = 3$–4 h) measured in the presence of the RNA polymerase inhibitor actinomycin D (5 μg ml^{-1}) was not affected by 12 h carbachol treatment (Figure 5). The rate of muscarinic m_2 receptor gene transcription was also measured by the nuclear RNA run-on assay. The data showed that there is a basal expression of m_2 muscarinic mRNA that was not affected by carbachol stimulation (Figure 6).

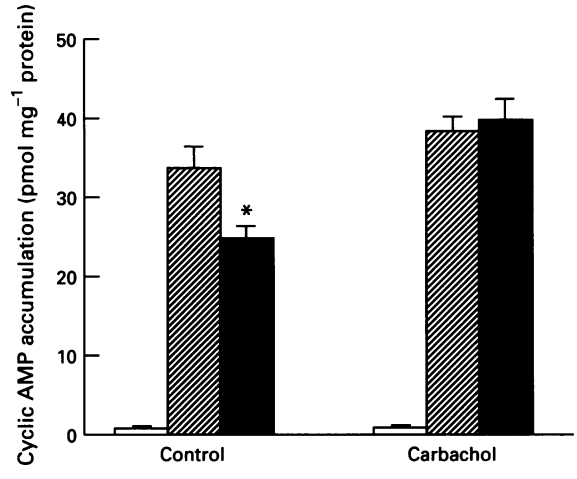


Figure 2 Effect of carbachol treatment on forskolin-induced accumulation of cyclic AMP in HEL 299 cells. Cells were treated for 24 h with 1 mM carbachol. After this incubation period, cells were washed and then incubated with the phosphodiesterase inhibitor zardaverine for 20 min. Cyclic AMP accumulation was measured after 10 min stimulation with vehicle (open column), forskolin (hatched column), and forskolin plus carbachol (solid column). The data shown are the mean ± s.e.mean of four independent experiments performed in triplicate.

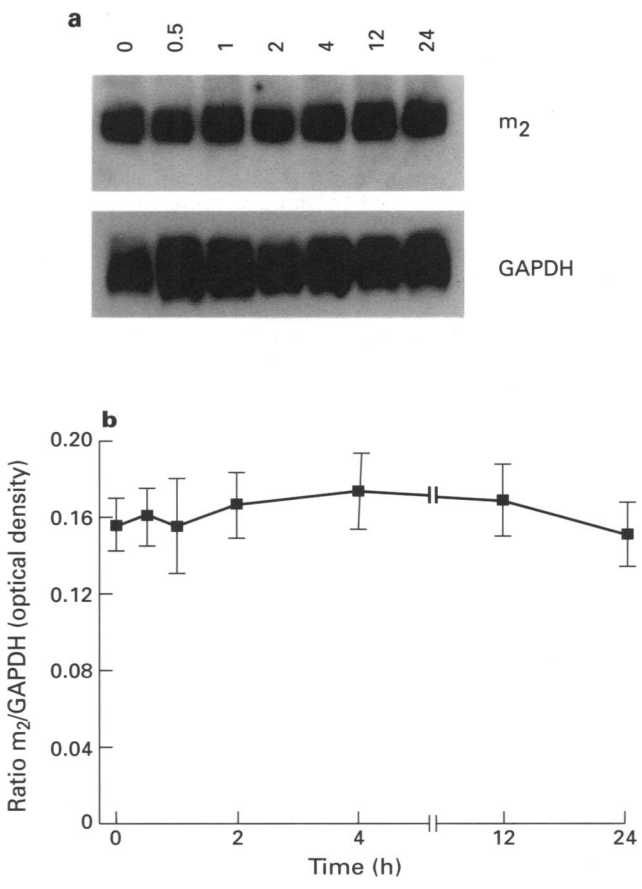


Figure 3 Effect of carbachol (1 mM) on the steady-state levels of muscarinic m_2 mRNA in HEL 299 cells. (a) Shows a representative Northern blot analysis performed with the human m_2 and rat GAPDH cDNAs as indicated in Methods; (b) shows the densitometric measurement of the Northern blot data. To account for difference in loading or transfer of the RNA, the m_2 signal was corrected for that of the GAPDH. The data shown are the mean ± s.e.mean of at least 3 independent experiments.

Discussion

While much progress has been made in recent years in defining the pharmacology and functional role of the muscarinic receptors, less is known about their regulation. In this study, we investigated the effect of homologous stimulation on the gene expression of M_2 muscarinic receptor in HEL 299 cells. The principal conclusion that emerges from the present study is that M_2 muscarinic receptors underwent down-regulation and functional desensitization following carbachol stimulation without any changes in steady-state levels of m_2 mRNAs or rate of transcription of the gene.

Specific [^{3}H]-NMS and [^{3}H]-QNB binding to muscarinic receptors in HEL 299 cell membranes was saturable and best described by the interaction of the radioligands with a single population of high affinity binding sites. The ratio of [^{3}H]-

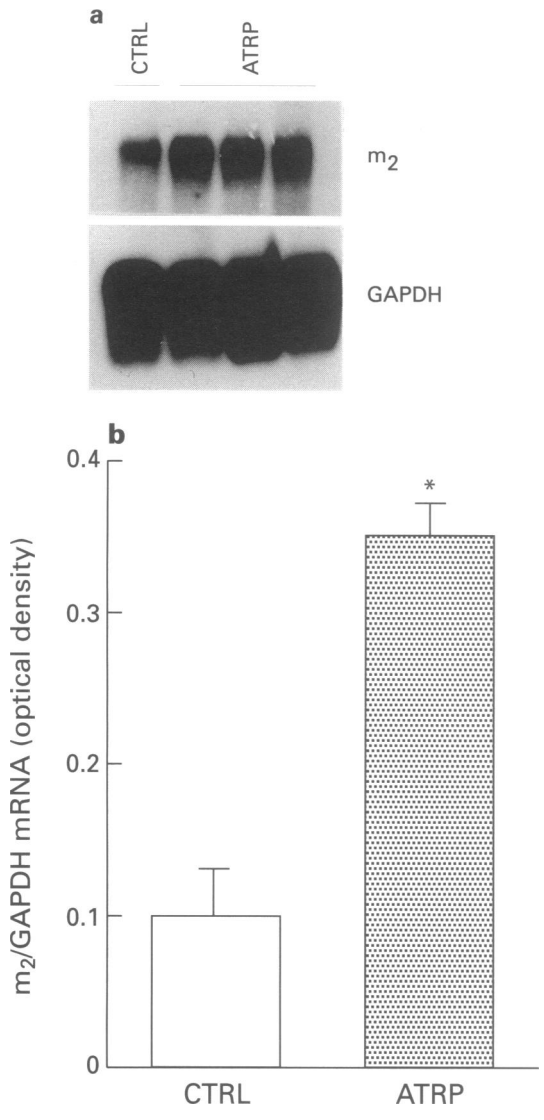


Figure 4 Effect of atropine on the steady-state levels of m_2 muscarinic receptor mRNA. Cells were treated with atropine (1 μM) for 24 h. After this incubation period, cells were washed twice with HBSS and mRNAs were extracted for Northern blot anaylsis. (a) Represents Northern blot analysis performed with the human m_2 and rat GAPDH cDNAs as indicated in Methods; (b) shows the densitometric measurement of the Northern blot data. The data shown are the mean ± s.e.mean of at least 4 independent experiments.

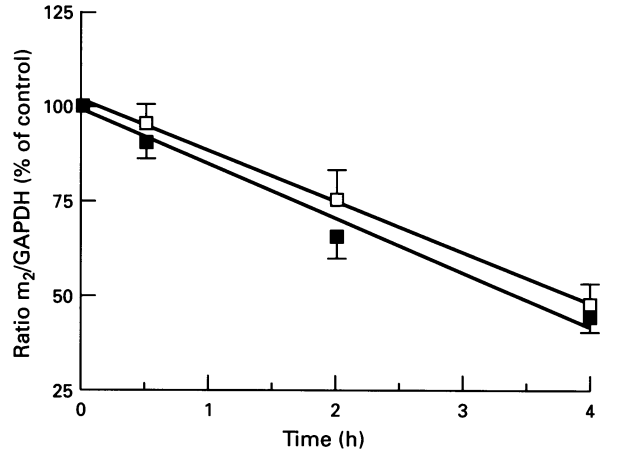
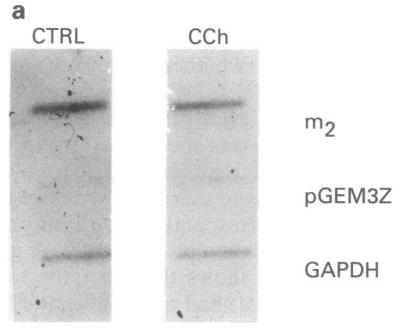


Figure 5 Degradation rate of m_2 muscarinic receptor mRNA. Cells were treated with vehicle or carbachol (1 mM) for 12 h. After this incubation period, cells were washed and actinomycin D (5 μg/ml^{-1}) was added for the time indicated: (■) actinomycin D; (□) carbachol plus actinomycin D. Messenger RNAs were extracted and evaluated for m_2 receptor expression by Northern blot. Data are the mean ± s.e.mean of three independent experiments.



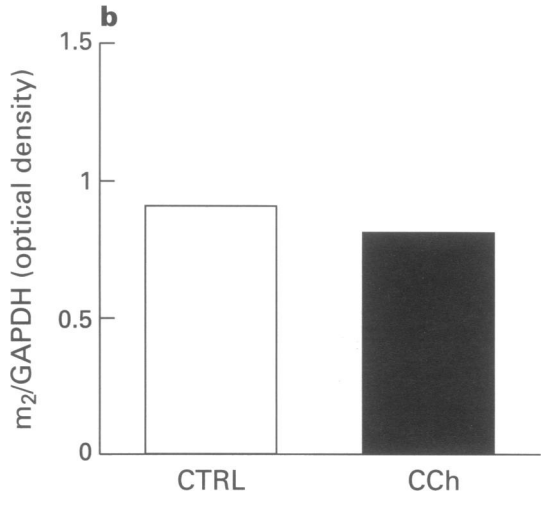


Figure 6 Relative transcription rate of m_2 muscarinic receptor gene in control (CTRL) and carbachol (CCh)-treated cells. (a) Nuclei were prepared 18 h after the addition of carbachol and incubated with [^{32}P]-UTP for 30 min at 27°C [^{32}P]-RNAs were isolated and hybridized to immobilized plasmid DNA (10 μg/slot) without any insert (pGEM3Z) or containing either rat GAPDH (GAPDH) or human m_2 muscarinic receptor cDNA (m_2) inserts. (b) Shows densitometric scanning of the autoradiograms. The rate of m_2 muscarinic receptor gene transcription is expressed relative to that of the GAPDH.

QNB to [^{3}H]-NMS binding sites was 1.2. This result suggests that muscarinic receptors in HEL 299 cells can be equally recognised by tertiary and quaternary antagonists. Similar results have been reported in SK-N-SH neuroblastoma cells (Fisher, 1988) and in M2LKB2-2 cells transfected with the m_2 muscarinic receptor gene (Wei *et al.*, 1994) where lipophilic and hydrophilic antagonists have been shown to label similar proportions of muscarinic receptors. Northern blot analysis on isolated messenger RNA revealed expression of the m_2-muscarinic receptor transcript with no evidence of other muscarinic receptor subtype mRNAs in HEL 299 cells. This result is in agreement with previous data obtained from the same cell line (Koman *et al.*, 1990; Rousell *et al.*, 1995).

Carbachol induced a time-dependent decrease in the number of muscarinic receptors measured with the hydrophilic ([^{3}H]-NMS) and the lipophilic ([^{3}H]-QNB) ligands without any change in the affinity of the remaining binding sites. This result suggests that the detected receptor down-regulation is due to a decrease in receptor density and not a result of the presence of residual agonist in the binding assay. The down-regulation of [^{3}H]-NMS binding sites in HEL 299 cells was rapid during the

first 2 h of carbachol stimulation (Figure 1). This first phase might represent internalised receptors. Indeed, at early time periods, the loss of the lipophilic ligand [^{3}H]-QNB binding sites occurs at a slower rate than does the loss of [^{3}H]-NMS binding sites as a result of receptor sequestration. At later time points, M_2 receptors down-regulate to a comparable extent with reduction of 40% for [^{3}H]-QNB binding and 56% for [^{3}H]-NMS binding. This result agrees with data obtained in human astrocytoma cell line (Harden *et al.*, 1985; Thompson & Fisher, 1990) and in a fibroblast cell line transfected with the m_2 muscarinic receptor gene (Wei *et al.*, 1994). The down-regulation seen after long-term carbachol treatment is probably due to receptor degradation triggered by prolonged carbachol occupancy. Functional desensitization was assessed by measurement of intracellular cyclic AMP accumulation following 24 h carbachol stimulation. The results show that the down-regulation was accompanied by uncoupling of the M_2 muscarinic receptors after carbachol treatment (Figure 3). This result suggests that functional desensitization may accompany receptor down-regulation in agreement with published data (Wei *et al.*, 1994).

Many reports have described the relationship between phosphorylation and receptor down-regulation (El-Fakahany & Cioffi, 1990; Hosey, 1992). Yang *et al.* (1993) using mutational analysis have suggested that certain threonine residues in the carboxyl terminal domain of the muscarinic m_3 receptor are required for receptor down-regulation. In human JEG-3 cell line expressing the porcine m_2 receptor, the substitution of cytoplasmic tail tyrosine residue attenuated the rate and extent of agonist-induced down-regulation without any effect on agonist-induced sequestration (Goldman & Nathanson, 1994). Several protein kinases have been shown to phosphorylate the muscarinic receptors (Haga *et al.*, 1993). In HEL 299 cells, the phorbol ester 4β-phorbol 12,13 dibutyrate (PDBu) caused a time-dependent decrease in the steady-state levels of m_2-receptor mRNA and [^{3}H]-NMS binding which were significantly inhibited by the specific PKC inhibitor GF-109203X (Rousell *et al.*, 1995). A role of PKC in the carbachol-induced down-regulation is unlikely since the profile of carbachol and PDBu-induced down-regulation of M_2 muscarinic receptors is quite different. This result would suggest that the mechanism underlying carbachol-induced down-regulation does not involve PKC. Similar results were obtained by Lai *et al.* (1990) in N1E-115 neuroblastoma cells and by Kopp *et al.* (1990) in HT-29 human colon carcinoma cells. An attractive and alternative candidate to PKC is the β-adrenoceptor kinase (βARK). It has been shown that human m_2 receptor serves as a substrate for the β-ARK1 (Kameyama *et al.*, 1993) and β-ARK2 isoforms (Richardson *et al.*, 1993) in an agonist-dependent manner. Moreover, in rat cerebellar granule cells, pretreatment with an antisense oligodeoxynucleotide against the mRNA encoding β-ARK attenuated the desensitization by carbachol (Contera *et al.*, 1993).

While a large body of data suggests that the rapid phase of carbachol-induced internalisation and desensitization of the receptors may be due to receptor phosphorylation, the mechanism of long-term carbachol-induced down-regulation remains to be elucidated. To assess whether the down-regulation of muscarinic M_2 receptors in HEL 299 cells is mediated through change in m_2 receptor gene transcription, the level of cellular m_2 mRNA was measured by Northern blot analyses following carbachol treatment. Over the time course investigated, no significant difference in the level of m_2 mRNA expression by carbachol was seen. Under the same conditions the level of m_2 mRNA expression was up-regulated by atropine or by the long-acting muscarinic antagonist Ba 679 BR (Haddad *et al.*, 1994) for 24 h (data not shown). Longer incubation periods up to 48 h did not produce any changes in m_2 mRNA. Few reports have investigated the effect of agonist treatment on the gene expression of muscarinic receptors. In cerebellar granule cells (Longone *et al.*, 1993; Fukamauchi *et al.*, 1993), chicken heart (Habecker & Nathanson, 1992) and CHO cells transfected with the m_1 receptor gene (Lee *et al.*, 1994) a decrease in the level of muscarinic receptor mRNAs was observed following carbachol treatment. Similar results were obtained *in vivo* following elevation of tissue acetylcholine levels by 10 day's injection with diisopropylfluorophosphate (Zhu *et al.*, 1991).

In HEL 299 cells, we were unable to demonstrate any changes in the level of m_2 muscarinic mRNA following carbachol treatment. The reasons for this discrepancy are not clear but may include the nature of the receptors present or the differential mechanisms of regulation of their expression. We have also investigated the effect of carbachol on m_2 muscarinic receptor mRNA stability. The results presented in Figure 5 show that the m_2 mRNA half-life was not affected by carbachol treatment. Similar results were obtained in IMR-32 cells where carbachol treatment had no effect on the steady state levels or stability of the m_3 receptor mRNA (Lee & Fraser, 1995). To determine directly whether carbachol induces a change in the transcription rate of the m_2 receptor gene, nuclear RNA run-on assay was carried out. The rate of muscarinic m_2 receptor gene transcription was normalized to that of the GAPDH gene and showed no difference between control and 18 h carbachol exposure. These data suggest that the decrease in the level of M_2 muscarinic receptors following prolonged carbachol treatment is not due to a decrease in the receptor synthesis as a consequence of the decrease in its mRNA but would result from an increase in the rate of receptor degradation triggered by prolonged agonist occupancy. In support of this conclusion, it has been shown that specific phosphorylation sites in the carboxyl tail are involved in the down-regulation of the mammalian m_2 (Goldman & Nathanson, 1994) and m_3 receptors (Yang *et al.*, 1993). The cellular mechanisms involved in this process are yet to be investigated.

In summary, we have shown that in human embryonic lung fibroblasts, prolonged carbachol exposure induced receptor desensitization as well as down-regulation which was not mediated through changes in the steady-state levels of m_2 mRNAs, stability of the message or alteration in the rate of transcription of the m_2 receptor gene.

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References

CAULFIELD, M.P. (1993). Muscarinic receptors, characterization, coupling and function. *Pharmacol. Ther.*, **58**, 319–379.

CONTERA, J.G., MCLESKEY, S.W., HOLOPAINEN, I., XU, J. & WOJCIK, W.J. (1993). Muscarinic m2 receptors in cerebellar granule cell cultures from rat: Mechanism of short-term desensitization. *J. Pharmacol. Exp. Ther.*, **265**, 433–440.

EL-FAKAHANY, E.E. & CIOFFI, C.L. (1990). Molecular mechanisms of regulation of neuronal muscarinic receptor sensitivity. *Memb. Biochem.*, **9**, 9–27.

FISHER, S.K. (1988). Recognition of muscarinic cholinergic receptors in human SK-N-SH neuroblastoma cells by quaternary and tertiary ligands is dependent upon temperature, cell integrity, and the presence of agonists. *Mol. Pharmacol.*, **33**, 414–422.

FUKAMAUCHI, F., SAUNDERS, P.A., HOUGH, C. & CHUANG, D-M. (1993). Agonist-induced down-regulation and antagonist-induced up-regulation of m2- and m3-muscarinic acetylcholine receptor mRNA and protein in cultured cerebellar granule cells. *Mol. Pharmacol.*, **44**, 940–949.

GOLDMAN, P.S. & NATHANSON, N.M. (1994). Differential role of the carboxyl-terminal tyrosine in down-regulation and sequestration of the m2 muscarinic acetylcholine receptor. *J. Biol. Chem.*, **269,** 15640–15645.

GREENBERG, M.E. & ZIFF, E.B. (1984). Stimulation of 3T3 cells induces transcription of the c-fos proto-oncogene. *Nature*, **311,** 433–438.

HABECKER, B.A. & NATHANSON, N.M. (1992). Regulation of muscarinic acetylcholine receptor mRNA expression by activation of homologous and heterologous receptors. *Proc. Natl. Acad. Sci. U.S.A.*, **89,** 5035–5038.

HADDAD, E.-B., MAK, J.C.W. & BARNES, P.J. (1994). Characterization of [^{3}H]BA 679 BR, a slowly dissociating muscarinic antagonist, in human lung: Radioligand binding and autoradiographic mapping. *Mol. Pharmacol.*, **45,** 899–907.

HAGA, T., HAGA, K., KAMEYAMA, K. & NAKATA, H. (1993). Phosphorylation of muscarinic receptors: Regulation by G proteins. *Life Sci.*, **52,** 421–428.

HARDEN, T.K., PETCH, L.A., TRAYNELIS, S.T. & WALDO, G.L. (1985). Agonist-induced alteration in the membrane form of muscarinic cholinergic receptors. *J. Biol. Chem.*, **260,** 13060–13066.

HOSEY, M.M. (1992). Diversity of structure, signaling and regulation within the family of muscarinic cholinergic receptors. *FASEB J.*, **6,** 845–852.

HULME, E.C., BIRDSALL, N.J.M. & BUCKLEY, N.J. (1990). Muscarinic receptor subtypes. *Annu. Rev. Pharmacol. Toxicol.*, **30,** 633–673.

KAMEYAMA, K., HAGA, K., HAGA, T., KONTANI, K., KATADA, T. & FUKADA, Y. (1993). Activation by G protein βγ subunits of β-adrenergic and muscarinic receptor kinase. *J. Biol. Chem.*, **268,** 7753–7758.

KOMAN, A., DURIEU-TRAUTMANN, O., COURAUD, P-O., STROSBERG, A.D. & WEKSLER, B.B. (1990). Modulation of muscarinic-receptor expression in human embryonic lung fibroblasts by platelet-derived growth factor. *Biochem. J.*, **270,** 409–412.

KOPP, R., MAYER, P. & PFEIFFER, A. (1990). Agonist-induced desensitization of cholinergically stimulated phosphoinositide breakdown is independent of endogenously activated protein kinase C in HT-29 human colon carcinoma cells. *Biochem. J.*, **269,** 73–78.

LAI, W.S., ROGERS, T.B. & EL-FAKAHANY, E.E. (1990). Protein kinase C is involved in desensitization of muscarinic receptors by phorbol esters but not by receptor agonists. *Biochem. J.*, **267,** 23–29.

LEE, N.H. & FRASER, C.M. (1995). Regulation of muscarinic receptor expression by changes in mRNA stability. *Life Sci.*, **56,** 899–906.

LEE N.H., EARLE-HUGHES, J. & FRASER, C.M. (1994). Agonist-mediated destabilization of m1 muscarinic acetylcholine receptor mRNA. Element involved in mRNA stability are localized in the 3′-untranslated region. *J. Biol. Chem.*, **269,** 4291–4298.

LONGONE, P., MOCCHETTI, I., RIVA, M.A. & WOJCIK, W.J. (1993). Characterization of a decrease in muscarinic m2 mRNA in cerebellar granule cells by carbachol. *J. Pharmacol. Exp. Ther.*, **265,** 441–446.

LOWRY, O.H., ROSEBOROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurement with the folin phenol reagent. *J. Biol. Chem.*, **193,** 265–275.

MALOTEAUX, J.M. & HERMANS, E. (1994). Agonist-induced muscarinic cholinergic receptor internalization, recycling and degradation in cultured neuronal cells. *Biochem. Pharmacol.*, **47,** 77–88.

MCKINNEY, M., STENSTROM, S. & RICHELSON, E. (1984). Muscarinic responses and binding in a murine neuroblastoma clone (N1E-115). *Mol. Pharmacol.*, **26,** 156–163.

MUNSON, P.J. & RODBARD, D. (1980). Ligand: a versitile computerized approach to characterization of ligand binding systems. *Anal. Biochem.*, **107,** 220–239.

RICHARDSON, R.M., KIM, C., BENOVIC, J.L. & HOSEY, M.M. (1993). Phosphorylation and desensitization of human muscarinic cholinergic receptors by two isoforms of the β-adrenergic receptor kinase. *J. Biol. Chem.*, **268,** 13650–13656.

ROUSELL, J., HADDAD, E.-B., MAK, J.C.W. & BARNES, P.J. (1995). Transcriptional down-regulation of m2 muscarinic receptor gene expression in human embryonic lung (HEL 299) cells by protein kinase C. *J. Biol. Chem.*, (in press).

THOMPSON, A.K. & FISHER, S.K. (1990). Relationship between agonist-induced muscarinic receptor loss and desensitization of stimulated phosphoinositide turnover in two neuroblastomas: methodological considerations. *J. Pharmacol. Exp. Ther.*, **252,** 744–752.

WEI, H.-B., YAMAMURA, H.I., & ROESKE, W.R. (1994). Down-regulation and desensitization of the muscarinic M_1 and M_2 receptors in transfected fibroblast B82 cells. *Eur. J. Pharmacol.*, (*Mol. Pharamcol. Section*), **268,** 381–391.

WESS, J. (1993). Molecular basis of muscarinic acetylcholine receptor function. *Trends Pharmacol. Sci.*, **14,** 308–313.

YANG, J., LOGSDON, C.D., JOHANSEN, T.E. & WILLIAMS, J.A. (1993). Human m3 muscarinic acetylcholine receptor carboxyl-terminal threonine residues are required for agonist-induced receptor down-regulation. *Mol. Pharmacol.*, **44,** 1158–1164.

ZHU, S.-Z., WANG, S.-Z., ABDALLAH, E.A.M. & EL-FAKAHANY, E.E. (1991). DFP-induced regulation of cardiac muscarinic receptor mRNA in vivo measured by DNA-excess solution hybridization. *Life Sci.*, **48,** 2579–2584.

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