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Characterization of a Decrease in Muscarinic m_2 mRNA in Cerebellar Granule Cells by Carbachol

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ABSTRACT

Studies involving carbachol (100 μM) treatment of cerebellar granule cells for 1, 3, 6, 9, 12 and 24 hr show a decrease in the mRNA encoding for the muscarinic m_2 receptor. The response was transient, decreasing m_2 mRNA by 25 to 50% in 6 and 9 hr, respectively. The data presented in this work were quantified by ribonuclease protection assay, using a [^{32}P]-cRNA probe corresponding to nucleotide +1138 to 1650 of the rat m_2 muscarinic receptor. Because cerebellar granule cells express muscarinic m_2 and m_3 receptors, we tested whether the carbachol-mediated decrease in m_2 mRNA resulted from a homologous or heterologous activation of muscarinic receptors. At a 1 μM concentration, methoctramine specifically blocked the muscarinic m_2 receptor and reversed carbachol's action. These data suggested that carbachol acts *via* a possible homologous activation of muscarinic m_2 receptors. The half-life of the receptor mRNA measured in the presence of actinomycin D with and without carbachol were similar. Because carbachol treatments decrease the steady-state levels of m_2 mRNA without changing the half-life of the message, we suggest that a carbachol treatment induces a decrease in the transcription of the gene for the muscarinic m_2 receptor.

Activation of cholinergic muscarinic m_2 receptors that inhibit adenylyl cyclase induces a rapid receptor desensitization ($T_{1/2}$ of 20 min) in primary cultures of cerebellar granule cells (Contrera *et al.*, 1993). Because all muscarinic receptors belong to a family of receptors that span the plasma membrane seven times and couple to guanine nucleotide binding (G) proteins, the mechanism of desensitization for muscarinic m_2 receptors may be similar to that of other receptors of the same family. A model receptor could be the *beta*$_2$ adrenergic receptor (Lohse *et al.*, 1990; Hadcock and Malbon, 1988; Collins *et al.*, 1989). Briefly, the mechanism of desensitization for the *beta*$_2$ adrenergic receptor, as it is understood today, proceeds in two steps. The first and most rapid step involves the phosphorylation of the receptor by BARK and protein kinase A (Benovic *et al.*, 1989; Lohse *et al.*, 1990). This phosphorylation converts the receptor to a low-affinity state. In addition, receptor sequestration also occurs rapidly. The second and slower step involves a change in the mRNA content that encodes the *beta*$_2$ adrenergic receptor. The events that regulate gene transcription and translation of mRNA are still not completely understood for the *beta* adrenergic receptor, although the increased formation of cyclic AMP is important. It has been reported that the gene for the *beta*$_2$ adrenergic receptor might be regulated by a CRE, a *cis*-acting DNA sequence located upstream from the promoter region. The addition of lipophilic cyclic AMP analogs to cell cultures expressing *beta*$_2$ adrenergic receptors induces a long-term decrease in mRNA encoding the *beta*$_2$ adrenergic receptor and mimics the agonist-mediated response (Hadcock and Malbon, 1988; Collins *et al.*, 1989).

Short-term treatment of cerebellar granule cell cultures with Carb initiates a mechanism of rapid desensitization of muscarinic m_2 receptors similar to the mechanism described for *beta*$_2$ adrenergic receptors. After long-term receptor stimulation by agonist, changes in mRNA-encoding muscarinic receptors also appear to be similar to the changes in mRNA-encoding *beta*$_2$ adrenergic receptors. In addition, stimulation of *beta*$_2$ adrenergic receptors increases the content of mRNA-encoding *alpha*$_1$ adrenergic receptors, showing a cross-regulation between two subtypes of the same receptor family (Morris *et al.*, 1991). In fact, in cerebellar granule cells, activation of muscarinic receptors with Carb decreases the content of both muscarinic m_2 and m_3 mRNAs, which are two of the five possible muscarinic receptor subtypes (m_1 to m_5) expressed in brain (Fukamauchi *et al.*, 1991; McLeskey and Wojcik, 1990); moreover, no detectable amount of m_5 mRNA is present in cerebellar granule cell cultures (unpublished observations). There is the possibility that these two muscarinic receptor subtypes may interact and thereby mediate the agonist-induced decrease in m_2 mRNA. In

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ABBREVIATIONS: BARK, *beta* adrenergic receptor kinase; CRE, cyclic AMP responsive element; Act D, actinomycine D; Carb, carbachol; CTP, cytidine triphosphate.

this report, we attempt to identify whether the regulation of m_2 mRNA results from homologous stimulation of muscarinic m_2 receptors and to further characterize the Carb-induced decrease in muscarinic m_2 mRNA content in cerebellar granule cell cultures from rat.

Methods

Primary cell cultures of rat cerebellar granule cells. Primary cell culture of rat cerebellar granule cells was prepared as previously described (Thangnipon *et al.*, 1983; McLeskey *et al.*, 1990). Briefly, these cultures were derived from cerebella dissected from 8-day-old rat pups. The cerebella were incubated for 10 min at 37°C with 0.025% trypsin and triturated with a Pasteur pipet to dissociate the cells. These cells were plated onto poly-L-lysine coated 10-cm Petri dishes (2.4×10^7 cells/dish). Cultures were grown in basal Eagle medium supplemented with 10% fetal calf serum, 25 mM potassium chloride, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 50 μg/ml gentamicin. Cultures were placed in a humidified environment containing 6% CO_2/95% air for 8 to 9 days before being used in various experiments. Within 24 hr of preparing the cultures, 10 μM cytosine arabinoside was added to the culture medium. All steps in the cell culture preparation were performed in a class 100 hood/environment.

Extraction of RNA. RNA was extracted using the guanidium thiocyanate/phenol-chloroform method with some modifications (Chomczynski and Sacchi, 1987; Thompson and Sommercorn, 1992). Briefly, the cells were scraped in a solution containing 4 M guanidium thiocyanate, 25 mM sodium citrate, pH 7, 0.1 M 2-mercaptoethanol and 0.5% sarcosyl. For every 1 ml of solution, 0.1 ml of 2 M sodium acetate, 1 ml of phenol (saturated with water) and 0.4 ml of a mixture of chloroform-isoamyl alcohol (49:1) were added to separate proteins and DNA from RNA. After centrifugation (10,000 × *g* for 20 min), the aqueous phase (which contained the RNA) was collected and 2.5 volumes of ethanol were added, mixed and centrifuged for 20 min at 10,000 × *g*. The precipitated nucleic acids were resuspended in 300 μl of 4 M lithium chloride and centrifuged at 15,000 × *g* for 15 min. The pellet was resuspended in 300 μl of 10 mM Tris, pH 7.4, 1 mM EDTA and 0.5% sodium dodecyl sulfate and an equal volume of chloroform-isoamyl alcohol (24:1) was added; the samples were mixed and centrifuged at 15,000 × *g* for 15 min. The aqueous phase was collected and 2 M sodium acetate (pH 5.2) was added at a volume 1/10 of the original sample volume. The RNA was pelleted, washed, dried and resuspended in diethyl pyrocarbonate-treated H_2O. The content of total RNA was determined by absorbance measurements at 260 nm.

Preparation of plasmid. cDNA fragment from the 3′ translated and a portion of the untranslated region (position + 1138 to 1650) from the m_2-cDNA (Gocayne et al., 1987) was removed using *Sma*I-*Pst*I and subcloned into pBluescript SK+ (Stratagene). The *in vitro* transcription was carried out on the *Sma*I linearized plasmid with 100 μCi of [^{32}P]CTP and 20 U of T7 polymerase at 37°C for 1 hr (Sambrook *et al.*, 1989). The length of the cRNA probe was 586 bases that comprises 70 bases of the plasmid polylinker region and 516 bases of the m_2 mRNA.

For the structural protein cyclophilin, two different cDNA were used and both provided similar results. One was a 645-base *Pst*I fragment from plasmid p1B15 (Milner and Sutcliffe, 1983; Danielson *et al.*, 1988) that was subcloned into the pGEM 4Z plasmid (Promega) to generate plasmid p15GI (Riva and Mocchetti, 1991). The *in vitro* transcription of this plasmid, linearized with *Nco*I, with T7 polymerase and 1 μCi of [^{32}P]CTP generates a 304-base [^{32}P]cRNA probe which comprises 294 bases of the cyclophilin mRNA and 10 bases of plasmid polylinkers. The other cyclophilin cDNA comprised positions 411 to 705 and was subcloned into the *Eco*RI-*Hin*dIII site of pGEM1 plasmid (Promega). This plasmid was linearized with *Eco*RI, and [^{32}P]cRNA probe was synthesized using SP6 polymerase and 1 μCi of [^{32}P]CTP. The probe (301 bases) comprised 294 bases of the cyclophilin mRNA and 7 bases of plasmid polylinkers. Cyclophilin cRNA was used as an internal standard to control for artifacts due to extraction of RNA from cerebellar granule cell cultures.

RNase protection assay. The m_2 and cyclophilin probes (2.5×10^5 cpm) and 40 μg of total RNA from cerebellar granule cells were hybridized overnight at 50°C in 20 μl of hybridization buffer composed of 80% formamide, 0.4 M NaCl, 1 mM EDTA and 40 mM 1,4-piperazine(ethanesulfonic acid), pH 6.4. RNase buffer (200 μl) containing 10 μg/ml RNase A and 200 U/ml RNase T1 was added to each tube and then incubated for 30 min at 25°C. The RNase mixture was inactivated by the addition of 20 μl of a mixture containing 10 mg/ml proteinase K and 20% sodium dodecyl sulfate. The RNase-protected hybrids were then precipitated with ethanol and separated on a 5% polyacrylamide, 8 M urea gel. Gels were dried under vacuum at 80°C and exposed to X-ray autoradiographic film with intensifying screens.

RNA calculation of m_2 mRNA was quantified by either scanning the protected fragment with a LKB laser densitometer or obtaining the optical density of a digitized image of the band with the SAMBA 4000 (Dynatech Laboratories, Chantilly, VA). The peak densitometry area of the autoradiogram of the m_2 mRNA was normalized by the peak densitometry area of the autoradiogram of the cyclophilin mRNA. Thus, the relative amount of m_2 mRNA was expressed as the ratio of arbitrary units of m_2 mRNA to arbitrary units of cyclophilin mRNA obtained within each experiment. Multiple experiments were averaged by first converting the ratio of m_2/cyclophilin mRNA in the control group to 100% for each experiment and normalizing the treatment groups to their respective control value. Hence, despite the possible varying recovery of RNA throughout all of the described steps, differences in m_2 mRNA values represent differences due to the pharmacological manipulation.

Calculations of m_2 mRNA half-life, rate constant and rate of decline. Act D was added to the cell cultures to block transcription. Thus, the rate of loss in m_2 mRNA content over time reflects the half-life of the mRNA. Data from each study were normalized to the control value and the control value was kept at 100% (the amount of m_2 mRNA present in the cell culture before the addition of actinomycin D). The results from these studies were analyzed by the Manual of Pharmacologic Calculations Computer program (Tallarida and Murray, 1992), which provides the fractional rate constant, k (hr^{-1}). The half-life, $T_{1/2}$ value (sec), was calculated by the following equation: $T_{1/2}$ (hr) = $0.693/k$ (hr^{-1}). The rate of decline was calculated from the following equation: rate of decline (% · hr^{-1}) = [m_2mRNA](% of control) · k (hr^{-1}).

Statistical analyses. Statistical differences between groups of parametric data were analyzed by either the Student's *t* test or the analysis of variance test (Tallarida and Murray, 1992). Nonparametric data were analyzed by the Kruskal Wallis test followed by the Mann Whitney *U* test, which determined differences between groups of a multiple group study (Roscoe, 1975). The individual m_2/cyclophilin mRNA ratios, which were normalized to percentages, were used in these analyses. Statistical significance was selected at the probability level of $P < .05$.

Results

Cerebellar granule cells express m_2 mRNA, as demonstrated by Northern blot analysis, and their content decreases after an exposure to a muscarinic receptor agonist (Fukamuchi *et al.*, 1991). We have used an RNase protection assay which possesses a greater sensitivity than Northern blot to further characterize the m_2 mRNA down-regulation. Initially, we attempted to verify the report by Fukamuchi *et al.* (1991) on the down-regulation of m_2 mRNA by Carb. The relative amount of m_2 mRNA was determined in primary cultures of cerebellar granule cells after treatment with 100 μM Carb. After 1-, 3-, 6-, 9-, 12- and 24-hr treatments with Carb, a statistically significant decrease in the mRNA content encoding the muscarinic m_2 receptor was observed at the 3rd, 6th and 9th hr (fig. 1). The

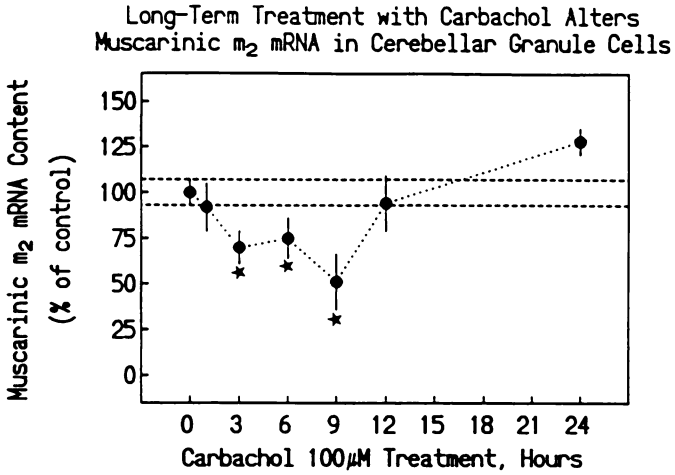

Fig. 1. Carb treatment (100 μM) decreases m_2 mRNA content in cerebellar granule cells. In order to average the different experiments, the control group in each experiment was normalized to 100%. Values are mean ± S.E. for an average of five experiments. The dashed line represents the control ± S.E. Statistical analysis showed the Carb treatment to be different than the control group ($P < .01$ by the analysis of variance. Subsequent comparisons between groups were performed by the Student's *t* test and this is represented as * $P < .05$.

maximal reduction in m_2 mRNA content appeared after the 9th hr of Carb treatment. The m_2 mRNA content returned to control values after 12 and 24 hr of continuous Carb treatment. The results are shown as the average of five experiments in which the relative amount of m_2 mRNA present in the control for each experiment was made 100% and the subsequent amounts measured after different Carb treatment times were calculated relative to the appropriate control. In the five experiments, the peak response to Carb varied from 3 to 9 hr. These experiments were originally performed without using the cyclophilin probe as an internal standard. Subsequent experiments included the cyclophilin riboprobe for a more precise determination of the relative amount of m_2 mRNA.

Ribonuclease protection assay, using total RNA extracted from cerebellar granule cells and [^{32}P]cRNA probes for muscarinic m_2 receptors and cyclophilin, shows two protected fragments of 516 bases (reflecting the m_2 mRNA) and 294 bases (reflecting the cyclophilin mRNA). Specificity of the m_2-cRNA probe was confirmed by showing the presence of m_2 mRNA in rat heart (fig. 2A). No protected fragment was observed in samples containing tRNA (data not shown). With increasing amounts of total RNA, the amount of each protected fragment reflecting m_2 and cyclophilin mRNA also increased (fig. 2 A and B). The ratio of m_2 mRNA to cyclophilin mRNA is similar for each amount of total RNA present during the RNase protection assay, indicating that the cyclophilin riboprobe would be an adequate internal standard for the relative quantification of m_2 mRNA in this assay (fig. 2B). To assume that the RNase protection assay was a quantitative method for measuring changes in the amount of muscarinic m_2 mRNA present within cerebellar granule cells, we used an amount of total RNA which was on the linear portion of the RNA curve, and ratios of m_2 mRNA to cyclophilin mRNA were calculated to account for the variability in RNA extraction.

Figure 3 shows that cycloheximide (30 μM, 6 hr) increases the m_2 mRNA content and that Carb (100 μM, 6 hr), even in the presence of cycloheximide, decreases the m_2 mRNA content by 47%. These results are similar to those shown in figure 1. In these studies with cycloheximide, the cyclophilin mRNA content did not change and all results are shown as ratios of m_2 mRNA content per cyclophilin mRNA content.

Primary cultures of cerebellar granule cells express both muscarinic m_2 and m_3 receptors and Carb shows no specificity for these subtypes. The decrease in m_2 mRNA content could result from Carb's specific stimulation of only muscarinic m_2 receptors, or muscarinic m_3 receptors, or from an activation by Carb of both receptor subtypes. To address this point, methoctramine (1 μM), which at this concentration blocks more than 80% of the muscarinic m_2 receptors and has no action at muscarinic m_3 receptors, was used (McLeskey *et al.*, 1990; McLeskey and Wojcik, 1990). As shown in figure 4, methoctramine alone (1 μM, 6 hr) did not alter the m_2 mRNA content present in these cell cultures, but it prevented the Carb (100 μM, 6 hr)-mediated decrease in m_2 mRNA. In order to detect the m_2 mRNA more readily, cycloheximide (30 μM, concurrently administered with Carb and/or methoctramine) was also included during the drug treatment.

Any change in steady-state levels of m_2 mRNA might reflect either a decrease in transcription of the gene for muscarinic m_2 receptors or a destabilization of the m_2 mRNA. The inhibitor of transcription, Act D (10 μg/ml), was added to the culture medium to block the transcription of the gene for muscarinic m_2 receptors, and the rate of decline and half-life of the m_2 mRNA were determined. Both rate of decline and half-life are thought to reflect the stability of the m_2 mRNA. The Act D treatment alone decreases m_2 mRNA with a $T_{1/2}$ of approximately 1 to 1.5 hr (table 1 and figure 5). This $T_{1/2}$ is similar to that reported by Collins *et al.* (1989) for the *beta*$_2$ adrenergic receptor expressed in DDT$_1$-MF-2 cells. If Act D was added simultaneously with Carb (100 μM) and the relative amounts of m_2 mRNA were determined after 1, 1.5 and 2 hr, the $T_{1/2}$ values of the m_2 mRNA were similar for cell cultures receiving Act D and cultures receiving Carb plus Act D (data not shown). When Act D was added to cell cultures already treated with Carb for 6 hr, the average $T_{1/2}$ of the m_2 mRNA was approximately 1.3 hr (table 1; fig. 5 shows one representative experiment of four). $T_{1/2}$ values for the control group (Act D given after a 6-hr vehicle treatment) and Carb-treated group (Act D given after a 6-hr Carb treatment) were not statistically different. Cerebellar granule cells treated with Carb for 6 hr also showed, after averaging four experiments, a statistically significant decrease by 45% in the amount of m_2 mRNA present in the cells before beginning the Act D treatment (table 1; a Carb-induced decrease in m_2 mRNA is also found in fig. 5). Because of the decreased amount of message, the average rate of decline calculated for the Carb plus Act D treatment was $23 \pm 9\% \cdot hr^{-1}$ and for the control, Act D treatment was $56 \pm 13\% \cdot hr^{-1}$ (table 1). The rates of decline in m_2 mRNA for the Carb plus actinimycine D treatment was 59% lower than the actinomycune D group ($P < .1$, Student's *t* test). Because the steady-state measurement of the m_2 mRNA decreases with a 6- to 9-hr treatment with Carb, we believe that a reduced transcriptional rate of the gene encoding for the muscarinic m_2 receptor might have occurred.

Discussion

In many instances, stimulation of neurotransmitter receptors by agonists either changes the mRNA encoding for that receptor (homologous regulation) or regulates mRNA content encod-

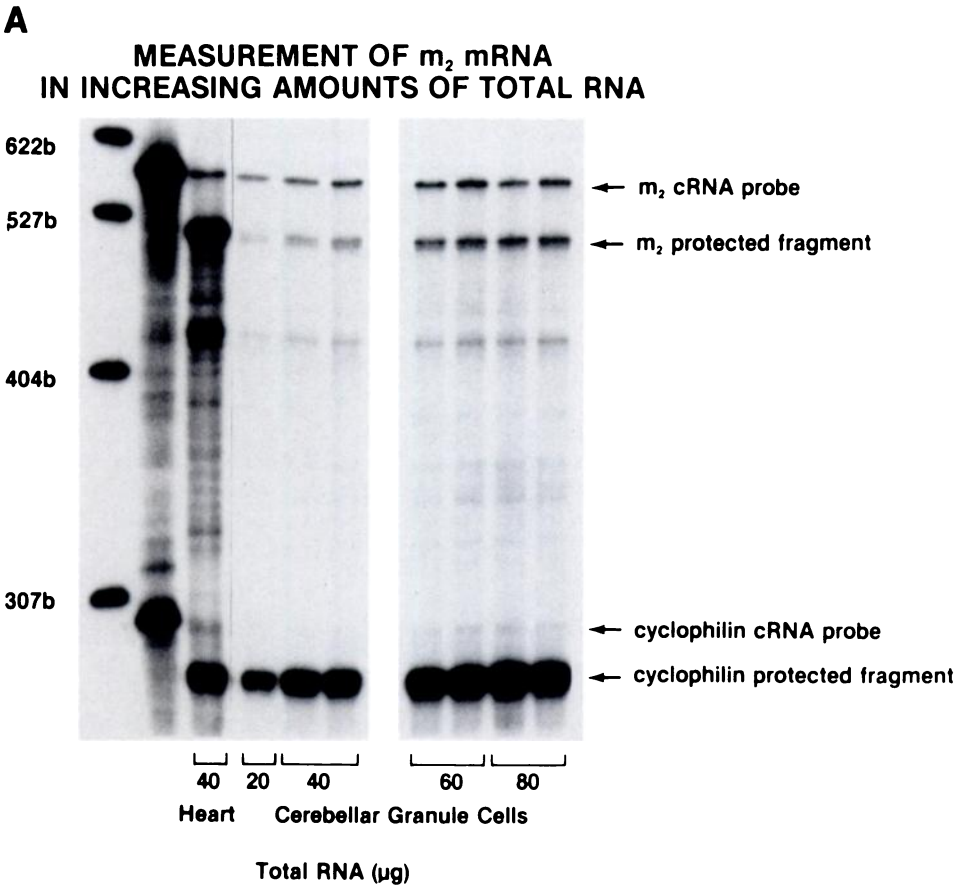

Fig. 2. Hybridization of [^{32}P]m_2-cRNA riboprobe to mRNA encoding for muscarinic m_2 receptors increases proportionately with increasing amounts of total RNA from primary cultures of cerebellar granule cells. A) A representative autoradiogram showing an increase in the protected fragment that reflects either the m_2 mRNA or cyclophilin mRNA with increasing amounts of total RNA. Quantitation of the autoradiogram is shown in panel B. B) Graph shows the amount (intensity units = optical density × square surface area) of m_2 mRNA (●) and cyclophilin mRNA (■) quantified by an image analyzer. The ratio of m_2 mRNA/cyclophilin mRNA (○) is also shown. This is one experiment representative of three.

ing for another neurotransmitter receptor (heterologous regulation). An example of both types of regulation exists in the DDT_1-MF-2 vas deferens clonal cells. In these cultures, long-term exposure to *beta* adrenergic receptor agonists both decreases mRNA content encoding for $beta_2$ adrenergic receptors (a form of homologous regulation) and increases mRNA encoding for $alpha_1$ adrenergic receptors (a form of heterologous regulation) (Hadcock and Malbon, 1988; Collins *et al.*, 1989; Morris *et al.*, 1991). In a somewhat similar manner, the steady-state amount of mRNA encoding for neuronal muscarinic m_2 and m_3 receptors decreased after a long-term treatment with Carb in primary cultures of cerebellar granule cells (Fukamuchi *et al.*, 1991). In that report, atropine and pirenzepine reversed Carb's action. However, neither Carb nor the two antagonists studied were able to descriminate between the two muscarinic receptor subtypes (McLeskey *et al.*, 1990; McLeskey and Wojcik, 1990). Because we were interested in understanding the type of regulation (homologous or heterologous) of mRNA encoding muscarinic m_2 receptors, we asked whether the activation of only the muscarinic m_2 receptor would result in a decreased content of m_2 mRNA. We have previously reported methoctramine (1 μM) to specifically antagonize muscarinic m_2 receptors without blocking muscarinic m_3 receptors. If the Carb-mediated decrease in m_2 mRNA resulted from an activation of only muscarinic m_2 receptors (homologous regulation), 1 μM methoctramine ought to block Carb's response. Alternatively, methoctramine might not interfere with Carb's action if the stimulation of muscarinic m_3 receptors were required (heterologous regulation). As shown in this report, methoctramine prevented the decrease in m_2 mRNA content induced by Carb. These data suggest that a specific activation of muscarinic m_2 receptor mediates the decrease in m_2 mRNA; thus, the regulation appears to be homologous.

Cycloheximide accumulates m_2 mRNA most likely by inhib-

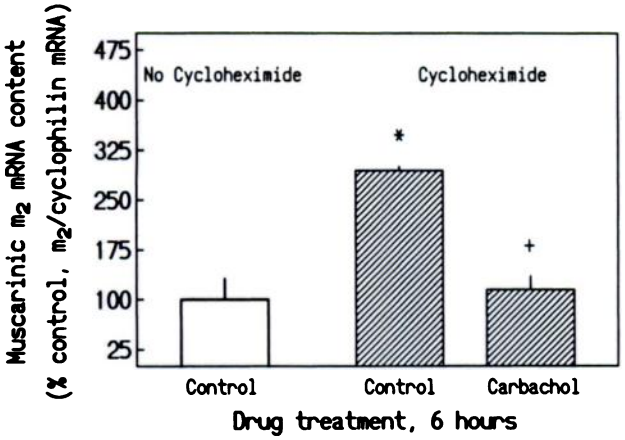

Fig. 3. Carb (100 μM) decreases the muscarinic m_2 mRNA content even in the presence of cycloheximide (30 μM). Cerebellar granule cell cultures were exposed to both Carb and cycloheximide for 6 hr and m_2 and cyclophilin mRNA were determined. The cyclophilin mRNA content was not affected by the cycloheximide treatment. Data represent the ratio of m_2 mRNA and cyclophilin mRNA autoradiographic bands and are the average of three experiments. In order to average the experiments, each experimental control was normalized to 100%. Values are shown as means ± S.E. * $P < .05$ when compared to control with no cycloheximide treatment; and + $P < .05$ when compared to the control with cycloheximide treatment using the Kruskal Wallis nonparametric test with multiple comparisons being performed by the Mann Whitney *U* test.

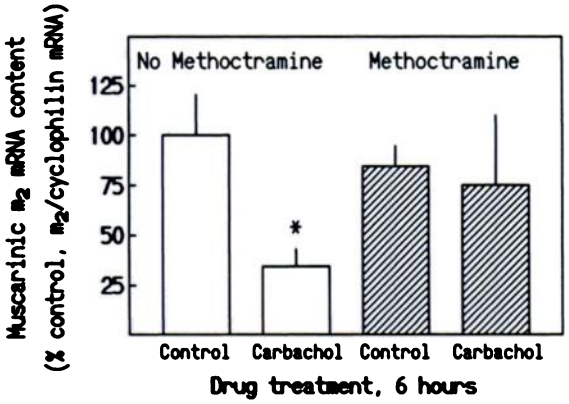

Fig. 4. Methoctramine (1 μM, 6 hr) blocks the Carb-induced decrease in m_2 mRNA content in cerebellar granule cells. Cycloheximide (30 μM, 6 hr) was included in this study because it increases the muscarinic m_2 mRNA content in cerebellar granule cells and, in its presence, Carb is still seen to decrease m_2 mRNA content. Neither cycloheximide nor Carb affected the cyclophilin mRNA content. The figure shows the ratio of muscarinic m_2 mRNA content to cyclophilin mRNA content and the results are the average of three experiments. In order to average the experiments, each experimental control was normalized to 100%. Values are shown as means ± S.E. * $P < .05$ when compared to control using the Kruskal Wallis nonparametric test with multiple comparisons being performed by the Mann Whitney *U* test.

iting the degradation of RNA. Carb, when added concomitantly with cycloheximide, still decreased m_2 mRNA. This finding suggests that Carb's action does not require *de novo* protein synthesis such as the stimulation of an inhibitory transcriptional factor to reduce the content of m_2 mRNA. This raises the possibility that activation of muscarinic m_2 receptors down-regulates muscarinic m_2 receptor gene expression. The mechanism mediating the decreased gene expression is unknown. It might be possible that a cyclic AMP-dependent process is involved, because muscarinic m_2 receptors are coupled to the inhibitory adenylate cyclase.

TABLE 1

Calculations of fractional rate constant, $T_{\frac{1}{2}}$ and rate of decline for m_2 mRNA in the presence and absence of 100 μM Carb

Results represent the average ± S.E. of four experiments. Statistical analysis of the $T_{\frac{1}{2}}$ values, fractional rate constants (k) and rate of decline values for the two groups showed no significant differences by the Student's t test, whereas statistical differences were found for the initial amounts of m_2 mRNA (%) by the t test.

Time = 6 hr	m_2 mRNA	$T_{\frac{1}{2}}$	k	Rate of Decline
	%	hr	hr^{-1}	$\%\cdot hr^{-1}$
Act D	100 ± 14	2 ± 0.6	0.55 ± 0.13	56 ± 13
Carb + Act D	55 ± 8*	1.5 ± 0.4	0.41 ± 0.11	23 ± 9

* $P < .05$ when compared to the Act D treatment.

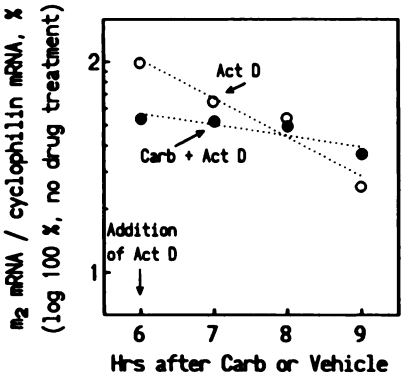

Fig. 5. Relative amounts of m_2 mRNA after Act D treatment in control cerebellar granule cell cultures (○) and 6-hr Carb-treated cell cultures (●). Total RNA was isolated at the indicated times and the amount of m_2 and cyclophilin mRNA was determined. The figure shows the logarithmic amounts of m_2/cyclophilin mRNA after 1, 2 and 3 hr with Act D and is one of four representative experiments. After 6 hr with Carb, the ratio of m_2 mRNA per cyclophilin mRNA was reduced by 47% (● at t = 6 hr). Calculations of the combined values from the four experiments are shown in table 1.

In this report, we also characterized the Carb-induced decrease in m_2 mRNA. A decrease in steady-state levels of m_2 mRNA may result from a decrease in transcription of the gene encoding for the muscarinic m_2 receptor or a decrease in stability of the m_2 mRNA. In order to address this issue, we treated cell cultures with Act D, an inhibitor of transcription. Interestingly, the half-life of the m_2 mRNA in the Act D-treated cells was short, from 1 to 1.5 hr, indicating a rapid expression and metabolism of this message. This observation may not be too surprising because the mRNA encoding for the *beta*$_2$ adrenergic receptors in DDT_1-MF-2 cells also has a short half-life of approximately 1.3 hr (Collins *et al.*, 1989).

In cerebellar granule cells treated with Carb for 6 hr, the half-life of the m_2 mRNA was 1.3 hr, which was similar to the control group containing Act D that was added to the cell cultures after a 6-hr vehicle treatment. Moreover, cerebellar granule cells treated with Carb for 6 hr showed a 45% decrease in the amount of m_2 mRNA present in the cells before beginning the Act D treatment. Because of this initial decreased amount of message, the rate of decline calculated for the Carb plus Act D treatment was statistically different from the rate of decline of m_2 mRNA for the control (Act D alone). These results suggest that the Carb treatment causes a decreased transcription of the gene for muscarinic m_2 receptors without affecting the stability of the m_2 mRNA. These observations agree with a recent report by Habecker and Nathanson (1992) which showed the m_2 mRNA content to decrease in embryonic chicken heart

cells after chronic Carb treatment. There also was no change in the half-life of the m_2 mRNA. Interestingly, the Carb-induced decrease in m_2 mRNA in chicken heart cells was maintained with continuous Carb treatment, whereas only a transient decrease in m_2 mRNA was found in cerebellar granule cells. It is possible that the signal(s) mediating the transient decrease in m_2 mRNA was short-lived.

Many similarities exist between the effects of long-term agonist treatment of either *beta*$_2$ adrenergic or muscarinic m_2 receptors. However, the *beta*$_2$ adrenergic receptor couples to the stimulatory adenylyl cyclase, whereas the muscarinic m_2 receptor couples to the inhibitory adenylyl cyclase. It is, however, unclear whether the mechanism underlying the transient decrease in m_2 mRNA in cerebellar granule cell cultures is similar to the mechanism mediating the change in *beta*$_2$ mRNA resulting from *beta* adrenergic receptor stimulation.

Acknowledgments

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References

BENOVIC, J. L., DEBLASI, A., STONE, W. C., CARON, M. G. AND LEFKOWITZ, R. J.: Beta-adrenergic receptor kinase: Primary structure delinates a multigene family. Science **246:** 235–240, 1989.

CHOMCZYNSKI, P. AND SACCHI, N.: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162:** 156–159, 1987.

COLLINS, S., BOUVIER, M., BOLANOWSKI, M. A., CARON, M. G. AND LEFKOWITZ, R. J.: cAMP stimulates transcription of the beta$_2$-adrenergic receptor gene in response to short-term agonist exposure. Proc. Natl. Acad. Sci. U.S.A. **86:** 4853–4857, 1989.

DANIELSON P. E., FORSS-PETTER S., BROWN M. A., CALAVETTA L., DOUGLAS J., MILNER R. J. AND SUTCLIFFE G. J.: p1B15: A cDNA clone of the rat mRNA encoding cyclophilin. DNA **4:** 261–267, 1988.

FUKAMAUCHI, F., HOUGH, C. AND CHUANG, D.-M.: Expression and agonist-induced down-regulation of mRNAs of m2- and m3- muscarinic acetylcholine receptors in cultured cerebellar granule cells. J. Neurochem. **56:** 716–719, 1991.

GOCAYNE, J., ROBINSON, D. A., FITZGERALD, M. G., CHUNG, F.-Z., KERLAVAGE, A. R., LENTES, K.-U., LAI, J., WANG, C.-D., FRASER, C. M. AND VENTER, J. C.: Primary structure of rat cardiac B-adrenergic and muscarinic cholinergic receptors obtained by automated DNA sequence analysis: Further evidence for a multigene family. Proc. Natl. Acad. Sci. U.S.A. **84:** 8296–8300, 1987.

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

HADCOCK, J. R. AND MALBON C. C.: Down regulation of beta-adrenergic receptors: Agonist-induced reduction in receptor mRNA levels. Proc. Natl. Acad. Sci. U.S.A. **85:** 5021–5025, 1988.

LOHSE, M. J., BENOVIC, J. L., CARON, M. C. AND LEFKOWITZ, R. J.: Multiple pathways of rapid beta$_2$-adrenergic receptor desensitization. J. Biol. Chem. **265:** 3202–3209, 1990.

MCLESKEY, S., FISCHOFER-HAHN, C., TAKAHASHI, K. AND WOJCIK, W. J.: Specificity of methoctramine in blocking muscarinic receptors which inhibit adenylate cyclase in cerebellar granule cells. Neuropharmacology **29:** 853–860, 1990.

MCLESKEY, S. W. AND WOJCIK, W. J.: Identification of muscarinic receptor subtypes present in cerebellar granule cells: Prevention of ^{3}H-propylbenzilylcholine mustard binding with specific antagonists. Neuropharmacology **29:** 861–868, 1990.

MILNER, R. T. AND SUTCLIFFE, J. G.: Gene expression in rat brain. Nucleic Acids Res. **11:** 5497–5520, 1983.

MORRIS, G. M., HADCOCK, J. R. AND MALBON, C. C.: Cross regulation between G-protein-coupled receptors: Activation of beta$_2$-adrenergic receptors increases alpha$_1$-adrenergic receptors mRNA levels. J. Biol. Chem. **266:** 2233–2238, 1991.

RIVA, M. A. AND MOCCHETTI, I.: Developmental expression of the basic fibroblast growth factor gene in rat brain. Dev. Brain Res. **62:** 45–50, 1991.

ROSCOE, J. T.: Fundamental Research Statistics for the Behavioral Sciences, Holt, Rinehart and Winston, Inc., Philadelphia, 1975.

SAMBROOK, J., FRITSCH, E. F. AND MANIATIS, T.: Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989.

TALLARIDA, R. J. AND MURRAY, R. B.: Manual of Pharmacologic Calculations with Computer Programs, version 4.2(B) software, Springer-Verlag, New York, 1992.

THANGNIPON, W., KINGSBURY, A., WEBB, M. AND BALAZS, R.: Observations on rat cerebellar cells *in vitro*: Influence of substratum, potassium concentration and relationship between neurones and astrocytes. Dev. Brain Res. **11:** 177–189, 1983.

THOMPSON, D. B. AND SOMMERCORN, J.: Use of a multiple S1 nuclease protection assay to monitor changes in RNA levels for type I phosphatase and several proto-oncogenes in response to insulin. J. Biol. Chem. **267:** 5921–5926, 1992.

CONTRERA, J. G., MCLESKEY, S. W., HOLOPAINEN, I., XU, J. AND WOJCIK, W. J.: Muscarinic m_2 receptors in cerebellar granule cell cultures from rat: Mechanism of short-term desensitization. J. Pharmacol. Exp. Ther. **265:** 433–440, 1993.

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