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Differential Regulation of Muscarinic Receptor mRNA Levels in Neuroblastoma Cells by Chronic Agonist Exposure: A Comparative Polymerase Chain Reaction Study

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SUMMARY

The human neuroblastoma line SH-SY5Y expresses three muscarinic receptor genes (m1, m2, and m3). In this study, we have investigated the effect of agonist exposure on the steady state levels of each muscarinic receptor transcript, using a comparative polymerase chain reaction (PCR) assay that allows changes in levels of very rare transcripts to be monitored. Northern blot analysis of cellular RNA revealed the presence of m3 mRNA, whereas PCR amplification of SH-SY5Y cDNA additionally revealed the presence of m1 and m2 transcripts. Cell surface muscarinic receptor number, as assessed by *N*-[^{3}H]methylscopolamine binding to whole cells, rapidly decreased to 42% of control levels within 1 hr of exposure to 100 μM carbachol; this was followed by a slower decline to 6% of control levels after 48 hr. Total receptor number, measured by binding of [^{3}H]quinuclidinyl benzilate, showed a much slower decline to 21% of control levels after 48 hr of treatment. Comparative PCR analysis showed that each muscarinic transcript was differentially regulated. The level of transcript encoding the major receptor population, the m3 mRNA, was rapidly elevated within 1 hr of agonist challenge and subsequently decreased to about 30% of prestimulation levels within 9 hr; this decrease was sustained for the time course of the experiment. m2 mRNA levels showed a transient increase followed by a decrease to 30% of prestimulation levels after 6 hr but, in contrast to the m3 transcripts, this depression was followed by a transient rise to 270% of prestimulation levels after 24 hr before declining to normal levels by 72 hr after stimulation. Exposure of cells to agonist clearly instigates a complex pattern of changes in levels of receptor and receptor mRNA; comparison of the relative time courses of these changes indicates that the decline in m3 transcripts precedes the loss of muscarinic receptor binding sites.

Activation of guanine nucleotide-binding protein-coupled receptors leads to changes in levels of second messengers and ion channel activity. Prolonged stimulation usually results in desensitization, followed by internalization and, ultimately, degradation (1, 2). Molecular cloning studies have demonstrated that many guanine nucleotide-binding protein-coupled receptors are encoded by discrete gene families, the individual members of which are highly homologous but frequently show diverse pharmacological profiles and patterns of expression.

Muscarinic receptors are part of this gene superfamily (3). At least five muscarinic receptor genes are known to be present in the human and rat genomes (4–7), each of which encodes a receptor with distinct pharmacological specificities (8–12) and patterns of expression (13–15). Although each muscarinic receptor gene is differentially expressed, many cells express an intimate mixture of muscarinic receptor transcripts (3, 16). Because activation of each muscarinic receptor subtype can transduce a unique set of responses, dependent upon the host cell type, the repertoire of receptor subtypes expressed by any given cell has a profound effect on the response of that cell to agonist challenge. In light of these observations, we were interested in investigating the effect of agonist stimulation on the levels of muscarinic receptor transcripts in individual cell populations and investigating the mechanisms involved in any such changes. Because neurotransmitter receptor genes are frequently expressed at very low levels in most neuroblastomas,[1] we were also interested in developing a quantitative reverse transcriptase-PCR protocol that would allow changes in rare transcripts to be monitored.

As a model system, we have used the SH-SY5Y neuroblastoma cell line (17); these cells (and their progenitor cell line, SK-N-SH) have been used extensively to examine muscarinic receptor pharmacology (18–22). Activation of SH-SY5Y cells with muscarinic agonists leads to a release of intracellular calcium (18, 19, 23) and a robust stimulation of phosphoinositide hydrolysis (18, 24, 25). Previous attempts to identify the muscarinic receptor subtype expressed by SH-SY5Y cells, using radioligand binding assays, have yielded equivocal results (18),

[1] M. C. Steel and N. J. Buckley, unpublished observations.

ABBREVIATIONS: PCR, polymerase chain reaction; NMS, *N*-methylscopolamine; QNB, quinuclidinyl benzilate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; kb, kilobase(s).

although most studies indicate that M3 is the predominant subtype (16, 22).

In the present study, we have used Northern blot analysis and a quantitative PCR procedure to identify the muscarinic transcripts expressed by this cell line and to monitor the changes in muscarinic receptor transcripts consequent to receptor activation.

Experimental Procedures

Cell culture. SH-SY5Y neuroblastoma cells (obtained from Dr. Stefan Nahorski, University of Leicester) were cultured on 10-cm dishes to a density of 10^7 cells/dish. Growth medium consisted of α minimum essential medium supplemented with 10% fetal calf serum, 2 mM glutamine, 60 μg/ml penicillin, and 100 μg/ml streptomycin. Cells were challenged with 100 μM carbachol for the times indicated. Growth medium was then withdrawn, 1 ml of 4 M guanidinium thiocyanate were added to the dish, and RNA was extracted using the protocol of Chomczynski and Sacchi (26). In the relevant experiments, atropine (1 μM) was added 30 min before addition of carbachol.

Binding studies. Radioligand binding studies were carried out on whole cells grown in 10-cm dishes. Cell membrane muscarinic receptor binding was assayed using [^{3}H]NMS (83 Ci/mmol; Amersham). Total muscarinic receptor binding was measured using [^{3}H]QNB. Nonspecific binding was measured in the presence of 1 μM atropine or 1 μM QNB. Care was taken to wash cells and membranes before labeling to remove any ligands that may have been present from previous treatments. Binding buffer consisted of 118 mM NaCl, 1.8 mM $CaCl_2$, 2.7 mM KCl, 0.81 mM $MgCl_2$, 1.0 mM Na_2HPO_4, 5.6 mM glucose, and 25 mM HEPES (pH 7.4). All binding assays were continued overnight at 4° and terminated by filtration through glass fiber filters. Protein assays were conducted using the Bio-Rad protein assay, according to the manufacturer's instructions.

RNA extractions and Northern blot analysis. Total RNA was extracted from cells according to the method of Chomczynski and Sacchi (26). Briefly, growth medium was withdrawn from SH-SY5Y cells and 1 ml of GnTC solution (4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% sarcosyl) was applied. The cell lysate was extracted with an equal volume of phenol (pH < 5.0) and purified according to standard protocols. Purity and quantitation were assessed by A_{260}/A_{280} ratios. Northern blot analysis was performed by electrophoresing 15-μg samples of cellular RNA on 1% denaturing agarose gels (containing 2% formaldehyde) and electroblotting the RNA onto nylon membranes (Genescreen; NEN).

Reverse transcription. Before reverse transcription, residual genomic DNA was removed by digestion with RQ DNase (Promega). RNA (10 μg) was precipitated and dissolved in 40 mM Tris·HCl (pH 7.9), 10 mM NaCl, 6 mM $MgCl_2$ RNAguard (17 units; Pharmacia) and RQ DNase (5 units; Promega) were added and digestion was carried out at 37° for 15 min. Reactions were terminated using phenol extraction or Strataclean resin (Stratagene). After DNase treatment, no signal could be seen in samples that were amplified without prior reverse transcription. RNA was precipitated with NaCl/ethanol and redissolved in 5 μl of H_2O. Samples (0.2–2 μg) of DNase-treated RNA were denatured and reverse transcribed in 20 μl of buffer composed of 50 mM Tris·HCl, 40 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 17 units of RNAguard, 2 μg of oligo $(dT)_{12-18}$ (United States Biochemicals), and 13 units of reverse transcriptase (avian myeloblastosis virus; Seikagaku America Inc). Incubation was carried out at 37° for 1 hr and stopped by incubation at 80° for 10 min. Reaction mixtures were not extracted and were stored at −20° before amplification.

PCR. PCRs and hybridizations were carried out using the following 24-mers and 48-mers, respectively (synthesized on Applied Biosystems synthesizers): m1-1069s, CTGGTCAAGGAGAAGAAGGCAGCT; m1-1518a, GTCTCTCTGGGCTGTCCAGGAAGG; m2-911s, GAAAACACAGTTTCCACTTCGCTG; m2-1170a, ATGATGAAAGCCAACAGGATAGCC; m3-1400s, ACCTCTGTCCTTCAAGGAGGCCAC; m3-1765a, CGCTTGTGAAAAATGACCGACTGT; m4-902s, ACCCAGAACACCAAGGAACGGCCA; m4-1559a, GGTACCTCACGGTGTCTGGGAGAC; m5-654s, ACAGAGAAGCGAACCAAGGACCTG; m5-1265a, TCTCTTTCGTTTGGTCATTTGATG; hprt-231s, CCTGCTGGATTACATTAAAGCACT; hprt-576a, CCTGAAGTACTCATTATAGTCAAG; m1-31a, GGCCACCGTCCAGGGACCCTTTCCTGGTGCCAAGACAGTGATGTTGGG; m1-1056a, ACTCAGGGTCCGAGCTGCCTTCTTCTCCTTGACCAGTGAGAAGGTCTT; m1-1299a, GGGCGCTTGGGGATCTTGCGCCAGCGCCTCTTGTCCCAGCGGCAGAGC; m2-49a, TCCAGCCACAAGGACAATAAATACCACTTCAAATGTCTTGTAAGGACT; m2-886a, AAGTGGAAACTGTGTTTTCATCCTGGGTTATCTCATCATCTCTCATA; m2-1076a, GATTGTCCTGGTCACTTTCTTTTCCCGGGATGGTGGAGGCTTCTTTTT; m3-7a, GATGTTGGGAAACAAAGGCGAGGTTGTACTGTTACTGTGCAAGGTCAT; m3-968a, TGTTGTTCCAACTGTCGCTGCTGCTGTGGTCTTGGTCCATCTGCTCGG; m3-1169a, GTGAAAAATGACCGACTGTCTCTGCTGGTACTGCTGTTTGCGCCTCTT; m4-10a, GTCACCAGGCGCACAGACTGATTGGCTGAGCTGCCATTGACAGGCGTG; m4-866a, GGTGTTCTGGGTGGCACTGCCACTGCCTGAGCTGGACTCATTGGAAGT; m5-641a, TGGAGGTCAGCCAGGTCCTTGGTTCGCTTCTCTGTCTCCCGGTAGATC; m5-1164a, CAGGGCATGATTTTCACCTTTCGACAGCCATTGTTAGTCTCCTGGGTC. Oligonucleotides were purified either by gel extraction from polyacrylamide gels or by passage of 'trityl-on' oligonucleotides through commercial reverse phase columns (Glen Research). Amplifications were carried out in 50 μl of buffer containing 10 mM Tris·HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$ 0.01% gelatin, 0.1% Triton X-100, 0.25 mM deoxynucleoside triphosphates, 50 pmol of primers, template corresponding to 2–200 ng of reverse-transcribed RNA, and 2 units of Taq polymerase (Promega). Amplification reactions intended for quantitation were 'spiked' with 1 μCi of [^{32}P]dATP (6000 Ci/mmol; NEN). Reaction mixtures were covered with mineral oil and amplifications were carried out in 0.5-ml polypropylene tubes in a Techne PHC-2 thermocycler. Cycles were as follows: 95° for 5 min, 60° for 30 sec, and 72° for 1 min, one cycle; 95° for 30 sec, 60° for 30 sec, and 72° for 1 min, 20–35 cycles; 95° for 30 sec, 60° for 30 sec, and 72° for 5 min, one cycle. Reactions were terminated with stop buffer and 25 μl samples were electrophoresed on 2% agarose (or 3% NUSIEVE/1% agarose) gels, containing 1 μg/ml ethidium bromide, and were viewed on a 300 nm UV transilluminator. For quantitative studies, gels were washed in 1× TBE (0.09 M Tris-borate, 2 mM EDTA, pH 8.0), primer bands were excised, and the gels were dried under vacuum (80° for 90 min) before exposure to Kodak XAR-5 film. After suitable exposure periods, autoradiographs were analyzed densitometrically using an LKB laser densitometer.

Probe labeling and hybridizations. Oligodeoxynucleotides (48-mers) were 3′-tailed with [^{32}P]dATP using terminal deoxynucleotidyl transferase (BRL). Tailing was carried out at 37° for 1 hr using a molar ratio of 10:1 dATP/oligonucleotide. Specific activities were >10^{10} dpm/μg, corresponding to an average tail length of >10 nucleotides. Amplified PCR products were purified by spin filtration through Centricon 100 columns and were labeled using random primers, DNA polymerase I (27), and [^{32}P]dATP (6000 Ci/mmol; NEN), to a specific activity of >10^9 dpm/μg. Hybridizations were carried out for 2–18 hr at 37° in 4× SSPE (150 mM NaCl, 10 mM sodium phosphate, 1 mM EDTA, pH 7.4), 50% formamide, 5× Denhardts', 0.1% sodium dodecyl sulfate, 250 μg/ml sheared salmon sperm DNA, 250 μg/ml yeast tRNA, and blots were washed at a final stringency of 1× SSPE at 60°. Hybridizations using randomly primed probes were conducted overnight in 3× standard saline citrate (150 mM NaCl, 15 mM sodium citrate, pH 7.0), 10× Denhardts', 50 μg/ml sheared salmon sperm DNA, 1 μl/ml bacterial lysate, and blots were washed at a final stringency of 0.1× SSPE at 65°.

Results

Muscarinic receptor gene expression in SH-SY5Y cells. Radioligand binding studies using [^{3}H]NMs indicated a single class of binding sites (B_{max}, 200–350 fmol/mg of protein;

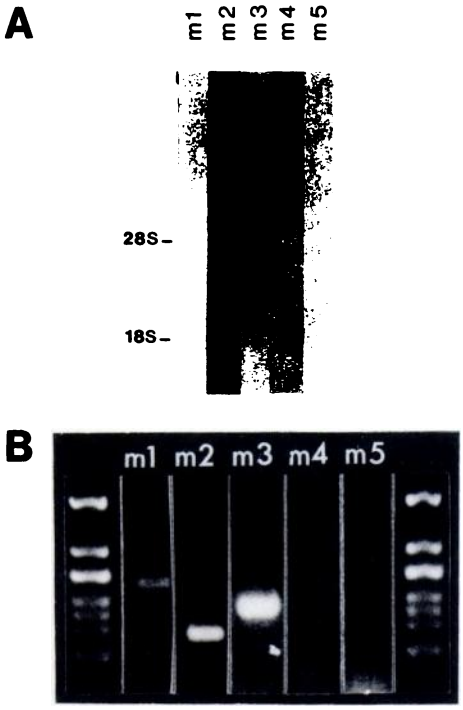

Fig. 1. Analysis of muscarinic receptor transcripts in SH-SY5Y cells. A, Northern blot analysis of SH-SY5Y RNA. Fifteen micrograms of total cellular RNA were electrophoresed and blotted as described in Experimental Procedures and were hybridized with a mixture of ^{32}P-tailed m1, m2, m3, m4, and m5 antisense oligodeoxynucleotides (see Experimental Procedures for sequences). Probe concentration was 1–2 × 10^6 dpm/ml, corresponding to a concentration of approximately 3 pmol/ml. Positions of the 28 S and 18 S ribosomal bands are indicated. Hybridizing bands can be seen corresponding to approximately 9, 5, and 4.5 kb. B, Reverse transcriptase-PCR analysis of reverse-transcribed SH-SY5Y mRNA. Two micrograms of total SH-SY5Y RNA were reverse transcribed and 200 ng were used for amplification, as described in Experimental Procedures. A total of 30 amplification cycles was used and half of the PCR product was electrophoresed through a 2% agarose gel containing 1 μg/ml ethidium bromide and was viewed on a 300-nm UV transilluminator. Size markers are indicated. m1, m2, m3, m4, and m5 amplification primers were m1-1069s/m1-1518a, m2-911s/m2-1170a, m3-1400s/m3-1765a, m4-902s/m4-1559a, and m5-654s/m5-1265a, respectively.

K_d, 104 pM). Similar studies using the selective M1 antagonist [^{3}H]pirenzepine revealed no specific binding (data not shown). Northern blot analysis of total RNA using sets of tailed oligonucleotides specific to each muscarinic receptor transcript revealed the presence of only m3 mRNA (Fig. 1A). Three transcripts were evident, corresponding to approximate lengths of 9, 5, and 4.5 kb. The 9-kb transcript was always the predominant transcript. All three transcripts were evident when the same blot was hybridized with each individual m3 probe, showing that the multiple transcripts were not a function of spurious hybridization by one of the oligonucleotides (data not shown).

All of the oligonucleotides used in this study generated positive signals when hybridized with or used to amplify the corresponding plasmid DNA. However, PCR analysis of SH-SY5Y RNA revealed m1 and m2 transcripts in addition to m3 mRNA (Fig. 1B). Because different primers were used to generate the various PCR reaction products, it was not possible to strictly quantitatively assess the relative amounts of m1, m2, and m3 transcripts by comparison of the ethidium bromide-induced fluorescence, although the consistent relative brightness of the m3 PCR band was in accord with the Northern blot data. Verification that the amplified DNA corresponded to the appropriate transcripts was provided by determination of the predicted length of the PCR fragments and by hybridization of blots of the PCR fragments with labeled nested oligonucleotides or, in some cases, by sequencing of the PCR product using a PCR thermocycling kit (Promega).

Down-regulation of receptor number. Chronic exposure of SH-SY5Y cells to 100 μM carbachol induced a time-dependent decrease in receptor number, as adjudged by radioligand binding studies. Over the time course studied, total receptor number ([^{3}H]QNB binding) decreased from 335 fmol/mg of protein (~20,000 receptors/cell) to 70 fmol/mg of protein (~4000 receptors/cell), when normalized to treatment with H_2O alone; $t_{1/2}$ was approximately 18 hr (Fig. 2, B and D). The reasons for the transient decrease in [^{3}H]QNB binding seen at 6 hr are unknown (Fig. 2D), but this was a consistent observation in all experiments. Fig. 2, A and C, shows the decrease in cell surface receptors (those labeled by [^{3}H]NMS) from 310 fmol/mg of protein (~16,000 receptors/cell) to 20 fmol/mg of protein (~1000 receptors/cell). Normalization of data to a null treatment was necessary because receptor number gradually changed during the course of the experiment (see Fig. 2, B and D). This is a phenomenon that we have noticed in many native and transfected cell lines; it appears to be a function of the length of time that the cells are maintained in culture, rather than a simple function of cell density.

Establishment of quantitative PCR conditions. The first step was to ascertain the range of amplification cycles that maintained an exponential relationship between cycle number and amount of amplified product. Fig. 3, A and B, shows that, between cycles 21 and 28, the amount of amplified product doubled with each amplification cycle. This relationship was constant, irrespective of the transcript that was under investigation. For convenience and clarity, we have shown only the data relating to a rare transcript (*hprt*) and to a more abundant transcript (m3). No difference was observed between the amounts of amplified products obtained using either one or two sets of primers. Having established the optimal cycle number for comparative analysis, we proceeded to examine the relationship between the initial amount of template and the amount of amplified product. Fig. 3, C and D, shows that the amount of amplified product was linearly proportional to the amount of starting template over a range of cDNA concentrations corresponding to 20–400 ng of reverse-transcribed RNA. Demonstration of linearity between the initial amount of cDNA template and the amount of amplified product is insufficient in itself, because this relationship does not take into account the efficiency of reverse transcription. Hence, there was the need to demonstrate the range of RNA concentrations over which a linear relationship existed between initial RNA concentration and the amount of amplified product. Fig. 3, E and F, shows that reverse transcription of 10–100 ng of RNA, followed by 26 cycles of amplification, maintained a linear relationship between the concentration of RNA and the amount of amplified product, i.e., a comparative PCR protocol was established.

Agonist regulation of muscarinic receptor mRNAs. Agonist-induced changes in the levels of m2 and m3 transcripts were examined using the comparative PCR procedures outlined above. The results of the PCR analysis can be seen in Fig. 4. It was not possible to accurately quantitate changes in the levels of m1, because this transcript was much less abundant than

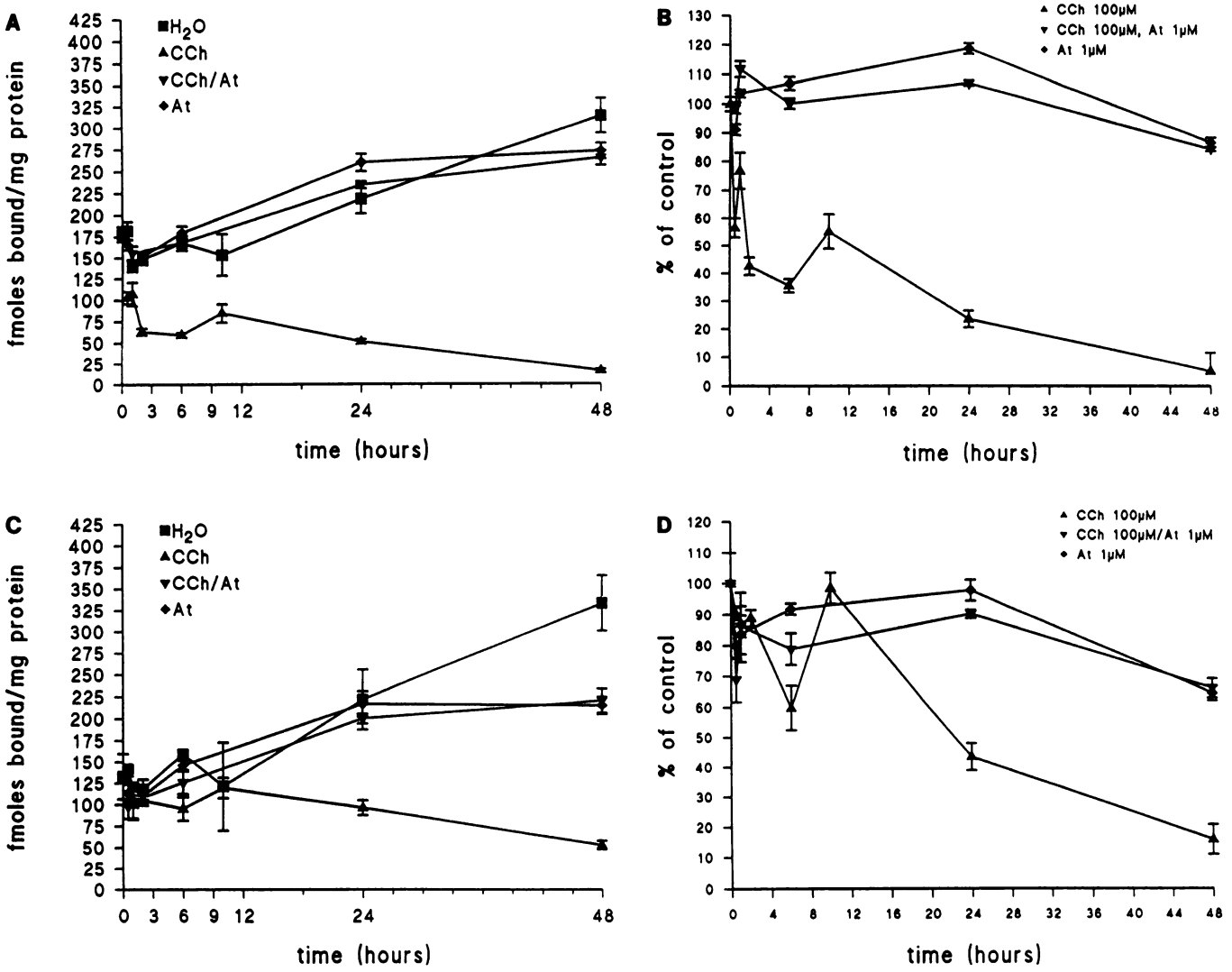

Fig. 2. Effect of carbachol treatment on levels of [^{3}H]QNB and [^{3}H]NMS binding. A, Change in amount of specifically bound [^{3}H]QNB as a function of time after challenge with 100 μM carbachol (▲), water (■), 100 μM carbachol plus 1 μM atropine (▼), or atropine alone (◆). B, Same data as in A, normalized to the amount of [^{3}H]QNB bound specifically by cells treated with water only. Data are expressed as a percentage of receptors expressed by cells treated with water only. C, Amount of specific [^{3}H]NMS binding in membranes prepared from the same cultures as used for the [^{3}H]QNB binding experiments. Results are similarly expressed. D, Same data as in C, normalized as in B. *Bars*, standard errors. All *points* are the mean of at least two independent experiments conducted in duplicate.

the m2 and m3 transcripts. Levels of m2 mRNA were not significantly reduced, compared with control levels, until 6 hr (although some reduction was observed after 3 hr of carbachol treatment). After 24 hr of incubation the level of m2 mRNA recovered and transiently exceeded by 270% the control levels, but after 48 hr m2 mRNA levels did not differ from control levels. In contrast, m3 mRNA levels were rapidly and transiently elevated after 1 hr of agonist treatment. Although the elevation failed to reach statistical significance, this phenomenon was consistently seen in independent experiments. However, after 6 hr levels were reduced to about 50% of control levels. This reduced level was maintained for the time course of the experiment. Atropine completely abolished the effects of carbachol, but treatment of cells with antagonist alone caused a small increase in the level of m2 mRNA and m3 mRNA after 7 hr of treatment (data not shown).

Discussion

We have established a comparative reverse transcriptase-PCR protocol in order to examine changes in levels of rare neurotransmitter receptor transcripts. This procedure has allowed us to monitor changes in the relative levels of two different muscarinic receptor transcripts in the SH-SY5Y neuroblastoma cell line; this procedure has also allowed us to detect two transcripts (m1 and m2) that were otherwise undetectable by conventional Northern blot analysis of cellular RNA.

Use of PCR to monitor changes in gene expression. We established a quantitative PCR protocol in order to examine changes in levels of rare transcripts that would be otherwise undetectable by RNA hybridization procedures, such as the m1 and m2 transcripts in the present study. Because of the inherent variations present within any individual PCR, quantitation is not possible without normalization to an internal standard. The constitutively expressed housekeeping *hprt* gene (28) was selected as a reference template for this purpose. Unless the amount of template is known by some other means (such as a nuclease protection assay), absolute quantitation is possible only if the same primers (and corresponding target sequences)

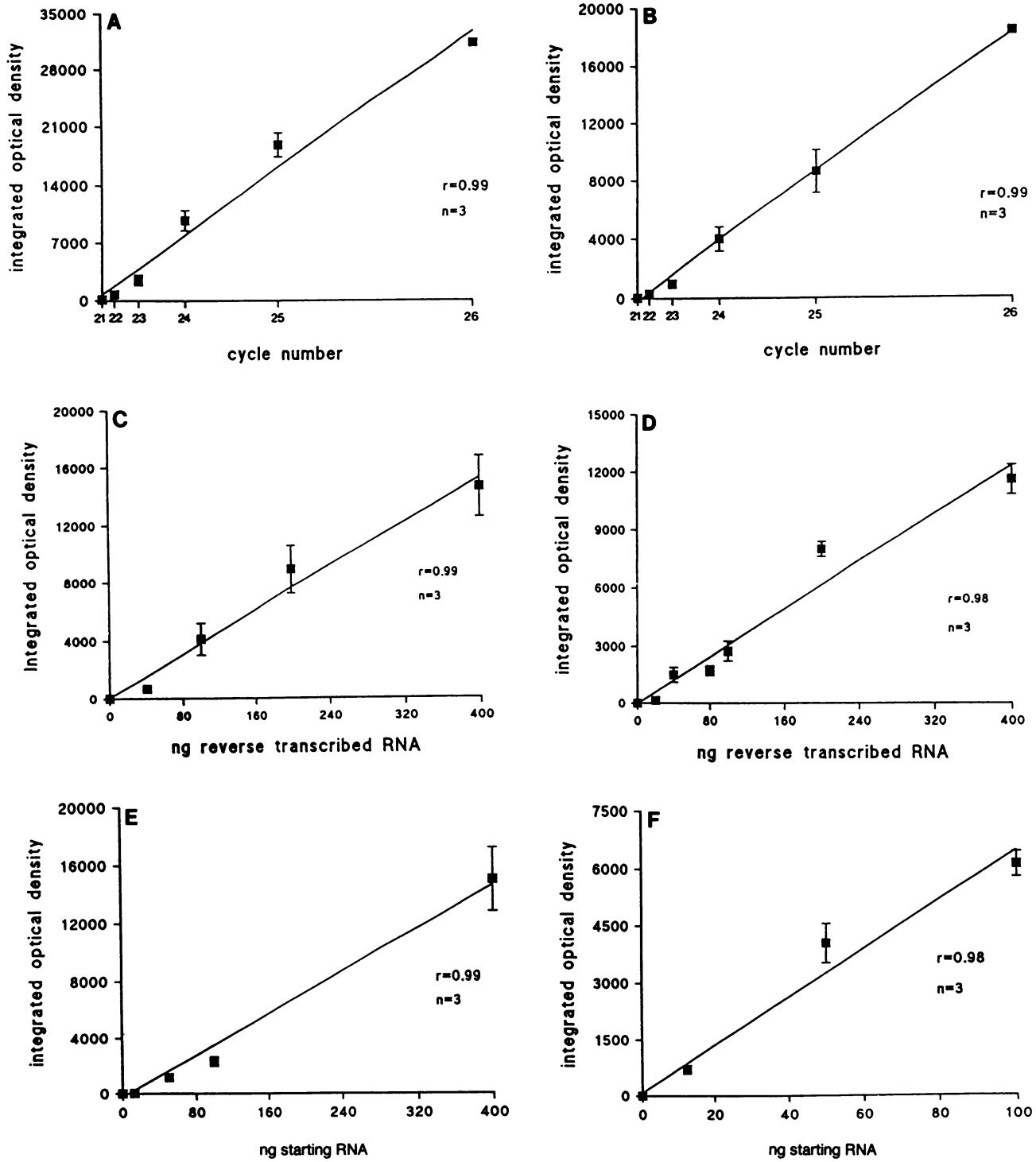

Fig. 3. Establishment of quantitative PCR conditions. Reverse transcriptions, PCRs, and measurements of amplified products were carried out as described in Experimental Procedures. A and B, Amount of amplified product as a function of cycle number, using m3 and *hprt* primers, respectively. For the sake of clarity, cycle number is shown relative to cycle 21 and is plotted on an exponential scale with a geometric constant of 2. The amount of amplified product is shown in arbitrary absorbance units. A, Relationship between product and cycle for a rare transcript (*hprt*); B, same relationship for a relatively abundant transcript (m3). In both cases, the relationship is linear, with a slope of unity, over the cycle range 21–28, thereby demonstrating a doubling of reaction product with each cycle number within this range. *Bars*, standard errors. All *points* represent the mean of three independent experiments carried out in duplicate. C and D, Amount of amplified product as a function of initial cDNA concentration. cDNA was reverse transcribed from 1 μg of total RNA and was diluted accordingly before amplification. Over the range of 20–400 ng of reverse-transcribed RNA, the plots are linear for m3 (C) and for *hprt* (D), as well as for all other transcripts examined (data not shown). PCRs were carried out over 26 cycles of amplification. All *points* represent the mean of three independent determinations carried out in duplicate. *Bars*, standard errors. E and F, Amount of amplified product as a function of initial RNA concentration. In this case, each *point* represents data gathered from individual reverse transcription reactions. A linear relationship exists between the amount of initial RNA and the amount of amplified product over a range of RNA between 20 and 400 ng. E and F, Data obtained with m3 and *hprt* primers, respectively. All *points* represent the means of at least two independent experiments carried out in duplicate. *Bars*, standard errors.

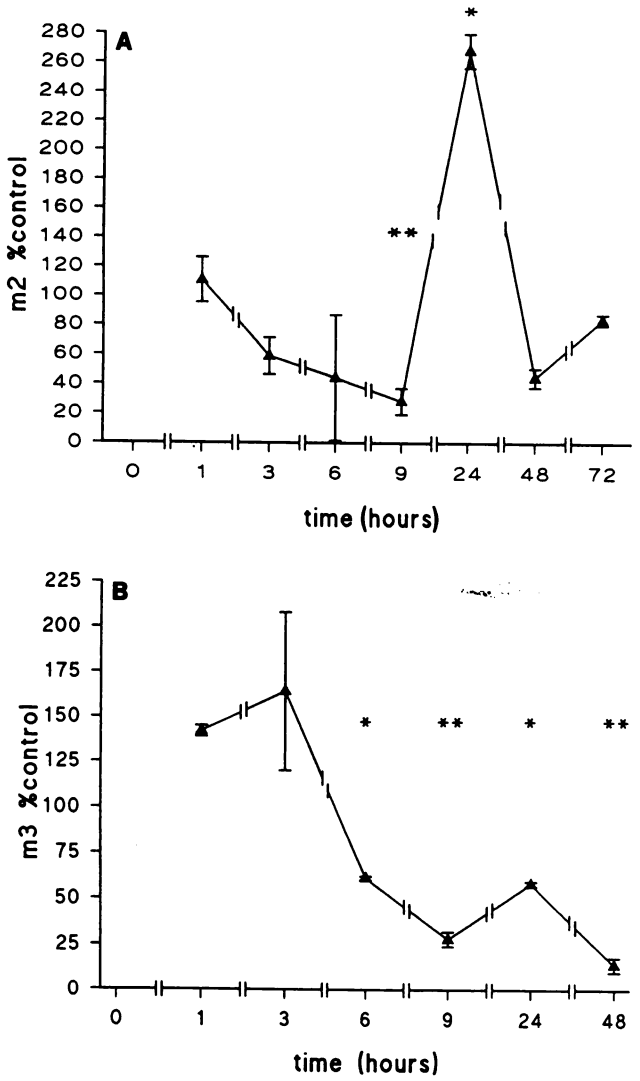

Fig. 4. Effect of carbachol on m2 and m3 mRNA levels. A and B, Levels of m2 and m3 amplified products, relative to the amount of coamplified *hprt* product. Results are presented as a percentage of the values obtained with water treatment only. All results are normalized to mRNA levels in cells treated only with water. * and **, Significant differences between experimental and control values at significance levels of $p > 0.05$ and $p > 0.001$, respectively. *Error bars*, standard errors.

are used to generate both of the amplified products; this has led to the construction of plasmids containing multiple priming sites that are used to 'spike' the amplification reaction (29). However, even this approach is not without its difficulties, because the rate of denaturation of primer/plasmid dimers and primer/cDNA dimers may differ considerably. The advantages of the protocol outlined here are principally its general applicability and the ease and directness of its quantitation. Because the densitometric analysis is performed directly on an autoradiograph of the dried gel, rather than on an autoradiograph of a blot of the dried gel (30, 31), no artifact is introduced due to inefficiencies in blotting or in subsequent hybridization of the blot with a radiolabeled probe.

It is not uncommon for mixed sets of primers to interfere with the amplifications, even when individual pairs of primers function normally; this phenomenon is possibly due to inappropriate primer/dimer formation and is usually resolved by using alternative sets of primers. Using the *hprt* and muscarinic receptor primers described in this study, we have noted no such interference, and the amounts of amplified target or reference product were the same irrespective of the presence or absence of the other set of primers.

Expression of muscarinic receptor genes by SH-SY5Y cells. Most radioligand binding studies have indicated that SH-SY5Y cells express a population of M3 receptors (22, 24) that are coupled to release of intracellular calcium, opening of calcium channels, and activation of phosphoinositide hydrolysis, although some studies have reported the existence of a subpopulation of M1 receptors (18), based upon their ability to bind the selective muscarinic antagonist pirenzepine. However, all such binding studies are limited by the inability of any muscarinic antagonist to distinguish sufficiently among the five muscarinic receptor gene products (8). The current study shows that, in addition to the predicted presence of m3 mRNA, SH-SY5Y cells also express m1 and m2 mRNA. Because different primers were used to amplify the m1, m2, and m3 cDNAs, it is not possible to compare strictly the relative amounts of m1, m2, and m3 transcripts. Nevertheless, the amount of m3-amplified product was severalfold greater than those of the m1- or m2-amplified products. While this work was in progress, another study, using subtype-specific antisera, demonstrated a small population of m1 and m2 receptors in this cell line (16), thereby indicating that the transcripts identified in the present study are translated into receptor protein. The function of these m1 and m2 receptors remains unknown.

Regulation of muscarinic gene expression. Chronic agonist exposure frequently leads to subsequent loss of receptor (down-regulation) (2), usually monitored as a loss of antagonist binding sites. As yet few studies have examined changes in receptor mRNA levels consequent to receptor activation, and there are no reports of the relative changes of related transcripts encoding different receptor subtypes expressed in the same cell. Agonist-induced loss of muscarinic receptors has been reported in a number of cell lines, including the parental SK-N-SH line (32, 33); However, it is not known from these studies whether these receptor losses are accompanied by any change in mRNA levels.

The initial rate of agonist-induced receptor loss from the cell surface is very rapid, occurring within 30 min of agonist challenge. It is important to note that this represents sequestration of receptor from the cell membrane but not loss of total receptor, because binding of the hydrophobic muscarinic receptor ligand [^{3}H]QNB is unaffected. This initial loss of receptor is not accompanied or preceded by any parallel loss of m3 mRNA. The subsequent, more gradual, loss of receptor occurs over a much longer time scale and, in this case, is preceded by a parallel fall in m3 mRNA levels, although no causal linkage between the decline in mRNA and receptor levels has yet been demonstrated.

A series of recent studies have examined changes in β-adrenergic receptor mRNA levels in response to receptor activation. These studies have revealed a complex picture. Agonist challenge of DDT$_1$MF-2 cells results in a 40% loss of β-adrenergic receptors after 1 hr; at this time β-adrenergic receptor mRNA levels are unchanged but they decline to similar levels after 16 hr of stimulation (34). Loss of mRNA levels is apparently due to a decrease in mRNA stability (34, 35). No change in the rate of transcription was noted in this report, but in another study (36) agonist stimulation of β-adrenergic receptors

on DDT$_1$MF-2 cells caused a transient rise in transcription and in mRNA levels that was not accompanied by any change in receptor levels. The maximal rise of mRNA levels occurred within 1.5 hr of stimulation, followed by a decline that was maximal after 24 hr. Taken together, these studies imply that transient rises in β-adrenergic receptor mRNA levels may occur as a result of stimulation of transcription, but these changes are not translated into changes in protein levels. As is the case in the present study, the initial decline in receptor numbers preceded the loss of mRNA. Interestingly, activation of HT-29 cells with forskolin causes a transient surge in α_2-adrenergic receptor mRNA levels superimposed on the background of a more gradual rise in mRNA levels; again, the transient surge in transcript levels is not accompanied by a parallel peak in receptor levels (37).

It is clear that transcripts encoding the m2 and m3 mRNAs are all regulated by agonist exposure, but equally clear is the fact that each transcript is differentially regulated. The only responses to muscarinic receptor activation that have been reported in SH-SY5Y cells are a stimulation of phosphoinositide hydrolysis and a biphasic increase in intracellular calcium; the initial phase is due to a release of intracellular calcium, whereas the second phase is consequent to opening of plasma membrane Ca^{2+} channels. Although these responses seem to be activated by stimulation of the major M3 receptor population, it is difficult to know whether activation of the minor populations of m1 and m2 receptors contributes to these intracellular changes or whether those receptors are coupled to alternative signal transduction pathways. Consequently, at present we do not know whether the changes in m2 mRNA levels occur as a result of m3 receptor activation or are a direct consequence of m1 and m2 receptor activation.

In light of these observations, it will be interesting to use recently derived muscarinic receptor-subtype specific antisera to see how the changes in transcript levels reported here are translated into changes in protein level. Clearly, there is a complex interplay controlling the level of expression of the different muscarinic receptor genes in the same cell. The mechanisms regulating these interactions, the relative contributions of transcriptional and post-transcriptional processes, and the consequences of these changes are currently under investigation.

The quantitative reverse transcriptase-PCR method described here is general in its applicability, as long as the appropriate controls are performed to ensure that specific amplification occurs under conditions that produce a linear relationship between the test template and reference template and their corresponding amplified products. In addition to being useful for detecting relative changes in low levels of rare transcripts, such as m1 and m2 in the present study, it is equally applicable to studying changes in transcripts expressed by very small amounts of tissue, such as embryonic brain regions.[2]

Acknowledgments

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