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Agonist-Induced Down-Regulation and Antagonist-Induced Up-Regulation of m_2- and m_3-Muscarinic Acetylcholine Receptor mRNA and Protein in Cultured Cerebellar Granule Cells

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SUMMARY

Cerebellar granule cells express m_2- and m_3-muscarinic acetylcholine receptors (mAChRs) and their corresponding mRNA with m_3-mAChR being the predominant receptor subtype. After stimulation with the mAChR agonist, carbachol, m_2- and m_3-mAChR mRNA levels were decreased in a time- and concentration-dependent manner with the maximal down-regulation at 2 and 8 hr, respectively. Immunoprecipitation studies revealed that amounts of m_2- and m_3-mAChR protein also decreased at 8 and 24 hr, respectively. The carbachol-induced down-regulation of m_3-mAChR mRNA was associated with a decrease in the transcription rate, but a substantial enhancement of the mRNA stability. Upon removal of carbachol after treatment for 8 hr, the levels of m_3-mAChR mRNA and mAChR binding sites returned to their original values with a $t_{1/2}$ of approximately 80 min and 6 hr, respectively. The carbachol-elicited loss of m_2- and m_3-mAChR mRNA was blocked by their corresponding receptor subtype-specific antagonists, AF-DX 116 (m_2-selective) and 4-diphenylacetoxy-*N*-methylpiperidine methiodide (4-DAMP) (m_3-selective), and was concurrent with an increase in c-*fos* mRNA levels. Exposure of granule cells to the nonselective mAChR antagonist, atropine, caused a time- and concentration-dependent increase in the level of both m_2- and m_3-mAChR mRNA and mAChR binding sites. At 24 hr, immunoprecipitable m_3-mAChR protein was predominantly increased. The atropine-induced up-regulation of m_3-mAChR mRNA was concurrent with a marked enhancement of the mRNA stability and its transcription rate. The elevated levels of m_3-mAChR mRNA and binding sites declined to their untreated values after the removal of atropine. Treatment with AF-DX 116 and 4-DAMP also produced an increase in the level of m_2- and m_3-mAChR mRNA and their corresponding immunoprecipitable receptor protein. These results demonstrate that the mAChR agonist and antagonist induce a down- and up-regulation of mAChR expression, respectively, through receptor-mediated mechanisms in cerebellar granule cells. Moreover, at least for m_3-mAChR mRNA, the agonist- and antagonist-induced effects are reversible and associated with corresponding changes in the transcription rate of this receptor mRNA species.

The mAChR mediates many of the actions of acetylcholine in the central nervous system. The mAChR belongs to a large superfamily of structurally related G-protein-coupled membrane receptors linked to several effector systems. Molecular cloning studies have revealed the existence of at least five different mAChR subtypes designated as m_1, m_2, m_3, m_4, and m_5 (1–3). These receptor subtypes have distinct pharmacological properties, mRNA sizes, tissue distributions, and second messenger coupling (see Refs. 4–8 for a review). It is believed that m_1-, m_3-, and m_5-mAChRs are predominantly coupled to phosphoinositide hydrolysis, arachidonic acid release, cAMP elevation, and M-current inhibition (9–12), whereas m_2- and m_4-mAChRs mediate the inhibition of adenylate cyclase activity (11, 12).

mAChRs are known to be modulated *in vivo* by treatments with receptor agonists and antagonists. For example, chronic infusion of mice with the agonist, oxotremorine, results in the development of tolerance to mAChR-mediated behavioral responses and the subsequent loss of mAChR binding sites in several brain regions (13). Chronic inhibition of acetylcholinesterase by injection of diisopropylfluorophosphate also reduces the number of mAChR binding sites in the rat brain (14). Conversely, repeated administration of an mAChR antagonist, atropine, significantly increases the number of mAChR binding sites in the neocortex and hippocampus (15). Because of the complexity of neuronal circuits and the heterogeneity of cell types in the brain, primary cultures of neurons and neuronal cell lines (16–18) have been widely used as models for studying the mechanisms underlying the *in vivo* effects of drugs on the adaptation of mAChRs.

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ABBREVIATIONS: mAChR, muscarinic acetylcholine receptor; QNB, quinuclidinyl benzilate; AF-DX 116, 11-[[2-[(diethylamino)-methyl]-1-piperidinyl] acetyl]-5,11-dihydro-6*H*-pyrido[2,3-*b*][1,4]benzodiazepin-6-one; 4-DAMP, 4-diphenylacetoxy-*N*-methylpiperidine methiodide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; kb, kilobase; PSS, physiological saline solution; SDS, sodium dodecyl sulfate.

Primary cultures of cerebellar granule cells are an almost homogeneous population of neurons (purity >92%) when prepared from 8-day-old rats (19). *In vitro* these cells differentiate into glutamatergic neurons (20), and express m_2- and m_3-mAChR mRNAs (21) and their corresponding receptors (22) which are coupled to adenylate cyclase (23) and phosphoinositide hydrolysis (24), respectively. Stimulation with the mAChR agonist, carbachol, results in time-dependent desensitization of carbachol-induced phosphoinositide hydrolysis and a decrease in mAChR binding sites which follows the onset of desensitization (24, 25). In a preliminary report, we showed that stimulation of cerebellar granule cells with carbachol induced a time-dependent loss of m_2- and m_3-mAChR mRNAs (21); these effects were blocked by atropine which, by itself, induced a significant increase in m_3-mAChR mRNA levels. In this study, we used this primary neuronal culture to further characterize these phenomena and explore possible molecular mechanisms underlying the agonist- and antagonist-induced changes in m_2- and m_3-mAChR mRNA levels. We have also performed quantitative immunoprecipitation of mAChRs using subtype-specific antibodies to compare the effects of agonist and antagonist treatment on receptor protein levels to the changes that we observed in mRNA levels.

Experimental Procedures

Materials. [^{3}H]QNB (44.3 Ci/mmol) and [^{32}P]UTP (3000 Ci/mmol) were purchased from New England Nuclear (Boston, MA). 4-DAMP was obtained from Research Biochemicals Inc. (Natick, MA). AF-DX 116 was kindly donated by Boehringer Ingelheim Co. (Ridgefield, CO). Tissue culture reagents were products from GIBCO Laboratories (Grand Island, NY). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted. The antisera selective for m_1-, m_2-, m_3-, m_4-, or m_5-mAChRs were kindly supplied by Dr. Barry B. Wolfe, Department of Pharmacology, Georgetown University School of Medicine. The m_3-mAChR antibody was directed against a synthetic peptide coding for an 18 amino acid domain of the carboxyl-terminal sequence (26). The m_1-, m_2-, m_4-, and m_5-mAChR antibodies were directed against the third intracellular loop of these mAChRs (27–29).

Cell culture. Primary cultures were prepared from 8-day-old Sprague-Dawley rats as described by Gallo *et al.* (30). Briefly, cerebella from 20 rats were pooled and chopped into 400-μm cubes. Cells were dissociated by trypsinization (0.025%) at 37° for 15 min and then triturated in Krebs-Ringer bicarbonate buffer containing 0.01% DNase and 0.05% soybean trypsin inhibitor. The dissociated cells were resuspended in basal modified Eagle's medium containing 10% fetal calf serum, 2 mM glutamine, 50 μg/ml gentamicin, and 25 mM KCl, and then plated onto 35-mm (3×10^6 cells) or 60-mm ($9–10 \times 10^6$ cells) Costar dishes precoated with poly-L-lysine. After 24 hr in culture at 37° in an atmosphere of humidified air containing 6% CO_2, β-cytosine arabinoside (10 μM) was added to inhibit the replication of non-neuronal cells.

Drug treatments. After 8 days in culture, cells were treated with the indicated drugs by the addition of at least a 100-fold concentrated stock solution to the culture medium. Control cultures always received the vehicle used to dissolve the drug. For time-course studies, drugs were added sequentially such that all cultured cells were harvested at the same time on the 8th day *in vitro* for quantification of mRNA and mAChR protein (see below).

Northern blot hybridization. Cells grown in 60-mm dishes were lysed with a solution containing guanidinium thiocyanate and total RNA was isolated by centrifugation through a 5.7 M cesium chloride gradient, as described by Chirgwin *et al.* (31). An aliquot of one-tenth of each sample was reserved for electrophoresis on 1% agarose gels for quantification of total cellular RNA. For this purpose, the RNA was allowed to migrate approximately 5 mm from the sample well, and the total RNA was quantified by image analysis (using software developed by Wayne Rasband at the National Institute of Mental Health), or by laser densitometry (LKB Bromma, Sweden) of photographic negatives of the ethidium bromide-stained RNA bands. The remaining RNA was denatured, electrophoresed on 1% agarose-formaldehyde gels, and then blotted to nitrocellulose. The RNA blot was prehybridized at 42° to decrease nonspecific binding, and then hybridized with ^{32}P-labeled fragments from mAChR cDNA clones (1×10^6 cpm/ml) (m_2-, 0.5-kb *Ava*I fragment of human Hm2p9; m_3-, 0.7-kb *Stu*I-*Nhe*I fragment of rat Rm3p8), generously provided by Dr. Tom I. Bonner (Laboratory of Cell Biology, National Institute of Mental Health, Bethesda, MD). These fragments constitute a portion of the i3 region, the large cytoplasmic loop between transmembrane regions 5 and 6 of these receptors. Hybridization was also performed using cDNA probes for murine c-*fos* or chicken β-actin; both were gifts from Dr. Craig B. Thompson (Howard Hughes Medical Institute, Ann Arbor, MI). The same RNA blot was routinely hybridized with the probes for m_2-mAChR, m_3-mAChR, c-*fos*, and β-actin. Washing was performed three times with 2 × SSC (1 × SSC = 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0) containing 0.1% SDS for 5 min at room temperature, followed by two or more high stringency washes at 56° for 15 min in 0.1 × SSC containing 0.1% SDS in the case of m_3-mAChR, c-*fos*, and β-actin, but at 48° for the human m_2-mAChR. Quantification of the specific radiolabeled bands was performed with a βetagen betascope blot analyzer (Waltham, MA), by laser densitometry, or by an image analysis of autoradiograms. Levels of mAChR mRNAs have been normalized to total cellular RNA and to the appropriate control group as indicated.

Measurement of run-off gene transcription in isolated nuclei. Cells from a 75-cm^2 flask were used for the isolation of nuclei. Nuclei were prepared as described by Greenberg *et al.* (32). The isolated nuclei were resuspended in 50 mM Tris-HCl, pH 8.3, 40% glycerol, 5 mM $MgCl_2$, and 0.1 mM EDTA, and stored at −70°. Nuclei (210 μl) were incubated for 30 min at 30° with 300 μCi of [^{32}P]UTP, 0.28 mM ATP, GTP, and CTP in a buffer of 44 mM Tris-HCl, pH 8.3, 6.67 mM $MgCl_2$, 167 mM KCl, and 15.6 mM 2-mercaptoethanol. Total RNA was then isolated by the method of Chirgwin *et al.* (31). The radiolabeled RNA pellet ($1–2 \times 10^7$ cpm) was dissolved in 1 ml of hybridization solution (0.1% bovine serum albumin, 0.1% ficoll, 0.1% polyvinylpyrrolidone, 250 μg/ml yeast tRNA, 25 mM sodium phosphate buffer, pH 6.7, 0.1% SDS, 1 mM EDTA, 50% formamide in 5 × SSC) and hybridized at 42° for 48 hr with 5 μg of the pSP64 plasmid immobilized onto a nitrocellulose membrane (as a control) or with an immobilized plasmid containing inserts of the rat m_3-mAChR cDNA or the chicken β-actin cDNA. The filters were washed with 2 × SSC and 0.1% SDS at 42° for 1–2 hr and autoradiographed.

mAChR binding assays. Eight-day-old cerebellar granule cells grown in 35-mm dishes were washed three times with 1 ml of physiological saline solution (PSS) (118 mM NaCl, 4.7 mM KCl, 3.0 mM $CaCl_2$, 1.2 mM MgCl, 1.2 mM KH_2PO_4, 0.5 mM EDTA, 10 mM glucose, and 20 mM HEPES, pH 7.4) and then incubated at 37° for 75 min in 1 ml of PSS containing 0.5 nM [^{3}H]QNB. The binding reaction was terminated by aspirating the reaction mixture followed by three rapid washings with 2 ml of PSS; 0.5 ml of 3% SDS was then added to solubilize cellular proteins. The protein solutions were transferred to vials, scintillation mixture added, and the radioactivity was counted. Nonspecific binding was measured in the presence of 1 μM atropine and was less than 10% of total binding.

Immunoprecipitation assays. Procedures for immunoprecipitation of [^{3}H]QNB-labeled mAChRs solubilized from membranes were essentially as described by Wall *et al.* (27). Briefly, cerebellar granule cells grown in 150-mm dishes were treated with drugs as indicated. Cells were then washed four times with prewarmed PSS and lysed in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.4). The isolated membranes were incubated with 2.0 nM [^{3}H]QNB for 45 min at 32° in TE buffer containing the protease inhibitor mixture (27). The labeled mAChRs were then solubilized with TE buffer containing 1% digitonin

and 0.2% cholic acid. The solubilized receptors (25–200 fmol of [^{3}H]QNB-labeled receptors or 2.5–20 × 10^3 dpm) were incubated with mAChR subtype-specific antisera (0.6 mg of protein/ml) in TE buffer for 48 hr at 4°. Bound [^{3}H]QNB was separated from free [^{3}H]QNB by Sephadex G-50 column chromatography, and the labeled receptor-antibody complex was isolated by incubation with Pansorbin for 2 hr at 4°. The percentage of immunoprecipitation was determined by dividing the amounts of tritium detected in Pansorbin pellet by the sum of the tritium in the supernatant and pellet. Nonspecific immunoprecipitation was performed using preimmune serum and was less than 1% of total labeled mAChRs. Confirming the reported selectivity of these antibodies (26, 28), we found that m_2- and m_3-receptor antisera completely precipitated [^{3}H]QNB-labeled receptors solubilized from A9 cells transfected with m_2- and m_3-receptor genes, respectively, whereas these antisera cross-precipitated less than 1% of the other receptor subtype derived from the transfected cells (data not shown).

Results

Agonist-induced changes in mAChR mRNAs. To assess the relative proportion of m_2- and m_3-mAChR binding sites expressed in cerebellar granule cells, specific binding of [^{3}H]QNB to mAChRs in intact cells was displaced by increasing concentrations of m_2-selective AF-DX 116 or m_3-selective 4-DAMP. Both drugs displaced [^{3}H]QNB binding in a biphasic manner (Fig. 1). The displacement curves were fitted to binding equations for two independent binding sites, using the Marquardt-Levenberg polynomial fitting algorithm provided by the program Sigma plot. At receptor subtype-selective concentrations, maximal displacement of [^{3}H]QNB binding by AF-DX 116 (1 μM) and 4-DAMP (0.1 μM) were approximately 20% and 80% of the total, respectively. These results indicate that m_3-mAChR is the predominant receptor subtype expressed in these cerebellar neurons. The levels of m_2- and m_3-mAChR mRNAs were determined by Northern blot hybridization. Under high stringency wash conditions, ^{32}P-labeled fragments of m_2- and m_3-mAChR cDNAs remained hybridized to an mRNA band of 5.2 and 4.5 kb, respectively, and the degree of hybridization was linear with 16 μg or more of total RNA (data not shown). The amount of total RNA used in our hybridization studies was routinely 7–8 μg, which was well within the linear range of the m_2- and m_3-mAChR cDNA probes for their corresponding mRNAs.

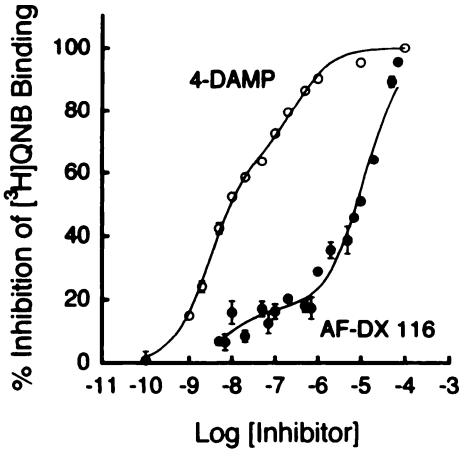

Fig. 1. Displacement of [^{3}H]QNB binding to intact cerebellar granule cells by 4-DAMP and AF-DX 116. Binding conditions were as described in Experimental Procedures except that 4-DAMP and AF-DX 116 were included at the indicated concentrations to displace [^{3}H]QNB binding. Specific binding was obtained by subtracting nonspecific binding (measured in the presence of 10 μM atropine) from total binding and was approximately 90% of total binding. Specific binding of the control was 263 fmol/dish of 3 × 10^6 cells. The specific activity of [^{3}H]QNB was 105 dpm/fmol. The displacement curves were fitted to binding equations for two independent binding sites using the Marquardt-Levenberg polynomial fitting algorithm provided by Sigma plot 4.0 (Jandel Scientific, Corte Madera, CA). The K_i values for 4-DAMP were 5.23 × 10^{-10} and 5.51 × 10^{-8} M for KiH (m_3) and KiL (m_2), respectively, with an m_3/m_2 selectivity ratio of 105. The K_i values for AF-DX 116 were 1.05 × 10^{-9} M and 1.65 × 10^{-6} M for KiH (m_2) and KiL (m_3), respectively, with an m_2/m_3 selectivity ratio of 1571. The *error bars* indicate the range of duplicate sample from a representative experiment. The error bars for many of the points are not visible because they are within the symbols. The experiment has been repeated three times with similar results.

As we have previously reported (21), stimulation of cerebellar granule cells with carbachol resulted in a time-dependent loss of m_2- and m_3-mAChR mRNAs (Fig. 2). The maximal decrease of m_2- and m_3-mAChR mRNA occurred at 2 and 8 hr after carbachol stimulation, respectively. After stimulation with carbachol for 2 hr, the level of m_2-mAChR mRNA was decreased in a concentration-dependent manner with an EC_{50} of 5 ± 0.4 μM (n = 3), whereas m_3-mAChR mRNA levels remained unchanged in the concentration range of carbachol (10^{-6} to 10^{-2} M) tested (Fig. 3A). To investigate whether a change in the mRNA level of the proto-oncogene c-*fos* accompanied the down-regulation of m_2- and m_3-mAChR mRNAs, we also measured c-*fos* mRNA levels in the same blot derived from carbachol-treated cultures. A correlation between m_2-mAChR mRNA down-regulation and the up-regulation of c-*fos* mRNA (2.2 kb) was observed. Treatment with carbachol for 8 hr resulted in a concentration-dependent increase of c-*fos* mRNA as well as a decrease of both m_2- and m_3-mAChR mRNA. Total cellular RNA and β-actin mRNA were unchanged under these experimental conditions (Fig. 3B).

To investigate whether carbachol-induced m_3-mAChR mRNA down-regulation was associated with changes in mRNA stability, cells were pretreated with 100 μM carbachol for 2 hr, actinomycin D was then added to arrest further RNA synthesis,

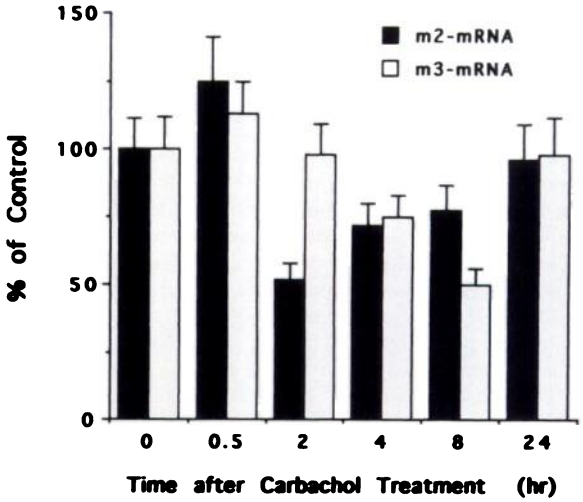

Fig. 2. Time course of carbachol-induced changes in mAChR mRNAs. Cells after 8 days in culture were treated with 100 μM carbachol for the indicated times. Levels of mAChR mRNA were then measured by Northern blot hybridization. The mRNA results have been normalized to total RNA at each time point, although their total RNA levels did not vary significantly. The same blot was used for hybridization to m_2- and m_3-mAChR cDNA probes. The data presented are the means ± standard error the means of three independent experiments performed with duplicate measurements. The data are expressed as change from the 0 time point at which drug was added.

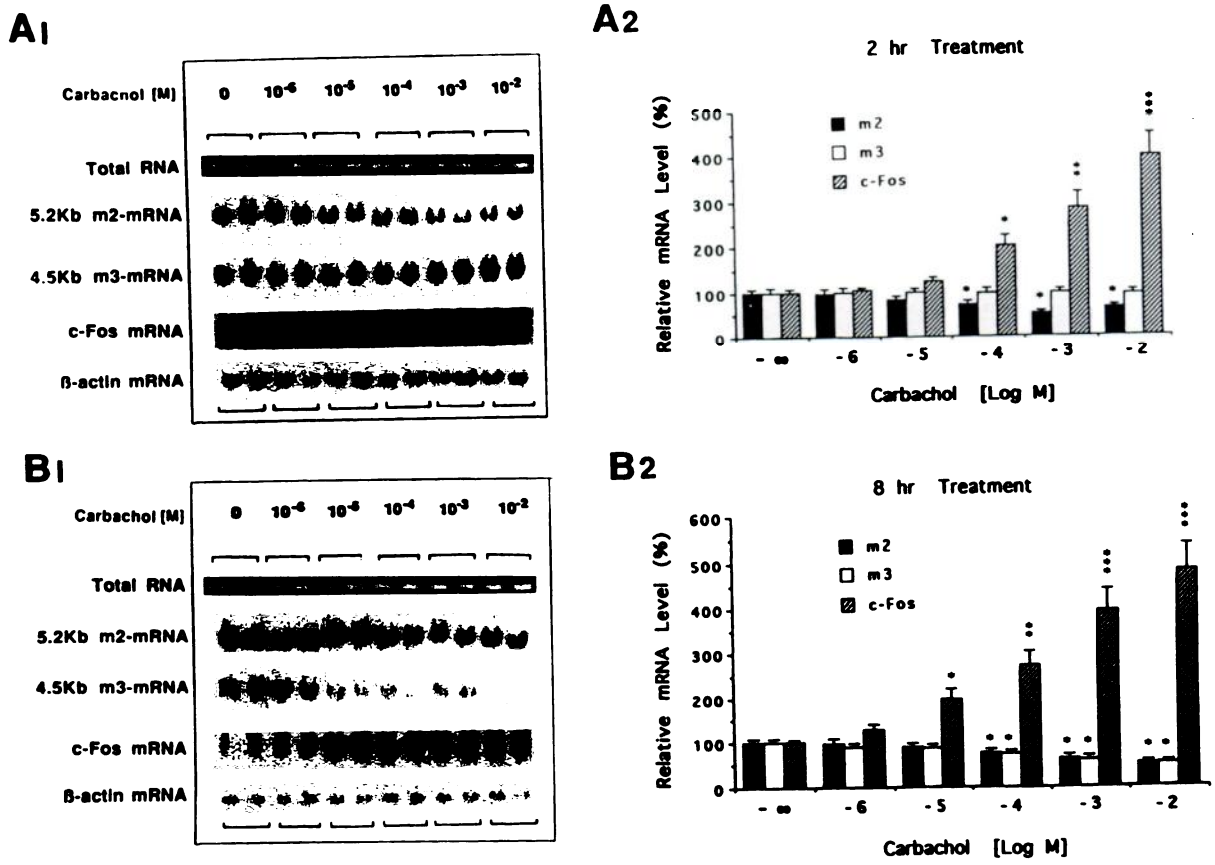

Fig. 3. Dose-effect relationship for carbachol-induced changes in mRNA. Cells were treated with the indicated concentrations of carbachol for either 2 hr (A) or 8 hr (B). mRNA levels for c-*fos*, m_2-mAChR, m_3-mAChR, and β-actin were then determined. The mRNA results were derived from the same RNA blot and have been normalized to total RNA in each sample. The RNA blots are shown in A1 and B1. The results in A2 and B2 are quantified data representing the means ± ranges of duplicate samples from a typical experiment which has been repeated three times with nearly identical results. *$p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, when compared with their untreated control group (Student's *t* test).

and the remaining m_3-mAChR mRNA was quantified at various times after actinomycin D treatment. The level of m_3-mAChR mRNA declined with a half-life ($t_{1/2}$) of approximately 40 min in untreated cells and about 80 min in carbachol-treated cells (Fig. 4A). Treatment with both cycloheximide and carbachol markedly enhanced the stability, suggesting that protein synthesis is involved in m_3-mAChR mRNA degradation. Moreover, the nonselective mAChR antagonist atropine (1 μM) also enhanced the m_3-mAChR mRNA stability with a $t_{1/2}$ of approximately 180 min (Fig. 4B).

To determine directly whether agonist-induced down-regulation of m_3-mAChR mRNA is due to a decreased transcriptional rate, nuclear RNA run-off analyses were performed. The results of these experiments were normalized to the rate of transcription for the β-actin gene, which remained virtually constant. The rate of m_3-mAChR gene transcription was decreased to 59.3 ± 6.1%, and increased to 230 ± 29% ($n = 3$) in nuclei from cells treated with carbachol (100 μM) or atropine (1 μM) for 8 hr, respectively (Fig. 5). These results indicate that the decrease in the abundance of m_3-mAChR mRNA following carbachol stimulation is primarily the result of a decrease in the rate of transcription rather than an increase in the degradation rate.

The reversibility of carbachol-induced down-regulation of mAChR mRNA and binding sites was examined (Fig. 6). Cells were pretreated with carbachol for 8 hr to maximally decrease m_3-mAChR mRNA. The agonist was then removed and cells were cultured in the agonist-free medium. At 0 time after drug removal, m_3-mAChR mRNA and mAChR binding sites were down-regulated by approximately 34% and 48%, respectively, while m_2-mAChR mRNA was only marginally decreased. In a time-dependent manner, m_3-mAChR mRNA and mAChR binding sites were restored to their original levels with a $t_{1/2}$ of approximately 80 min and 6 hr, respectively.

Antagonist-induced up-regulation of mAChR mRNAs. Exposure of cerebellar granule cells to atropine (1 μM) resulted in a time-dependent increase in the levels of both m_2- and m_3-mAChR mRNA and mAChRs assessed by [^{3}H]QNB binding to granule cell monolayers (Fig. 7A). At 8 hr, the levels of m_2-mAChR mRNA, m_3-mAChR mRNA, and mAChR binding sites were approximately 140, 200, and 235% of the control, respectively, and the up-regulation was sustained for at least until 72 hr after atropine treatment. The levels of all three parameters were increased in parallel by an 8-hr treatment with increasing concentrations of atropine (1–1000 nM) (Fig. 7B). Higher concentrations of atropine failed to produce a further increase (data not shown). The m_2-mAChR-selective antagonist AF-DX 116 and the m_3-mAChR-selective blocker 4-DAMP were examined for their ability to up-regulate mAChR mRNAs. AF-DX 116 (1 μM) induced an increase in m_2- but not m_3-mAChR mRNA levels, whereas 4-DAMP (0.1 μM) up-regulated m_3- but not m_2-mAChR mRNA levels after treatment of cells for 8 hr

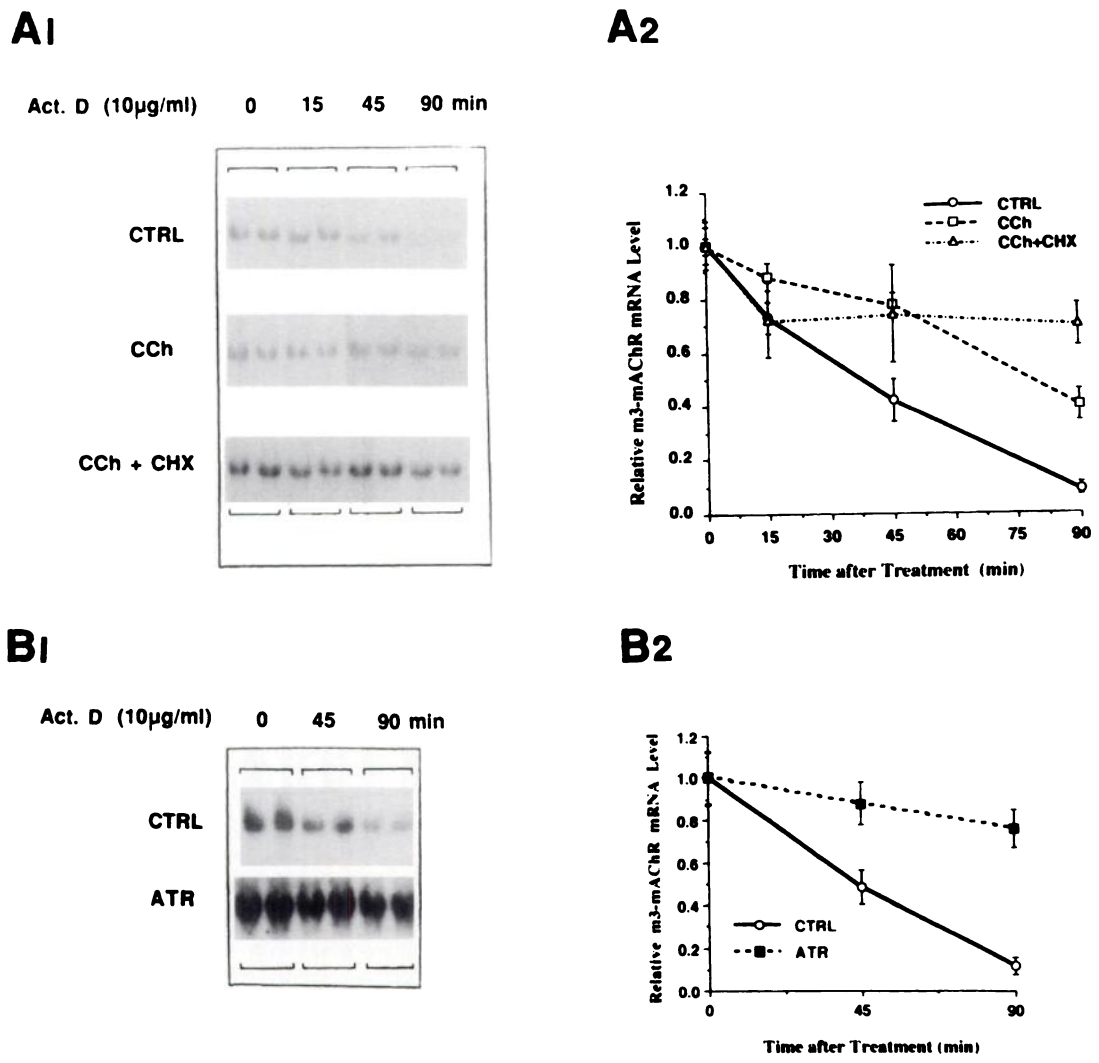

Fig. 4. Measurement of the stability of m_3-mAChR mRNA in cerebellar granule cells. A, Cells were cultured for 2 hr in the absence or presence of 100 μM carbachol (*CCh*) or CCh + 10 μM cycloheximide (*CHX*). Actinomycin D (*Act. D*) (10 μg/ml) was then added to the culture medium, and m_3-mAChR mRNA was quantified after the indicated times. Data have been normalized to amounts of total RNA in each lane and are expressed as mRNA levels relative to the time at which actinomycin D was added. Please note that the pretreatment with carbachol in the presence of cycloheximide markedly up-regulated m_3-mAChR mRNA. B, Cells were cultured for 8 hr in the absence or presence of 1 μM atropine (*ATR*). Actinomycin D was then added to the culture medium, and m_3-mAChR mRNA was quantified at indicated times. Pretreatment with atropine markedly increased the level of m_3-mAChR mRNA. Data shown are the means ± ranges of duplicated samples from an experiment which has been repeated twice with nearly identical results.

(Fig. 8). AF-DX 116- and 4-DAMP-induced up-regulation of mAChR mRNAs was associated with no significant change in c-*fos* mRNA levels, even though carbachol treatment produced an increase in c-*fos* mRNA at this time point (Fig. 8). In addition, AF-DX 116 completely blocked carbachol-induced down-regulation of m_2-mAChR mRNA and partially attenuated the carbachol-elicited c-*fos* mRNA increase. In contrast, 4-DAMP completely blocked carbachol-induced down-regulation of m_3-mAChR mRNA as well as the up-regulation of c-*fos* mRNA. AF-DX 116 did not inhibit carbachol-induced m_3-mAChR mRNA down-regulation and 4-DAMP did not affect the m_2-mAChR mRNA down-regulation, indicating again the selectivity of these two subtype-specific muscarinic antagonists. To determine the recovery of mAChR mRNA and binding site levels after atropine treatment, cells were pretreated with 1 μM atropine for 8 hr to up-regulate mAChR mRNAs and binding sites followed by the removal of the antagonist. The up-regulated m_3-mAChR mRNA and mAChR sites (and to a much lesser extent m_2-mAChR mRNA) declined to their control values between 8 and 24 hr after the removal of atropine (Fig. 9).

Agonist- and antagonist-induced regulation of mAChR proteins. The technique of immunoprecipitation developed by Wolfe and co-workers (26–29) was employed to quantify m_2- and m_3-mAChR protein in cerebellar granule cells after drug treatment. Antisera selective for m_2- and m_3-mAChRs immunoprecipitated their expected [^{3}H]QNB-labeled receptor protein solubilized from granule cell membranes. This immunoprecipitation was dependent on the concentration of m_2- and m_3-receptor antisera with the m_3-receptor antisera being more potent. At the saturating concentration of both antisera (0.6 mg/ml), 24.18 ± 6.80% and 74.19 ± 14.30% of the total labeled mAChRs were immunoprecipitated by the m_2- and m_3-selective antisera, respectively (data not shown). Moreover,

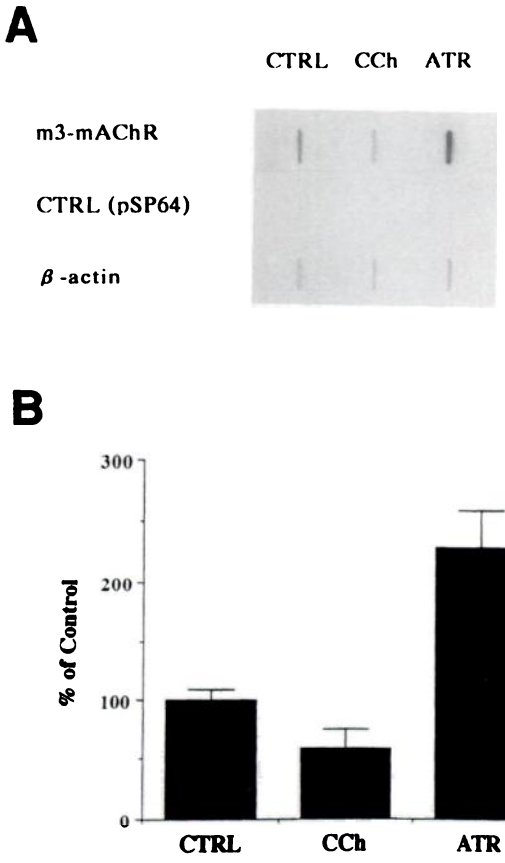

Fig. 5. Relative transcription rate of the m_3-mAChR gene in control (*CTRL*), carbachol-, or atropine-treated cerebellar granule cells assessed by nuclear run-off transcription assays. A, Nuclei were prepared 8 hr after addition of carbachol (100 μM) or atropine (1 μM) and incubated with [^{32}P]UTP for 30 min at 30°. The ^{32}P-RNA was isolated and hybridized to plasmid DNAs (5 μg/slot) containing either m_3-mAChR or β-actin cDNA inserts, or to the plasmid DNA without any insert (pSP64). B, Densitometry of autoradiograms. m_3-mAChR transcription rates are expressed relative to the β-actin transcription rate which was not significantly changed by carbachol or atropine treatment. The data shown are means ± standard error of three independent experiments.

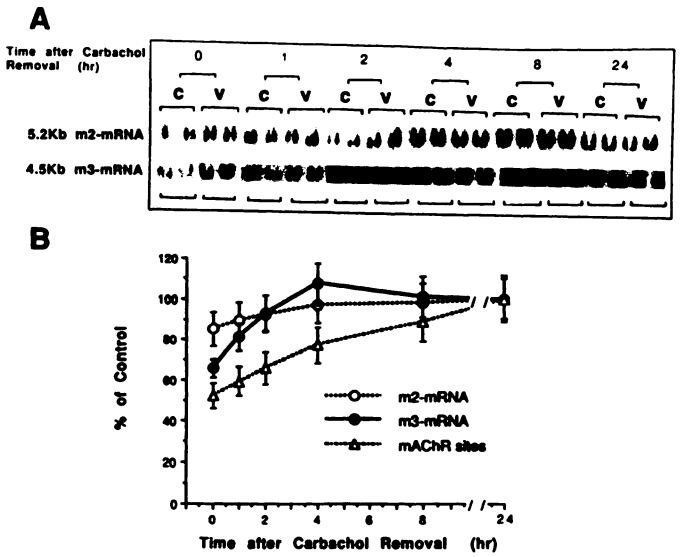

Fig. 6. Recovery of carbachol-induced down-regulation of mAChR mRNA and binding sites. Cells were pretreated with 100 μM carbachol (*C*) or vehicle (*V*) for 8 hr, washed three times to remove the agonist, and then fresh growth medium was added. The growth medium contained 10% fetal calf serum dialyzed to prevent neurotoxicity from glutamate present in the nondialyzed serum. At various time points after removal of the agonist, m_2-mAChR mRNA, m_3-mAChR mRNA, and mAChR binding sites were quantified. Please note that the 8-hr carbachol pretreatment resulted in a down-regulation of m_2-mAChR mRNA, m_3-mAChR mRNA, and mAChR binding sites by 15, 34, and 48%, respectively. The RNA blots are shown in A. The result in B are quantified data expressed as the means ± ranges of the untreated control of duplicate determinations. The mRNA results were always normalized to total RNA in each sample. The experiment was repeated three times with similar results. The 100% value for mAChR binding sites was 251 fmol/dish of 3×10^6 cells.

antisera selective for m_1-, m_4-, and m_5-mAChRs did not significantly precipitate labeled receptors derived from granule cells.

To examine whether carbachol-induced differential down-regulation of m_2- and m_3-mAChR mRNA resulted in corresponding changes in the receptor proteins, immunoprecipitation assays were performed using cells treated with the agonist (Fig. 10). Total [^{3}H]QNB-labeled mAChR was decreased to approximately 71% and 58% of the untreated value at 8 and 24 hr after carbachol treatment, respectively. Immunoprecipitable m_2-mAChR protein was decreased at 8 hr (from 24.31 ± 3.81% to 10.27 ± 2.82% of the total labeled receptors in untreated cells), but returned to the untreated value at 24 hr (23.01 ± 3.92%). In contrast, m_3-mAChR protein was virtually unaffected at 8 hr (from 72.52 ± 7.4% to 61.80 ± 7.23%) but markedly decreased at 24 hr (35.86 ± 6.73%). The effects of antagonist treatment for 24 hr on mAChR protein levels were also investigated using immunoprecipitation. Atropine treatment significantly increased the level of m_3-receptor protein (from 75.20 ± 12.61% to 126.96 ± 24.99% of the total labeled receptors in untreated cells); however, the increase in m_2-receptor protein (from 23.23 ± 2.55% to 35.72 ± 4.55%) did not reach statistical significance (Fig. 11A). Treatment with the m_2-receptor selective AF-DX 116 markedly increased m_2-receptor levels (from 22.97 ± 3.54% to 56.71 ± 8.23%), but did not affect m_3-receptor protein (from 74.37 ± 10.02% to 78.7 ± 10.09%) (Fig. 11B). Conversely, the m_3-selective 4-DAMP up-regulated m_3-receptor protein (from 73.09 ± 11.06% to 124.66 ± 19.51%) with no significant change of m_2-receptor protein levels (22.88 ± 5.03% to 24.87 ± 5.00%) (Fig. 11C).

Discussion

In this study we have demonstrated that carbachol-induced down-regulation of m_2- and m_3-mAChR mRNAs in cerebellar granule cells was time- and concentration-dependent (Figs. 2 and 3). This down-regulation was associated with a reduction of mAChR binding sites (21) and was blocked by the m_2- and m_3-mAChR-selective antagonists (Fig. 8). Immunoprecipitation studies revealed that m_2- and m_3-mAChR protein was decreased at 8 and 24 hr, respectively, after carbachol stimulation (Fig. 10). It is noteworthy that at 8 hr m_3-mAChR mRNA was markedly down-regulated, while the down-regulated m_2-mAChR mRNA was returning toward the untreated level. Thus the loss of m_2- and m_3-mAChR protein follows the down-regulation of their respective receptor mRNA. This delay in changes in receptor protein as compared with mRNA may reflect both the inherent difference in stabilities of mAChR protein and mRNA and the lag in time between translation and expression of fully functional receptor protein. The recovery of m_2- but not m_3-mAChR protein at 24 hr could be due to more rapid down-regulation and subsequent recovery of m_2-receptor mRNA (Fig. 2). The down-regulation of mAChR

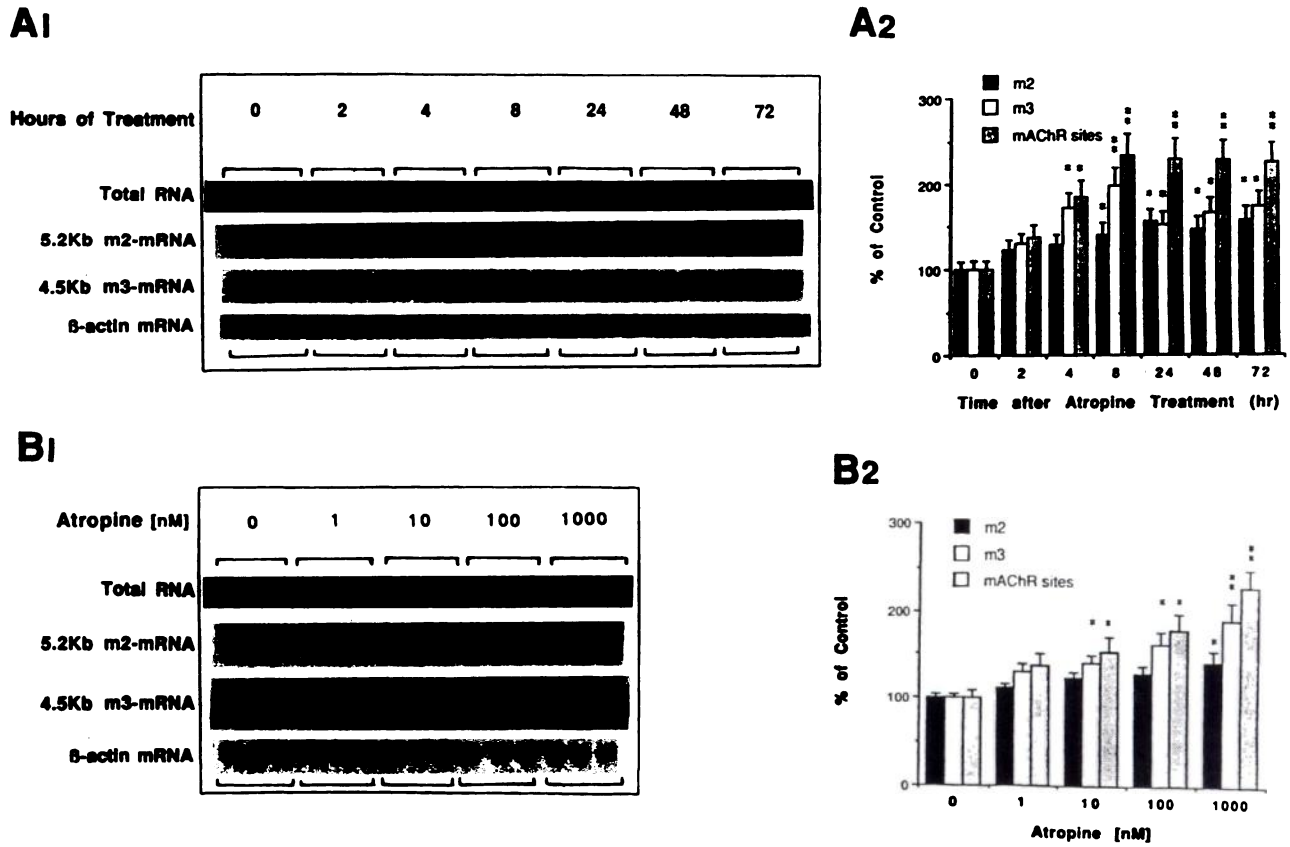

Fig. 7. Atropine-induced up-regulation of mAChR mRNA and binding sites. Cells were treated with 1 μM atropine for the indicated times (A) or exposed to the indicated concentrations of atropine for 8 hr (B). Levels of m_2-mAChR mRNA, m_3-mAChR mRNA, β-actin mRNA, and mAChR binding sites were then quantified. mRNA levels were normalized to total RNA in each lane. The results in A2 and B2 are quantified data of the mRNA blots in A1 and B1 and represent means ± standard error of triplicate samples. The experiment has been repeated three times with nearly identical results. $^*p < 0.05$; $^{**}p < 0.01$, when compared with their untreated control group (Student's t test). The 100% values for mAChR binding sites were 262 fmol/dish of 3×10^6 cells ($n = 6$).

mRNAs and binding sites was reversible upon the removal of the prestimulating carbachol (Fig. 6). At least in the case of m_3-mAChR, the mRNA loss induced by carbachol cannot be attributed to mRNA destabilization. In fact, the $t_{1/2}$ of m_3-mAChR increased from approximately 40 to 80 min after carbachol treatment (Fig. 4). The $t_{1/2}$ value of 80 min was comparable with that obtained from the recovery of m_3-mAChR mRNA after removal of the prestimulating agonist (Fig. 6). The marked increase of m_3-mAChR mRNA stability in cells treated with both carbachol and cycloheximide suggests that continued protein synthesis is required for mRNA degradation. A similar conclusion was drawn in studies of the mRNA stability of tumor necrosis factor-α and the proto-oncogene, c-*myc* (33). It has been reported that persistent carbachol stimulation induces down-regulation of m_1-mAChR mRNA in transfected cells (34) and m_2- and m_4-mAChR mRNAs in chick heart cells (35). In the latter study, the down-regulation of m_2- and m_4-receptor mRNAs was associated with no change in the $t_{1/2}$ of these mRNA species. The carbachol-induced stabilization of m_3-receptor mRNA observed in the present study differs from their results and could be a phenomenon related to the particular mAChR subtype and/or cell type studied.

Treatment of cells with an mAChR antagonist atropine, on the other hand, resulted in a time- and concentration-dependent increase in the levels of m_2- and m_3-mAChR mRNAs, and the mAChR binding sites (Fig. 7). After 24 hr of atropine treatment, the immunoprecipitable m_3-mAChR protein was markedly increased, while the m_2-mAChR was not (Fig. 11A). The atropine-induced up-regulation of m_2- and m_3-mAChR mRNA was reversed by washing out the antagonist (Fig. 9). Treatment with the receptor subtype-selective antagonists, AF-DX 116 and 4-DAMP, induced an increase in the respective receptor subtype mRNA and protein (Figs. 8 and 11). The up- and down-regulation of mAChR mRNAs and their binding sites demonstrated in this *in vitro* study could be the molecular basis of the changes in mAChR binding sites in the brain of experimental animals chronically treated with atropine (15), oxotremorine (13), or an acetylcholinesterase inhibitor (14, 36). It is of interest to note that the m_3-mAChR protein is the major subtype to be increased in the brain after chronic atropine treatment of rats—an observation consistent with our results obtained with cerebellar granule cells.

Nuclear run-off assays demonstrated that the transcription rate of m_3-mAChR at 8 hr after exposure to carbachol (100 μM) or atropine (1 μM) was about 60% or 230% of the control, respectively (Fig. 5). The lack of change in β-actin mRNA under these experimental conditions indicates that the change in m_3-mAChR mRNA was not due to general changes in transcription. These results suggest that, at least for m_3-mAChR, alteration in the transcription rate is likely to be a major mechanism for the mRNA regulation after agonist or antagonist treatment. Because of a relatively weak signal in the change

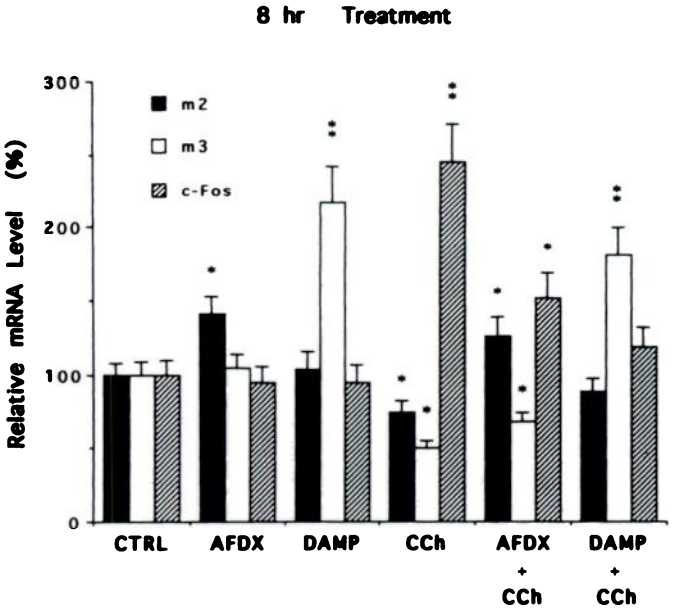

Fig. 8. Effects of mAChR subtype-specific antagonists on mRNA levels. Cells were treated with the indicated drugs for 8 hr. Levels of mRNAs for m_2-mAChR, m_3-mAChR, β-actin, and c-*fos* were determined. The results are quantified data of mRNA blots and represent the means and ranges of duplicate samples. The mRNA levels were normalized to total RNA in each sample and expressed as percentage of the untreated control group. The concentrations of AF-DX 116, 4-DAMP, and carbachol used were 1 μM, 0.1 μM, and 100 μM, respectively. The experiment has been repeated three times with similar results. *, $p < 0.05$; **, $p < 0.01$, when compared with their untreated control group (Student's *t* test).

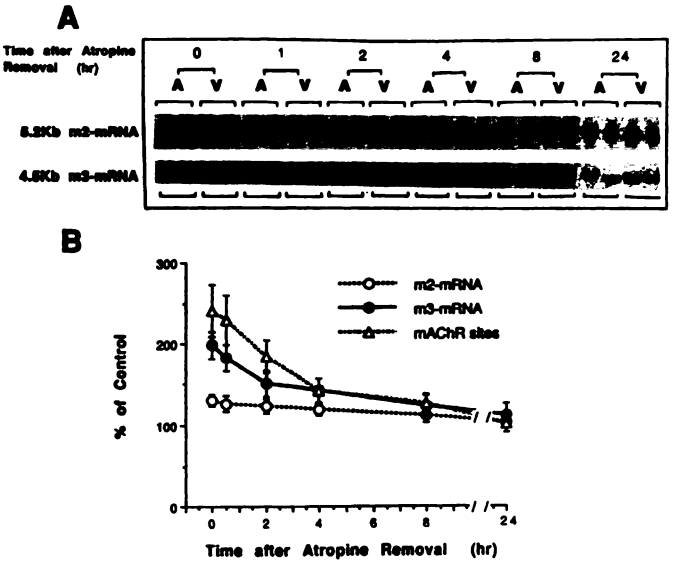

Fig. 9. Recovery of atropine-induced up-regulation of mAChR mRNA and binding sites. Cells were pretreated with 1 μM atropine (A) or vehicle (V) for 8 hr and then washed three times to remove the atropine; the cells were then cultured with growth medium containing 10% dialyzed fetal calf serum. At various times after atropine washout, m_2-mAChR mRNA, m_3-mAChR mRNA, and mAChR binding sites were assessed. Please note that the 8-hr atropine exposure up-regulated the level of m_2-mAChR mRNA, m_3-mAChR mRNA, and mAChR binding sites to 130, 198, and 241% of the control, respectively. Data are expressed as percentage of the untreated control group and are the mean and ranges of duplicate samples from an experiment reproduced three times. The 100% value for mAChR binding sites was 279 fmol/dish of 3×10^6 cells.

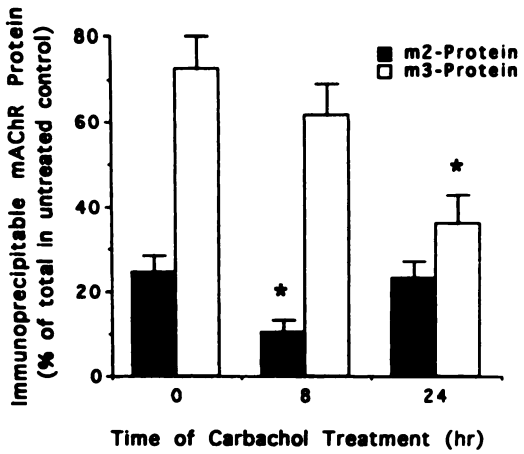

Fig. 10. Carbachol-induced differential down-regulation of immunoprecipitable m_2- and m_3-mAChR proteins. Cells grown in 150-mm dishes were treated with 100 μM carbachol for 8 or 24 hr. Membranes were then prepared and labeled with [^{3}H]QNB. The solubilized labeled receptors were then immunoprecipitated with m_2- or m_3-receptor antisera. Please note that carbachol treatment for 8 or 24 hr progressively decreased total [^{3}H]QNB-labeled mAChRs. Immunoprecipitable m_2- and m_3-mAChR proteins at 8 and 24 hr were expressed as percentage of total labeled receptors solubilized from membranes of untreated cells. Data shown are means ± SEM of three independent experiments. *$p < 0.05$, when compared with percentage for the respective receptor subtype in untreated cells.

of m_2-mAChR mRNA induced by either carbachol or atropine at this time point, the transcription rate and stability of m_2-mAChR mRNA have not been assessed.

In the case of carbachol-induced m_2-mAChR mRNA down-regulation, the concomitant increase in c-*fos* mRNA (Figs. 3 and 8) is consistent with a possible role of c-*fos* in the negative regulation of m_2- and possibly m_3-mAChR mRNAs. c-*fos* might, for example, be coupled to another proto-oncogene, Jun-B, to serve as a transcriptional repressor (37). The level of c-*fos* mRNA was unchanged under conditions in which m_2- and m_3-mAChR mRNAs are up-regulated by AF-DX 116 and 4-DAMP, respectively (Fig. 8). It is conceivable that mAChRs in cerebellar granule cells are tonically activated due to spontaneous synaptic activity between contacted neurons. A persistent blockade of this spontaneous activity by the mAChR antagonists would then produce an up-regulation of mAChR mRNAs. Alternatively, it could be that subtype-specific antagonists are involved in the modulation of second messenger pathways that have not yet been discovered, resulting in an enhanced transcription and/or stability of mAChR mRNAs. In this context, it is noteworthy that significant effects of β-adrenergic receptor antagonists on β-adrenergic receptor number (38) and receptor mRNA level (39) have been reported. The molecular mechanism underlying the down-regulation by the agonist and up-regulation by the antagonists of mAChR mRNA and protein are under investigation.

The results of immunoprecipitation of labeled mAChRs (Figs. 10 and 11) and displacement of [^{3}H]QNB binding to intact cells by subtype-specific antagonists (Fig. 1) confirm Northern blot (21) and receptor binding (22) data that cerebellar granule cells express only m_2- and m_3-mAChRs. Our present results further demonstrate the m_3 is the predominant receptor subtype expressed, comprising approximately 80% of total receptor population. Surprisingly, in subtype antagonist binding experiments using intact cells, we found that AF-DX 116 had

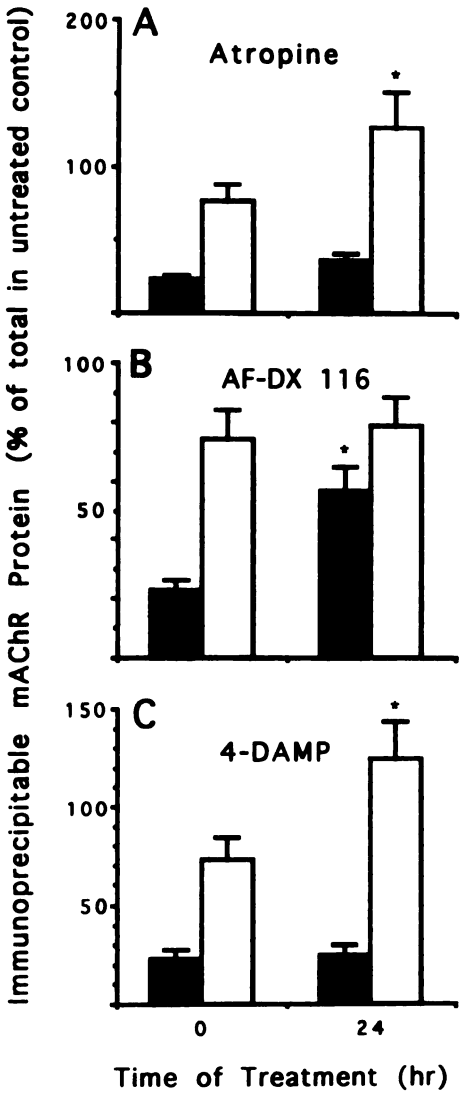

Fig. 11. Antagonist-induced differential up-regulation of immunoprecipitable m_2- and m_3-mAChR proteins. Cells were treated with 1 μM atropine (A), 1 μM AF-DX 116 (B), or 0.1 μM 4-DAMP (C) for 24 hr. After extensive washings, cells were lysed and membranes were prepared. Immunoprecipitation of [^{3}H]QNB-labeled mAChRs using m_2- or m_3-receptor antisera was then performed. Please note that treatment with atropine, AF-DX 116, or 4-DAMP all significantly increased total [^{3}H]QNB-labeled mAChRs. Immunoprecipitable m_2- and m_3-mAChR proteins after antagonist treatment were expressed as percentage of total labeled receptors solubilized from membranes of untreated cells. Data shown are means ± standard error of three independent experiments. *$p < 0.05$, when compared with percentage for the respective receptor subtype in untreated cells. *Solid bars*, m_2-mAChR protein; *open bars*, m_3-mAChR protein.

a much higher selectivity for m_2-mAChRs, and 4-DAMP had a higher selectivity for the m_3 receptors (Fig.1) than previously described (for a review, see 5 and 40). The observations that 1 μM AF-DX 116 and 0.1 μM 4-DAMP selectively up-regulated m_2- and m_3-receptors, respectively (Figs. 8 and 11), suggest that the high selectivities of these two antagonists are biologically meaningful. We have performed binding assays on membranes prepared from granule cells and obtained K_i values consistent with those found in the literature (data not shown). These observations suggest that disruption of cerebellar granule cells to produce membranes used in binding assays causes the loss of factors which influence the binding selectivity of muscarinic antagonists. Experiments are in progress to characterize what such factors might be.

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