The Journal of Neuroscience, April 1994, 14(4): 2106–2116

A Single Transmitter Regulates Gene Expression through Two Separate Mechanisms: Cholinergic Regulation of Phenylethanolamine *N*-Methyltransferase mRNA via Nicotinic and Muscarinic Pathways

Marian J. Evinger,[1] Paul Ernsberger,[2] S. Regunathan,[3] T. H. Joh,[3] and D. J. Reis[3]

[1]Departments of Pediatrics and of Neurobiology & Behavior, SUNY at Stony Brook, Stony Brook, New York 11794-8111, [2]Departments of Medicine and of Neuroscience, Case Western Reserve University, School of Medicine, Cleveland, Ohio 44106, and [3]Department of Neurology and Neuroscience, Cornell University College of Medicine, New York, New York 10021

ACh regulates the gene encoding phenylethanolamine *N*-methyltransferase (PNMT) in bovine adrenal chromaffin cells. In addition to stimulating catecholamine release from these cells, cholinergic agents elevate transcription of the PNMT gene. Carbachol, which activates both nicotinic and muscarinic receptors, produces 12–19-fold increases in PNMT mRNA and a 22-fold increase in epinephrine release. Selective nicotinic and muscarinic antagonists (hexamethonium and atropine) each partially reduce carbachol-stimulated increases in PNMT mRNA while a combination of both eliminates >90% of the carbachol response, thus indicating that separable nicotinic and muscarinic components contribute to the cholinergic increase in PNMT mRNA. Muscarine alone produces a dose-dependent increase (mean sixfold) in steady state PNMT mRNA levels and stimulates the rate of transcription fivefold. Only atropine and the m3–m4-selective muscarinic antagonist 4-diphenylacetoxy-4-methylpiperidine (4-DAMP) reduce the response to muscarine, strongly suggesting that the m4 receptor is crucial for PNMT mRNA activation. In these chromaffin cells, muscarine inhibits adenylate cyclase, antagonists bind with affinities characteristic of m4 receptors, and cDNA hybridization detects only m4 mRNAs (Fernando et al., 1991). Nicotine also induces a dose-dependent increase (mean of 8.5-fold) in PNMT mRNA levels. The importance of voltage-gated Ca^{2+} channels in the nicotine effect is demonstrated by the stimulatory effects of calcium ionophores on PNMT mRNA levels (two- to fivefold increase) and the ability of the L- and N-type channel blockers nifedipine and ω-conotoxin to decrease the nicotine response (by 60% and 40%, respectively). Nuclear "run-on" assays further reveal that nicotine enhances transcription of the PNMT gene (approximately fourfold). Thus, this study provides the first demonstration that both nicotinic and muscarinic stimulation modify genomic responses of bovine adrenergic chromaffin cells and identifies possible mechanisms.

[Key words: transcription, primary cultures, catecholamine genes, neural regulation, gene expression]

Stimulation of chromaffin cells of the adrenal medulla by preganglionic cholinergic neurons not only releases norepinephrine and epinephrine from the gland but, when prolonged, increases the activities, amounts, and mRNAs for several enzymes subserving catecholamine biosynthesis (Stachowiak et al., 1990a; Fossom et al., 1991). The epinephrine (adrenaline)-synthesizing enzyme phenylethanolamine *N*-methyltransferase (PNMT) is induced by cholinergic stimulation of the adrenal medulla (Wakade et al., 1988). While both of these actions of preganglionic nerve activity are mediated presynaptically by ACh, the class of cholinergic receptor responsible for each action is less certain. In some species—for example, cat, guinea pig, and rat—both nicotinic and muscarinic receptors can stimulate catecholamine secretion from adrenal medullary cells, while only nicotinic agonists are active in cow (reviewed in Carmichael, 1986). However, muscarinic receptor stimulation potentiates nicotinic effects on catecholamine release (Barron and Hexum, 1986).

Conflicting evidence exists regarding the ability of both receptors to induce production of the mRNA coding for PNMT (Evinger et al., 1988; Stachowiak et al., 1990a; Wan et al., 1991). It therefore is of considerable interest to resolve whether stimulated muscarinic and nicotinic receptors are each capable of activating the gene for PNMT. Since intracellular signal transduction pathways for adrenal muscarinic and nicotinic receptors differ substantially, this would suggest that two divergent intracellular pathways responding to the same transmitter must converge within the cell to activate a common gene.

In the present study we investigate whether selective stimulation of cholinergic muscarinic and nicotinic receptors on bovine adrenal chromaffin cells in culture can initiate transcription of the PNMT gene to promote accumulation of PNMT mRNA. We demonstrate that both receptors are capable of this action.

Materials and Methods

Chromaffin cell cultures. Chromaffin cells were established in primary culture by a modification of the method of Wilson (1987), as described in Ross et al. (1990). Briefly, freshly isolated medullae from bovine adrenal glands (Max Insel Cohen, Inc.) were dispersed in a solution of DNase I (Sigma; 50 μg/ml) and collagenase (type I; Worthington Biochemicals; 2 mg/ml) and then fractionated by density centrifugation on Renografin gradients. Cells were cultured at a density of 2.7×10^7 per 10 cm plate in Dulbecco's modified Eagle's medium:F12 medium supplemented with 10% fetal bovine serum, 10 mM HEPES, pH 7.4, and penicillin, streptomycin, and neomycin antibiotic (GIBCO/Bethesda Research Labs).

Northern analyses of chromaffin cell RNA. Total RNA was isolated from each of triplicate chromaffin cell cultures by incubation in 0.5%

Received Mar. 15, 1993; revised Aug. 27, 1993; accepted Sept. 29, 1993.

This work was supported by grants GM 46588 to M.J.E., HL44514 to P.E., and, in the initial phases of this study, HL 18974 to D.J.R.

Correspondence should be addressed to Marian J. Evinger, Ph.D., Department of Pediatrics, Health Sciences Center T-11-045, SUNY at Stony Brook, Stony Brook, NY 11794-8111.

NP-40 detergent and phenol extraction (Ausubel et al., 1991) with 10 μg/ml glycogen added to improve recovery. Northern analysis of 10–15 μg of total RNA following denaturing formaldehyde agarose gel electrophoresis was performed to detect the presence of specific mRNAs. Random-primed PNMT cDNA (Baetge et al., 1986) was used for detection of the 1100 nucleotide PNMT mRNA, while an 18 S rRNA (gift of R. Guntaka, Univ. Missouri) or α-tubulin cDNA (Carroll et al., 1991) was used for subsequent probing of blots to normalize for any variation in loading between samples. Hybridization was performed in 50% formamide, 2× SSC (standard saline citrate: 0.15 M NaCl, 0.015 M sodium citrate), 10 mM PIPES, pH 6.5, 1× Denhardt's solution, 100 μg/ml calf thymus DNA, and 50 μg/ml *Escherichia coli* tRNA at 42°C for 16–24 hr. Washing was performed at 55°C to 0.1× SSC. Following autoradiography with an intensifying screen, the resulting films were scanned with an LKB densitometer. Multiple exposures were performed to ensure that the autoradiographic signals were within the linear response range of the Kodak XAR film.

Transcription elongation assays. In vitro nuclear transcription run-on assays to measure the rate of PNMT gene transcription were performed as described by Evinger et al. (1992b). Nuclei were prepared from bovine chromaffin cell cultures treated for 1 hr prior to harvest by scraping and washing in phosphate-buffered saline. Cell lysis was accomplished by incubation in buffer A (10 mM Tris, pH 8.0, 3 mM $CaCl_2$, 2 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.3 M sucrose) for 5 min on ice, centrifugation, and resuspension in buffer A plus 0.5% NP-40 detergent for 5 min (0 °C). Nuclei were washed twice by centrifugation in buffer A and then resuspended in buffer B [50 mM HEPES, pH 7.0, 5 mM $MgCl_2$, 0.5 mM dithiothreitol, and 25% (v/v) glycerol] at 5×10^7 nuclei/ml.

Elongation assays and hybridizations were performed as previously described (Evinger and Joh, 1989; Evinger et al., 1992b). Transcription was initiated by the addition of nuclei ($2–5 \times 10^6$), allowed to proceed for 20 min at 22°C, and then terminated by digestion with DNase (2 μg/ml) for 30 min, followed by Proteinase K (100 μg/ml) and 0.1% SDS. Heterogeneous nuclear RNAs (hnRNAs) were extracted and precipitated prior to two successive rounds of DNase and Proteinase K digestion. Total cpm incorporated into hnRNAs was determined by trichloroacetic acid precipitation.

hnRNAs were hybridized (as described above) with each reaction containing duplicate filters of PNMT cDNA (or antisense cRNA) and, as an index of nonspecific binding, equal amounts of pGEM-3 DNA (or *E. coli* rRNA). Hybridization proceeded at 42°C for 48 hr. Filters were then washed twice in 0.3 M NaCl, 1 mM EDTA, 0.1% SDS, and then incubated with RNases A (20 μg/ml) and T_1 (10 U/ml) to digest unhybridized transcripts. Washing continued using progressively stringent conditions until no more counts were detectable in the eluate. All filters were washed at least 3 hr in 0.1× SSC at 65°C.

Catecholamine quantitation. Catecholamines were measured in chromaffin cells and in culture medium following 10 min of treatment (Regunathan et al., 1991b). Following alumina extraction, a μ-Bondpak C18 reverse-phase column (Millipore-Waters) fractionated catecholamines via isocratic elution using 0.1 M citrate/acetate buffer (pH 4.5) containing 1 mM sodium octyl sulfate and 2 mM EDTA. Flow rate was maintained at 0.8 ml/min at a column temperature of 35°C. Electrode potential was +0.65 V versus Ag/AgCl reference electrode; sensitivity was 1 nA full scale. Baseline separation was achieved for norepinephrine, epinephrine, and dopamine with retention times of 6, 8, and 14 min, respectively.

PNMT enzymatic activity was measured using ^{3}H-S-adenosyl methionine (^{3}H-SAM) as substrate with protein determined by the Lowry method (Evinger et al., 1992b).

Measurement of second messengers. Production of cAMP and cGMP was measured by radioimmunoassay as described in Regunathan et al. (1991a). Assays for cAMP and cGMP were carried out using the nonacetylated protocols with the Amersham radioimmunoassay kits.

Inositol phosphate hydrolysis was assayed by estimating the accumulation of inositol-1-phosphate in the presence of lithium according to the method of Berridge as described by Regunathan et al. (1991a). Cells (10^6) were prelabeled with 2 μCi of ^{3}H-inositol for 16 hr in cell culture medium. Following labeling and then washing in Krebs-Ringer bicarbonate buffer (KRB) containing 10 mM unlabeled inositol, cells were incubated in the presence of drugs for 30 min in KRB containing 10 mM lithium chloride. After extraction and then fractionation on an AG 1-X8 (Bio-Rad) column, inositol-1-phosphate was eluted using 0.2 M ammonium formate in 0.1 M formic acid. Radioactivity was measured by liquid scintillation counting. Results represent dpm of inositol-1-phosphate expressed as percentage of total dpm (inositol-1-phosphate + lipid dpm).

Table 1. Effect of cholinergic agonists on the release of catecholamines from bovine adrenal chromaffin cells

	Epinephrine (% of cell content)	Norepinephrine (% of cell content)
Control	0.95 ± 0.15	1.05 ± 0.21
Carbachol (100 μM)	21.3 ± 2.4*	29.3 ± 2.6*
Nicotine (10 μM)	19.5 ± 1.8*	27.3 ± 2.2*
Carbachol + hexamethonium (100 μM)	2.5 ± 0.5**	2.4 ± 0.6**
Nicotine + hexamethonium (100 μM)	1.5 ± 0.7**	2.1 ± 0.8**

The release of catecholamines from chromaffin cells in 10 min was measured in the presence of cholinergic agents. The content of catecholamines in the cells was 21.5 ± 2.6 and 17.8 ± 1.9 nmol/10^6 cells for epinephrine and norepinephrine, respectively. Values are mean ± SE from four or five experiments.

* $p < 0.001$ compared to control.

** $p < 0.001$ compared to carbachol or nicotine group.

^{3}H-QNB binding assay for muscarinic ACh receptor sites. Radioligand binding assays with ^{3}H-QNB (^{3}H-quinuclidinyl benzilate) for determination of specific binding to muscarinic receptor subtypes were performed as previously described (Haxhiu-Poskurica et al., 1993). Membrane pellets were slowly thawed and resuspended at a concentration of 100–120 μg protein/ml in Tris-HCl buffer. Assays were conducted in a total volume of 250 μl in polypropylene 96-well plates (Beckman Macrowell), and each well contained 125 μl of membrane suspension, 25 μl of radioligand, and 100 μl of drug or vehicle (water). Incubations were initiated by the addition of membrane suspension and were carried out for 60 min at 25°C. Nonspecific binding of ^{3}H-QNB was defined in the presence of 1.0 μM atropine. Incubations were terminated by vacuum filtration over prewetted Whatman GF/B glass fiber filters using a cell harvester (Brandel). The filters were washed four times with 5 ml of ice-cold Tris-HCl, transferred to minivials, covered with 4 ml of scintillation cocktail (Bio-Safe II, Research Products International), and counted at 50% efficiency for up to 5 min. Protein content of membrane suspension was determined by the Peterson (1977) method.

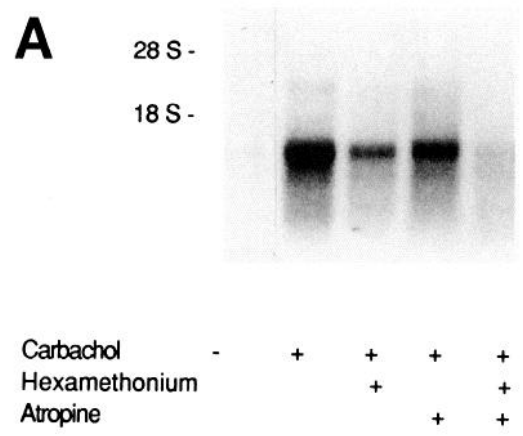
Data were obtained as dpm and transferred to the EBDA (Equilibrium Binding Data Analysis) program (McPherson, 1985) for initial processing, and then 4–10 experiments were analyzed simultaneously using the LIGAND program for nonlinear curve fitting (Munson and Rodbard, 1980). Protein assay data also were analyzed by nonlinear curve fitting (McPherson, 1985).

Reagents. Nicotine tartrate (±) (Sigma) was freshly prepared as a 5 mM stock. Muscarine and ω-conotoxin were purchased from Research Biochemicals, Inc. The calcium ionophore A23187 (Pharmacia) was prepared as a 100 μM stock in dimethyl sulfoxide. ^{3}H-QNB (40–60 Ci/mmol) was obtained from New England Nuclear (Boston, MA), stored at −20°C in ethanol, and diluted in water prior to assay. Stock solutions of most drugs were made in 0.01 M acetic acid and diluted in water immediately prior to assay. Pirenzepine (M_1 antagonist), methoctramine (M_2 antagonist), and 4-diphenylacetoxy-4-methylpiperidine (4-DAMP) (M_3/M_4 antagonist) were obtained from Research Biochemicals International (Natick, MA). AF-DX 116 was a gift from Boehringer-Ingelheim (Ridgefield, CT). Atropine, scopolamine, and other compounds were obtained from Sigma Chemical (St. Louis, MO). All other reagents were obtained from routine laboratory suppliers.

Results

Effects of carbachol on PNMT mRNA

In order to resolve whether a single transmitter, ACh, could act simultaneously through separate muscarinic and nicotinic receptor populations, the dual cholinergic agonist carbachol was first employed to ascertain whether PNMT mRNA levels respond to cholinergic stimulation. Exposure of primary cultures



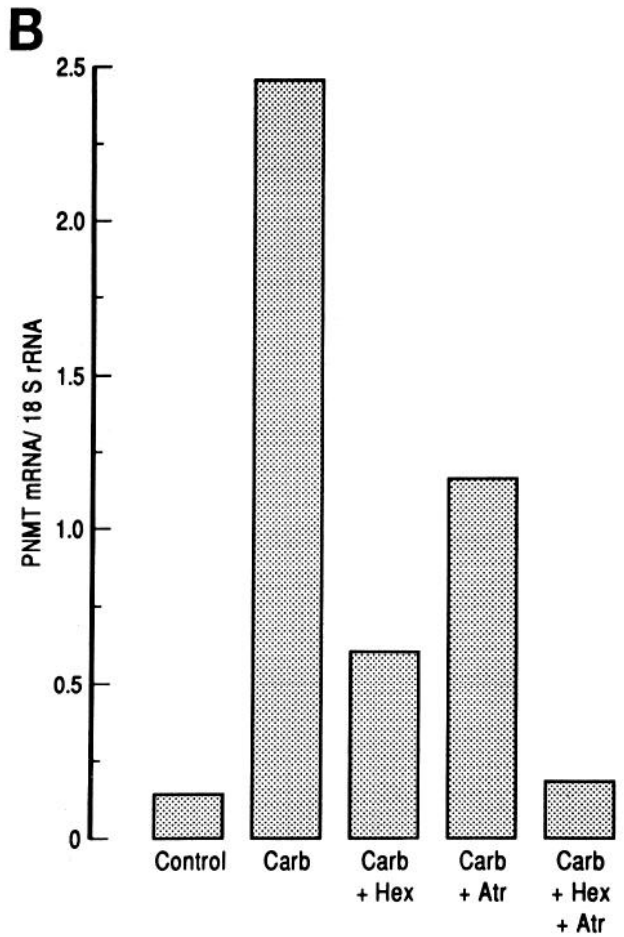


Figure 1. Cholinergic regulation of PNMT mRNA. To determine whether PNMT mRNA levels are regulated by cholinergic stimuli, chromaffin cell cultures were incubated with (1) media alone, (2) 200 μM carbachol, (3) carbachol + 10 μM hexamethonium (nicotinic antagonist), (4) carbachol + 1 μM atropine (muscarinic antagonist), or (5) carbachol + hexamethonium and atropine. *A,* Northern blots were probed first with PNMT cDNA and then with cDNA for 18 S rRNA. *B,* Changes in mRNA are expressed as the ratio of PNMT:18 S rRNA autoradiographic intensities.

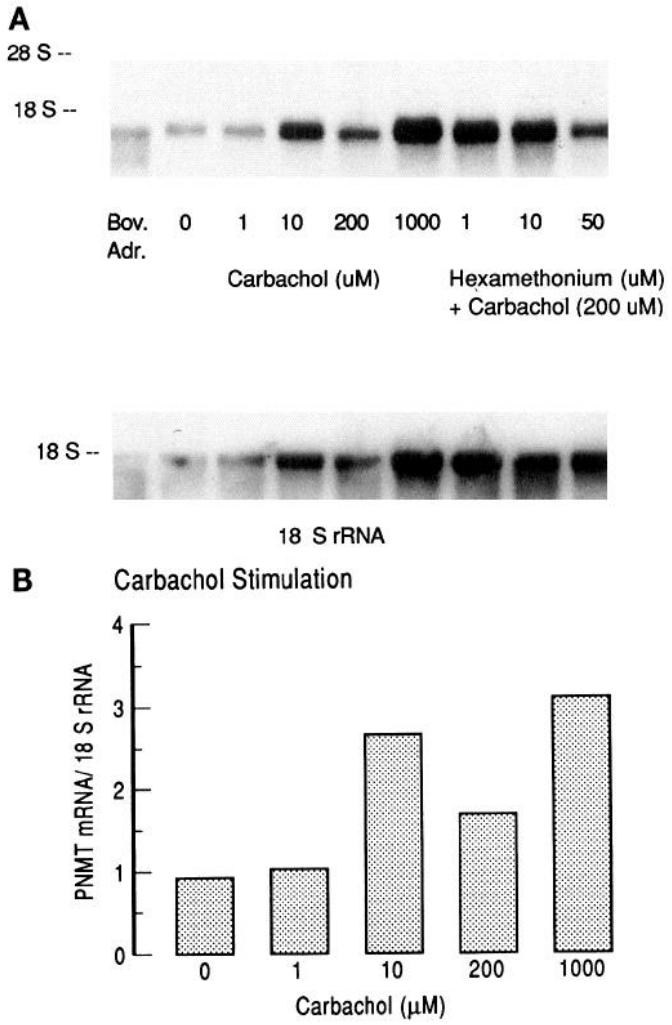
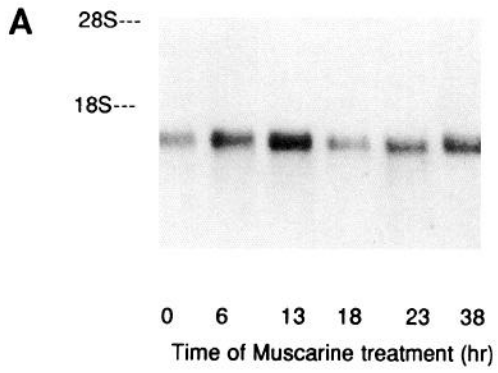


Figure 2. Nicotinic modulation of PNMT mRNA. *A,* Autoradiograms depicting the dose response of PNMT mRNA (*top*) and 18 S rRNA (*bottom*) in bovine adrenal chromaffin cell cultures after 18 hr of incubation in the presence of carbachol. The nicotinic component of this response is evidenced by the decreasing steady state levels of carbachol-stimulated PNMT mRNA in the presence of increasing concentrations of hexamethonium. *B,* Densitometric quantitation of the autoradiographic data for carbachol induction.

of bovine adrenal chromaffin cells to carbachol (100 μM) elicited a 22-fold and 28-fold increase of release into the medium of epinephrine and norepinephrine, respectively (Table 1). This same concentration of carbachol also increased the accumulation of PNMT mRNA 12–19-fold (mean of 13.4-fold for n = 6 experiments) after 16 hr of treatment as determined by Northern blot analysis (Fig. 1). The increase in PNMT mRNA elicited by carbachol was dose dependent, with a maximum effect obtained at 200 μM (Fig. 2). At higher doses, the response declined, an effect comparable to the reported reduction of catecholamine secretion, and presumably a result of receptor desensitization (Marley, 1988).

To establish that the responses were receptor mediated, cultures were treated with carbachol (200 μM) alone or with carbachol following incubation (20 min) with the nicotinic receptor antagonist hexamethonium bromide (10 μM) or the muscarinic receptor antagonist atropine (10 μM). Hexamethonium inhibited by 75% the carbachol-stimulated increase (18-fold) in PNMT mRNA (Fig. 1*B*), consistent with evidence that the major cho-



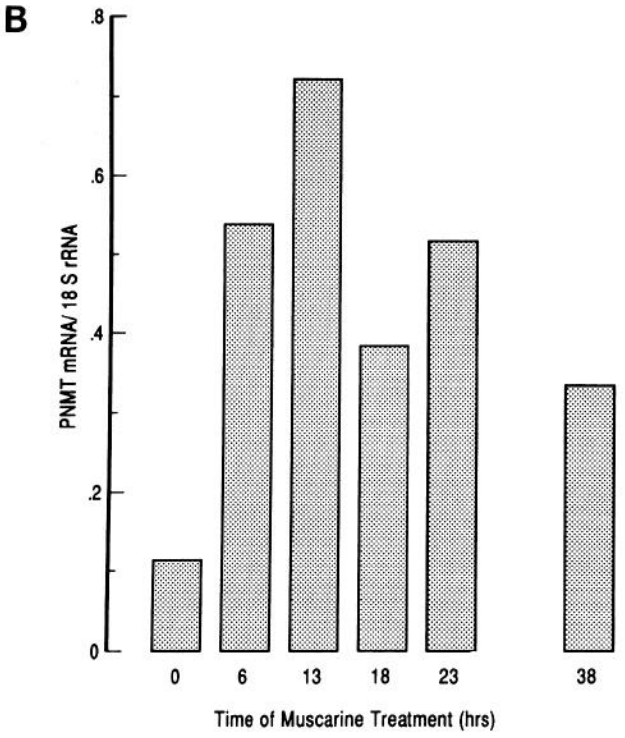


Figure 3. Muscarinic stimulation of PNMT mRNA. *A*, Northern blot analysis of the time course for muscarinic induction of PNMT mRNA in chromaffin cell cultures incubated with 100 μM muscarine. *B*, Densitometric quantitation of PNMT mRNA relative to 18 S rRNA reveals maximal accumulation is observed at 13 hr with response again approaching control levels after 38 hr.

linergic response in bovine chromaffin cells is mediated by nicotinic receptors (reviewed in Carmichael, 1986). Atropine reduced the response by 50%. Combined, hexamethonium and atropine reduced the response by > 90%. There was no change from control levels when cultures were treated with hexamethonium or atropine in the absence of carbachol. Thus, cholinergic stimulation of bovine chromaffin cells increases PNMT mRNA through actions on both nicotinic and muscarinic cholinergic receptors.

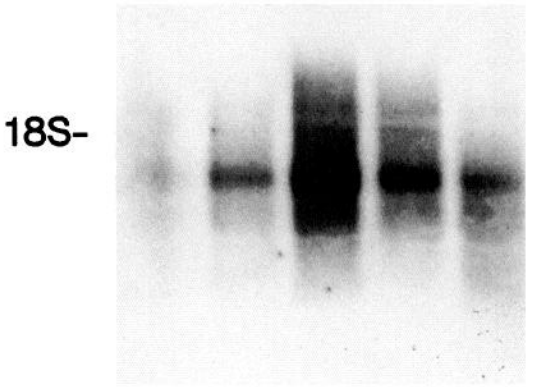


Figure 4. Inhibition of the muscarinic response by antagonists. Muscarine-stimulated levels of PNMT were compared to media-treated controls (*lane 1*) after a total of 20 hr in culture with muscarine present for 2 hr (*lane 2*) or 20 hr (*lane 3*). The PNMT mRNA response does not appear to desensitize but rather increases with extended exposure to muscarine. As seen previously (Fig. 1), 15 min of incubation with atropine prior to addition of muscarine (100 μM) blocks muscarinic-stimulated effects after 20 hr in culture (*lane 4*). The m3–m4-selective antagonist 4-DAMP (10 μM) added 15 min prior to muscarine appears to have a similar inhibitory influence on PNMT message levels after 20 hr in culture (*lane 5*).

Stimulation of muscarinic cholinergic receptors increases levels of PNMT mRNA

Muscarine chloride, a selective agonist at muscarinic cholinergic receptors, dose dependently increased PNMT mRNA, with maximal effects obtained at 100 μM and 50% maximal response at 25–30 μM (not shown). The increase is detectable at 1 hr, is maximally elevated at 12–16 hr, and requires >38 hr of treatment to return to unstimulated levels (Fig. 3). The increase in PNMT mRNA elicited by muscarine was directly related to the duration of exposure to the drug. For example, when chromaffin cells were exposed for 2 hr to 100 μM muscarine, followed by replacement with conditioned medium (from untreated cultures), the increase in PNMT mRNA was fourfold at 20 hr (Fig. 4, lane 2). In contrast, continuous exposure to muscarine for 20 hr resulted in a 12-fold increase in PNMT mRNA (Fig. 4, lane 3).

Muscarine elicited a mean maximal increase in PNMT mRNA of 6.3-fold (range, 3.9–12-fold; $n = 7$). There was greater experimental variability in response to muscarine than was produced by exposure to nicotine or by depolarization with potassium (see below). Magnitude of response also varied between commercial batches of muscarine. Choline chloride (100 μM) had no effect on PNMT mRNA levels (not shown), indicating that the effects of muscarine chloride were not attributable to the chloride counterion.

The catalytic activity of PNMT also increased following treatment with muscarine. Exposure to 100 μM muscarine increased PNMT enzymatic activity from 19.5 to 35.4 and from 5.85 to 10.61 pmol ^{3}H-SAM/mg protein in two separate experiments. However, since PNMT mRNA elevation precedes accumulation of enzyme protein, this mean 1.8-fold increase in enzymatic activity at 16 hr would be expected to be less than the maximum response occurring at 24–36 hr.

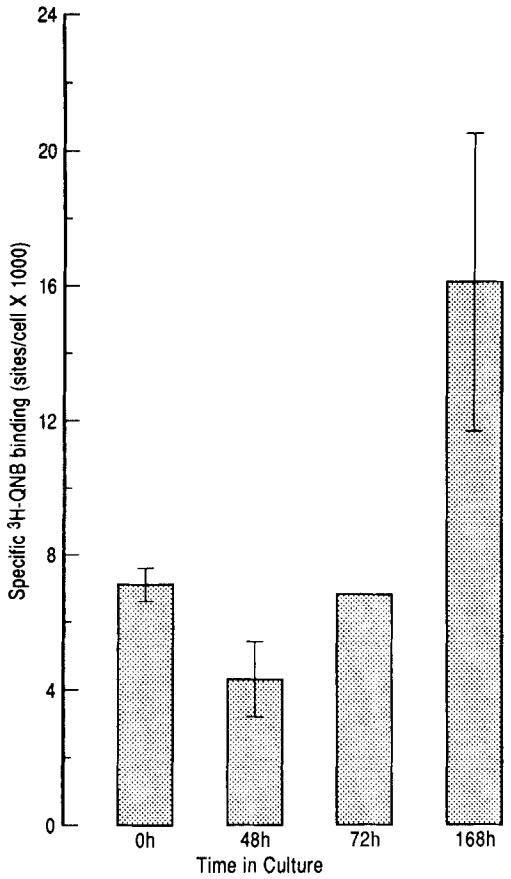


Figure 5. Specific ^{3}H-QNB binding to muscarinic receptors in membranes from chromaffin cell maintained for different lengths of time in culture. Chromaffin cell membranes were obtained immediately prior to culture, and after 48, 72, and 168 hr in culture. Chromaffin cell membranes were incubated with 0.2 nM ^{3}H-QNB for 60 min at 25°C. Nonspecific binding was defined in parallel incubations containing 1 μM atropine and was not affected by time in culture. Values (mean ± SEM) represent the average of two experiments, each conducted in duplicate.

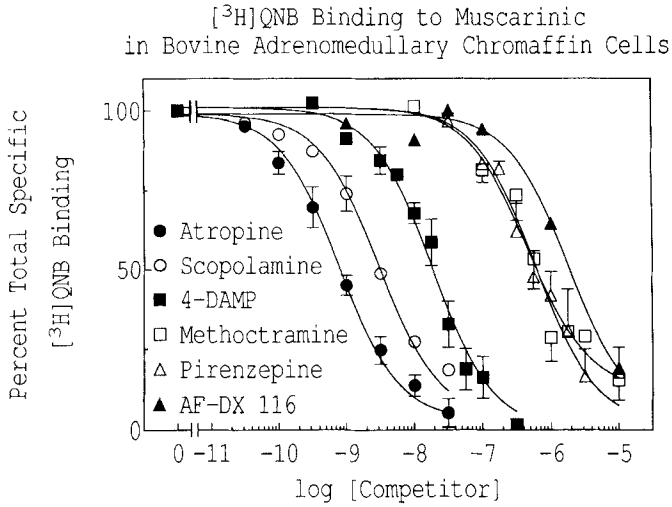


Figure 6. Dose-dependent inhibition by muscarinic antagonists of specific ^{3}H-QNB binding in membranes prepared from bovine adrenal medullary chromaffin cells. Data represent the mean ± SE from four or five experiments conducted in triplicate and are expressed as a percentage of total specific binding defined as the difference between total binding and that remaining in the presence of 1.0 μM atropine. Curves were generated by nonlinear curve fitting to a single-component logistic function (INPLOT, GRAPHPAD software). Each curve accounts for a minimum of 96% of the total variance in the data (r^2 values range from 0.961 to 0.996).

Muscarinic receptor subtype

Induction of PNMT message by muscarine is blocked by the nonselective muscarinic receptor antagonist atropine (1 μM) (Fig. 4, lane 4). Since hybridization analyses of muscarinic receptor mRNAs have established that bovine adrenal chromaffin cells express only the m4 muscarinic receptor subtype mRNA (Fernando et al., 1991), we investigated whether this subtype is the receptor mediating the induction of PNMT mRNA by muscarine.

Chromaffin cells were exposed for 15 min to the m3–m4-selective antagonist 4-DAMP, the m1-selective antagonist pirenzepine, or the m2 (cardiac)-selective antagonist AF-DX 116, before incubation with muscarine (100 μM). Pretreatment with 4-DAMP (Fig. 4, lane 5), but not with pirenzepine or AF-DX 116 (not shown), blocked by 80% the muscarine-stimulated increase in PNMT mRNA. A less than 10% change occurred when antagonists alone were tested. This result is consistent with the effect being mediated by muscarinic receptors of the m4 subclass.

Further confirmation of the presence of m4 receptors on chromaffin cells in culture is provided by receptor binding and second messenger analyses. Scatchard analyses of radioligand assays for binding of ^{3}H-QNB to chromaffin cell membranes revealed that the receptors are both high affinity (K_d = 300 ± 73 pM) and saturable (B_{max} = 58 ± 9 fmol/mg protein), with approximately 7600 ± 1200 sites per chromaffin cell. Comparable ^{3}H-QNB binding parameters were obtained from freshly isolated chromaffin cells (K_d = 480 ± 90 pM; B_{max} = 97 ± 14 fmol/mg protein), indicating that enzymatic dissociation does not cause loss of binding sites. Values represent the mean of two experiments using chromaffin cells and three experiments using adrenal medulla, with each conducted in triplicate. Moreover, this level of ^{3}H-QNB binding is maintained throughout one week in culture (Fig. 5). Importantly, muscarinic receptor expression appears to remain constant during the 3–4 d intervals needed to examine PNMT mRNA production.

Muscarinic receptors on bovine chromaffin cells displayed pharmacological characteristics of the m4 subtype identified by hybridization analyses (Fernando et al., 1991). Competition of specific antagonists for muscarinic sites with both ^{3}H-QNB (Fig. 6) and ^{3}H-*N*-methyl scopolamine (NMS) produced rank order of potencies with profiles that fit either the cloned m3 or m4 subtypes. The order of potency of muscarinic antagonists was established by measuring the affinity constants (K_i) and calculating the Hill slopes (n_H) for the following compounds (Fig. 6): scopolamine (0.37 ± 0.15 nM; n_H = 0.79 ± 0.13) = atropine (0.39 ± 0.02 nM; 0.92 ± 0.01) > 4-DAMP (2.97 ± 0.31 nM; 1.03 ± 0.09) > methoctramine (60 ± 6.8 nM; 0.98 ± 0.19) ≥ pirenzepine (79 ± 7.2 nM; 1.13 ± 0.22) > AF-DX 116 (890 ± 98 nM; 1.05 ± 0.10). These data are consistent with the m4 subtype. Further evidence for the m4 subtype is the strong correlation between antagonist binding affinities observed in bovine chromaffin cells with those reported for cloned m4 receptors in CHO cells (Hulme et al., 1990) or expressed in rabbit lung. When the negative log of K_i values calculated from antagonist binding for scopolamine, atropine, 4-DAMP, meth-

Table 2. Muscarine inhibits cAMP production in bovine chromaffin cells

	pmol cAMP per 10^6 cells
Control	11.0 ± 0.4
Forskolin	25.3 ± 1.7*
Muscarine	8.3 ± 0.63*
4-DAMP	10.8 ± 0.75*
Muscarine + forskolin	10.8 ± 1.6*
Muscarine + forskolin + 4-DAMP	26.8 ± 1.3*

cAMP production was measured in chromaffin cells in culture. Values are from four experiments. Cells were incubated with the following effectors for 10 min: forskolin (25 μM), muscarine (100 μM), 4-DAMP (10 μM). For multiple treatments, cells were incubated for (1) 15 min with muscarine prior to addition of forskolin for the final incubation of 10 min or (2) 15 min with 4-DAMP and then 15 min with muscarine prior to 10 min incubation with forskolin.

* $p < 0.001$ compared to control.

octramine, pirenzepine, and AF-DX 116 were plotted against affinity values for rat m4 clones transfected into CHO cells and tested in ^{3}H-NMS binding assays (Hulme et al., 1990) or for scopolamine and 4-DAMP in rabbit lung m4 receptor binding assays (Lazareno et al., 1990) (no data for cloned m4 receptors), the best fit by linear regression yielded a correlation coefficient of 0.991 ($P < 0.01$), a highly significant value. Hill transformation of the inhibition curves for each antagonist yields Hill coefficients (n_H) approximating 1, a fact also consistent with binding to a single receptor subtype.

The m4 muscarinic receptor is proposed to be coupled intracellularly to a mechanism that inhibits adenylate cyclase (Hulme et al., 1990). This is assayed as the ability of muscarine treatment to diminish forskolin-stimulated adenylate cyclase activity. The presence of this mechanism in bovine chromaffin cells was confirmed by measuring cAMP accumulation in homogenates of cells treated with forskolin (25 μM), either in the presence or in the absence of muscarine (100 μM). Forskolin treatment caused a doubling of cAMP content (from 11.0 to 25.3 pmol in 10^6 cells) within 10 min, while muscarine alone had no effect (8.3 pmol of cAMP) (Table 2). However, the presence of muscarine prior to addition of forskolin blocked the anticipated increase in cAMP (10.8 pmol of cAMP). Conversely, the m3–m4-selective antagonist 4-DAMP attenuated the inhibitory effect of muscarine, thereby yielding cAMP levels similar to those seen with forskolin treatment alone (26.8 pmol cAMP). These data further establish the presence of the m4 subtype muscarinic receptors on bovine chromaffin cells.

Effects of stimulation of nicotinic cholinergic receptors on PNMT mRNA

Exposure of chromaffin cells to nicotine (10 μM for 10 min) partially depletes the catecholamine content of adrenal chromaffin cells. Consistent with reports by others, epinephrine content was reduced on average 48% (from 271 to 115 nmol/mg protein in 10^6 cells), norepinephrine by 59% (from 297 to 121 nmol/mg protein), and dopamine by 32% (from 1970 to 1340 pmol/mg protein). Long-term (15 hr) exposure to nicotine also dose dependently increased PNMT mRNA with maximum effects, up to 10-fold obtained between 30 and 50 μM and with a decrease in response at higher doses (Fig. 7). The maximal increase occurred between 12 and 15 hr, followed by PNMT mRNA levels declining to approximately 20% of their maximal state

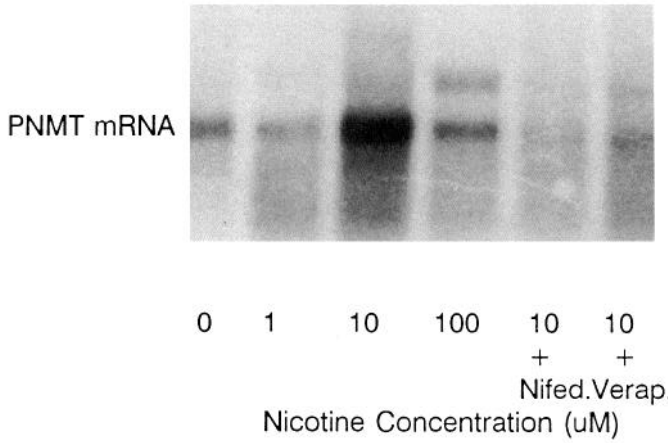


Figure 7. Nicotine-stimulated increases in PNMT mRNA involve Ca^{2+} channels. Increasing concentrations of nicotine were incubated with chromaffin cells for 24 hr. The calcium L-channel antagonists verapamil and nifedipine were tested for their ability to block the stimulatory effect of nicotine on PNMT mRNA. Total RNAs (12.5 μg) were prepared from chromaffin cell cultures treated for 24 hr with (*left to right*): nicotine at (1) 0, (2) 1, (3) 10, (4) 100 μM, (5) 10 μM + nifedipine (10 μM), and (6) 10 μM + verapamil (10 μM). When normalized to 18 S rRNA in each lane, the 10.5-fold increase in PNMT mRNA elicited by 10 μM nicotine is reduced by 70% with nifedipine and ~80% with the verapamil.

by 24 hr, and fully recovering by 30 hr (not shown). Also, consistent with the response seen for carbachol (Figs. 1 and 2), the nicotinic receptor antagonist hexamethonium (10 μM) inhibited over 90% of the PNMT response obtained with 50 μM nicotine.

Calcium effects on PNMT mRNA expression

Stimulation of nicotinic receptors activates voltage-gated calcium channels in adrenal chromaffin cells (Kley et al., 1987; Holz, 1988; Marley, 1988), resulting thereby in an influx of extracellular calcium sufficient to induce vesicular exocytosis and catecholamine release. Physiological and pharmacological data have suggested a mixture of multiple distinct calcium channels, specifically L-, N-, and (more recently) P-type channels (Jan et al., 1990; Duarte et al., 1993). We investigated whether comparable mechanisms are associated with the nicotine-stimulated increase in PNMT mRNA.

To assess whether increased calcium influx influences PNMT mRNA, cultures were incubated with the calcium ionophore A23187 or with the L-type calcium channel agonist BAY K 8644. The doses selected were those reported to increase enkephalin gene expression in the adrenal medulla (Kley et al., 1987). A23187 (10 nM) and BAY K 8644 (250 nM) (Fig. 8) increased PNMT mRNA three- and fivefold, respectively, relative to control cultures (all data normalized to 18 S rRNA) as shown by Northern analysis. Mean increases ($n = 3$ experiments) of 2.7- and 3.8-fold were obtained for A23187 and BAY K 8644. Also, previously (not shown), addition of 2 mM calcium to chromaffin cells maintained in calcium-depleted (<2 μM) medium elicited marked increases in PNMT (12.2-fold), tyrosine hydroxylase (9.8-fold), and enkephalin (4.9-fold) mRNAs but no changes in α-tubulin mRNA (Evinger et al., 1987). This study thus established that extracellular calcium can directly influence PNMT mRNA production.

The increase in PNMT mRNA elicited by nicotine was reduced 60–90% ($n = 3$ experiments) (Fig. 7) by nifedipine, an

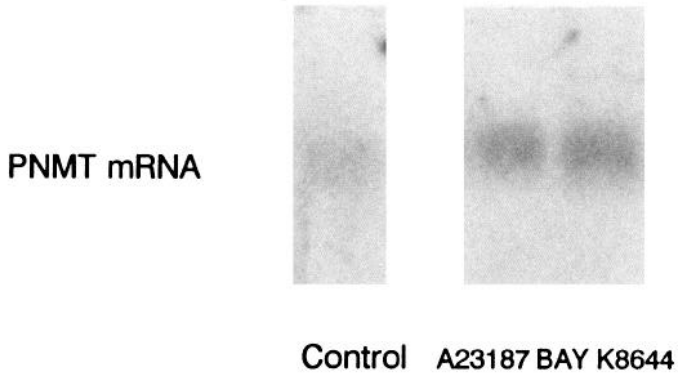


Figure 8. Effects of calcium influx on PNMT mRNA. The influx of extracellular calcium mediated by incubation of chromaffin cells with the calcium ionophore A23187 (10 nM) or the agonist BAY K 8644 (250 nM) resulted in increased levels of PNMT mRNA relative to saline-treated control cultures.

antagonist of the L-type calcium channel, and by 80% following treatment with verapamil, an antagonist of N- and L-type channels. ω-Conotoxin, an antagonist of N-type calcium channels, reduced the response by only 40% (data not shown). Tested individually, each of these antagonists was without effect on PNMT mRNA levels. However, while calcium channel antagonists to varying degrees blocked the major portion of the nicotine-induced increase in PNMT mRNA, in no instance was the entire effect abolished by blocking voltage-gated calcium influx, thereby suggesting the presence of an additional non-calcium component of the response.

Consistent with reports that other second messenger systems may also contribute to the cholinergic response, we confirmed in our chromaffin cell system that carbachol stimulates cAMP production in 10 min by twofold (from 5.36 ± 0.24 to 9.66 ± 0.36 pmol cAMP/mg protein, $n = 4$) and inositol phosphate turnover by approximately fourfold (from 3.6 ± 0.5% to 16.1 ± 3.7% inositol phosphate formed in 30 min, $n = 5$–6). In a separate experiment, ACh (50 μM, plus 50 μM 1-methyl-3-isobutylxanthine) induced a fourfold increase in cGMP in 5 min (from 23.5 ± 4.7 to 106 ± 5.0 pmol cGMP/mg protein, $n = 2$–3). As we previously established that dibutyryl cAMP and the phorbol ester 12-*O*-tetradecanyl-phorbol-13-acetate each induce PNMT mRNA (Carroll et al., 1991), we must yet resolve which of the multiple second messenger systems in bovine chromaffin cells influence PNMT mRNA expression as a result of nicotinic or muscarinic stimulation.

Effects of nicotine and muscarine on the rate of PNMT gene transcription

To determine whether muscarine or nicotine stimulate transcription of the PNMT gene, nuclear elongation "run-on" assays were performed to contrast the relative proportion of the transcriptional profile occupied by PNMT hnRNAs in chromaffin cells treated with nicotine (50 μM), muscarine (100 μM), or by depolarization with 50 mM KCl. The amount of ^{32}P-labeled hnRNA synthesized in nuclear preparations from chromaffin cells was hybridized to single-stranded PNMT cDNA or antisense cRNA fixed to nitrocellulose filters. Because incorporation of label into hnRNA and efficiency of hybridization vary from experiment to experiment, data from four separate determinations were compared as the n-fold induction in stimulated chromaffin cells relative to saline-treated control cultures. In each determination, nonspecific hybridization to an equivalent amount of pGEM or pUC 18 plasmid DNA (or *E. coli* rRNA for cRNA hybridizations) was subtracted. The results of representative run-on assays are depicted in Table 3. Experiment 1 includes both cDNA and cRNA probes for detection of PNMT transcripts. Each determination was run in duplicate. In experiment 2, α-tubulin hnRNA production was measured as an internal standard, as steady state levels of α-tubulin mRNA had previously been shown to remain constant with these treatments (Evinger et al., 1987).

Nicotine, muscarine, and K^+-mediated depolarization increased the rate of PNMT mRNA transcription. On average, nicotine stimulated the transcription rate 3.9-fold ($n = 3$), and muscarine 5.4-fold ($n = 4$), while depolarization was the most potent stimulus, increasing transcription rates 6.3-fold for the PNMT gene. The more pronounced effect of potassium paralleled the 8–12-fold increase in steady state PNMT mRNA following potassium treatment (Evinger et al., 1987). In experiment 2, expression of the α-tubulin gene remained relatively constant while that of the PNMT gene showed characteristic induction with these neural stimuli.

Discussion

Cholinergic innervation of the adrenal medulla mediates sympathetic responses to stressful stimuli with enhanced splanchnic nerve activity correlating to elevated release of catecholamines and opioid peptides. Moreover, repeated or chronic cholinergic stimulation results in elevated activity of the catecholamine-synthesizing enzymes tyrosine hydroxylase, dopamine β-hydroxylase, and PNMT, and in compensatory changes in the expression of these genes (e.g., Stachowiak et al., 1990a; Fossom et al., 1991; Kilbourne et al., 1992). Although Wan et al. (1991) reported that nicotine stimulates proenkephalin mRNA accumulation, they did not see an effect on PNMT mRNA levels with either nicotine treatment or potassium-mediated depolarization. Stachowiak et al. (1990a) did see a slight effect with nicotine but no response with muscarinic agents. In contrast, our data definitively established that cholinergic stimulation of bovine adrenal chromaffin cells markedly elevates PNMT mRNA accumulation, and, importantly, that both nicotinic and muscarinic stimulation independently influence production of this mRNA.

Our initial study defined the relative proportions of the cholinergic response to carbachol (a stable analog of ACh) as predominantly nicotinic but with a distinct muscarinic component. We have demonstrated that stimulation by carbachol of PNMT mRNA in cultured bovine adrenal chromaffin cell is dose dependent and receptor mediated, and that it achieves maximal levels 12–19 times control values by 12–16 hr after treatment. Selective inhibition of nicotinic receptors with hexamethonium blocked ~75% of the carbachol-stimulated induction, while preincubation with the general muscarinic antagonist atropine inhibited the response by ~50%. When combined the response was virtually abolished. Because carbachol is relatively nonselective with respect to receptor subclasses, we examined more specifically the relationship between cholinergic nicotinic and muscarinic receptors and the induction of PNMT mRNA by stimulation of each receptor with selective agonists.

Muscarinic receptors

The differing capacities of muscarinic and nicotinic receptors to stimulate catecholamine secretion among species are exem-

Table 3. Effect of neural stimuli on the rate of PNMT transcription

	Control	Nicotine	Muscarine	K^+
Experiment 1				
Total treatment counts hybridized				
PNMT cDNA	7991	8262	12,071	11,541
pGEM DNA	7143	4969	7176	5409
Specific counts hybridized				
PNMT	848	3693	4875	6132
Relative to control				
PNMT	1.0	4.4	5.8	7.2
Total treatment counts hybridized				
PNMT cRNA	4417	5290	5842	7743
E. coli	3963	3750	3670	5271
Specific counts hybridized				
PNMT	454	1540	2172	2472
Relative to control				
PNMT	1.0	3.4	4.8	5.44
Experiment 2				
Total treatment counts hybridized				
PNMT cDNA	2867	4592	3818	3684
α-Tubulin cDNA	3024	3441	3729	2684
pUC DNA	2700	2880	3275	2401
Specific counts hybridized				
PNMT	167	1712	643	1283
α-Tubulin	324	561	454	283
Relative to control				
PNMT	1	10.2	4.0	7.7
α-Tubulin	1	1.7	1.4	0.87

Chromaffin cell cultures were incubated for 1 hr at 37°C with PBS (Control), nicotine (50 μM), muscarine (100 μM), or KCl (50 mM), and then harvested for preparation of nuclei as described in Materials and Methods. *In vitro* nuclear run-on assays were performed, hnRNAs isolated, and hybridizations conducted. In experiment 1, hnRNAs were hybridized to denatured cDNAs (1 μg) fixed to nitrocellulose, with pGEM DNA (1 μg) serving as an index of nonspecific binding. Alternatively, antisense PNMT cRNA transcribed *in vitro* (1.1 μg) was bound to nitrocellulose; counts bound to *E. coli* rRNA (1.1 μg) were subtracted as nonspecifically bound cpm. Equal cpms (1.02×10^8) were added to each hybridization. In experiment 2, hnRNAs were hybridized to 1 μg of denatured cDNAs for PNMT and for α-tubulin and to pUC 18 DNA as the index of nonspecific binding. In this experiment, 1.5×10^8 cpms were added to each hybridization.

plified by the reports that muscarine is unable to mediate catecholamine release from bovine adrenal chromaffin cells (reviewed in Carmichael and Stoddard, 1989). However, distinct from its lack of effects on secretion, muscarine does stimulate production of PNMT mRNA. These effects are dose dependent, with maximal effects observed between 50 and 100 μM, and occur with a characteristic transient time course. Maximal levels of PNMT mRNA accumulate by 12 hr, are maintained for 6–8 hr, and then decline slowly for approximately 16–24 hr. Induction by muscarine depends upon time in contact with the cells; removal of the drug by media change arrests further increments in PNMT mRNA levels.

The effects of muscarine are receptor mediated since induction was blocked by treatment with the general muscarinic antagonist atropine (1 μM). Moreover, only antagonism of specific receptor subtypes was effective: the m3–m4-selective antagonist 4-DAMP blocked >60% of this effect at 10 μM (a concentration capable of blocking > 90% of the specific ^{3}H-NMS binding from adrenal medullary microsomal membranes), while antagonists selective for m1 or m2 subtypes were without effect.

Muscarine also stimulated the rate of PNMT gene transcription severalfold in nuclear run-on assays. While levels of induction vary more for muscarine treatment than for nicotine or potassium in the same study, the causes are likely to be technical in nature, attributable to differing potencies of commercial lots of muscarine from the same supplier or, alternatively, to trace protease contamination of collagenase that may selectively damage muscarinic receptors during cell isolation.

Bovine chromaffin cells express a population of muscarinic receptors that have been characterized pharmacologically and molecularly as consistent with the m4 subtype. Radioligand binding assays for muscarinic receptors in bovine chromaffin cells reveal that these receptors are high affinity and saturable with evidence for a single class of binding sites based upon analyses with ^{3}H-NMS (Fernando et al., 1991) and with ^{3}H-QNB (this study). The total density of muscarinic receptor binding sites is lower than that observed in many brain regions (Ernsberger et al., 1988) or in airway smooth muscle (Haxhiu-Poskurica et al., 1993), but in agreement with a previous report of ^{3}H-QNB binding in bovine adrenal medulla (Yamanaka et al., 1986).

The order of potency of ligand binding to chromaffin cell membranes with muscarinic antagonists is scopolamine = atropine > 4-DAMP > methoctramine ≥ pirenzepine > AF-DX 116, which is consistent with the m4 subtype (Hulme et al., 1990; Lazareno et al., 1990). Affinity constants for binding to bovine chromaffin cell membranes show high correlation with rabbit lung m4 (Lazareno et al., 1990; $r = 0.996$, $N = 5$), with

rat pheochromocytoma PC12 cells (Michel et al., 1989; $r = 0.954$, $N = 5$), and with NG108-15 neuroblastoma-glioma cells (Michel et al., 1989; $r = 0.950$, $N = 5$). We further suggest that a previous report of m1 receptors in bovine adrenal medulla, based on the relatively high affinity of pirenzepine (IC_{50} = 150 nM; Yamanaka et al., 1986), can be reinterpreted based on the relative similarity of binding properties of m1 and m4 subtypes (Hulme et al., 1990). In another report (Aguilar et al., 1992), the entire adrenal medulla, rather than the purified chromaffin cells used in our study, was utilized to demonstrate an m2-like receptor predominating in a mixture of two or three subtypes, thereby raising the possibility that other muscarinic subtypes may be expressed by nonchromaffin cells in this tissue.

Importantly, in agreement with our data, hybridization with specific cDNAs for each of the subtypes revealed a signal in bovine adrenal medullary A^+ RNA only with the m4 probe (Fernando et al., 1991). Also, consistent with the hypothesis that bovine adrenal medulla contains m4 muscarinic receptors, the affinity constants for cloned rat m4 receptors transfected into CHO cells (Hulme et al., 1990) have been compared with affinity constants obtained by iterative curve fitting with the LIGAND program for bovine chromaffin cells. The negative log values of the K_i for each of the six antagonists highly correlate between bovine chromaffin cells and the rat m4 clone ($r = 0.991$, $P < 0.01$), with a slope of the linear regression very close to 1 (1.03 ± 0.07). Moreover, each compound fell close to the line of identity, showing a high degree of agreement for affinity constants. These results strongly suggest that bovine adrenal chromaffin cells express a single subtype of muscarinic receptor binding sites and that this subtype is of the m4 class.

Nicotinic receptors

Nicotine, a well-characterized stimulus of catecholamine and enkephalin secretion in bovine chromaffin cells (Holz, 1988), induces membrane depolarization. Consequently, depolarization opens voltage-dependent Na^+ and Ca^{2+} channels. The ensuing large influx of extracellular Ca^{2+} elicits a cascade of events, including catecholamine secretion.

Nicotine elicits a rapid, dose-dependent, and significant elevation of PNMT mRNA with maximal accumulation following treatment of bovine chromaffin cells with 10–50 μM of the agent, a concentration that achieves maximal catecholamine release, enzyme activation (reviewed in Carmichael, 1986), and gene stimulation [including the immediate early gene c-*fos* in the PC-12 adrenal pheochromocytoma line (Greenberg et al., 1986) and proenkephalin mRNA in bovine chromaffin cells (Wan et al., 1991)]. The time course for induction of PNMT mRNA following nicotine treatment closely parallels that seen for other forms of neural stimulation: increases in PNMT mRNA are detectable after 1 hr of treatment, are sustained for approximately 15–16 hr, and then slowly decline over the course of an additional 24–36 hr. This is similar to the time course associated with accumulation of PNMT mRNA following reserpine treatment *in vivo* (Schalling et al. 1988; Evinger et al., 1990) or by potassium depolarization of bovine chromaffin cells *in vitro* (Evinger et al., 1987). The relatively tight time frame in which expression occurs may contribute to the disparity between our results and those of others with regard to the inducibility of PNMT mRNA by nicotine. By sampling mRNA at 8 and 24 hr after addition of nicotine, Wan et al. (1991) may have overlooked the peak production interval of PNMT mRNA, hence leading to their conclusion that nicotine does not stimulate PNMT mRNA accumulation.

To establish that the induction of PNMT mRNA by nicotine was receptor specific, we examined the effects of selective nicotinic antagonists on the response. Chromaffin cell nicotinic receptors (~15,000 per cell; Higgins and Berg, 1987) bind α-bungarotoxin with high affinity, cross-react with neutralizing antibodies to ganglionic nicotinic receptors, and show saturation kinetics comparable to those described in neural tissue (Higgins and Berg, 1987). Hexamethonium bromide, an inhibitor of peripheral nicotinic receptors, blocked both carbachol- and nicotine-stimulated increases in PNMT mRNA in a dose-dependent manner. Maximal inhibition of carbachol (200 μM) effects required 50 μM hexamethonium (Fig. 2), while 10–15 μM hexamethonium achieved blockade of the nicotine (50 μM) response.

We likewise investigated whether nicotinic receptor stimulation induces PNMT mRNA by increasing the influx of extracellular Ca^{2+}. That calcium could induce PNMT mRNA was demonstrated by treatment with both the calcium ionophore A 23187 (10 nM), which permits calcium entry through a non-channel-related mechanism, and BAY K 8644 (250 nM), an agonist acting at adrenal medullary voltage-gated calcium channels (Kley et al., 1987). We observed that both agents increased PNMT mRNA approximately threefold at the single concentration tested. Moreover, that calcium was relevant to the nicotine effect was established by the demonstration that chelation of extracellular calcium with EGTA at a concentration (1.8 mM) that sequesters free calcium in the media completely blocked the nicotine-stimulated increase in PNMT mRNA. However, this treatment has multiple, non-channel-related effects (including loss of cell attachment to the culture plates), thus leading us to pursue more channel-selective procedures.

We therefore examined the effects of treatment with specific calcium channel antagonists upon nicotinic induction of PNMT mRNA. Voltage-gated calcium channels on bovine chromaffin cells share attributes of L-, N-, and P-type channels based upon inhibition with the antagonists verapamil, nifedipine, ω-conotoxin, and agatoxin (Jan et al., 1990; Duarte et al., 1993), although controversy remains regarding the ability of ω-conotoxin to block calcium uptake (Regunathan et al., 1992; Duarte et al., 1993). Three of these agents were tested and shown to be effective in reducing the nicotine-stimulated induction of PNMT mRNA. The N+L-selective antagonist verapamil was the most potent, reducing induction by 80%. Nifedipine, an L-type–selective antagonist, was less effective, while ω-conotoxin was least effective, decreasing PNMT mRNA induction by 40%. These results argue for, but do not establish, that a mixed population of L- and N-type channels participates in mediating the effect, with L-type channels being either more potent or abundant. The ability of the more recently characterized P-type channels to influence PNMT message expression remains to be assessed. The fact that none of the selective calcium channel antagonists completely blocked the rise in PNMT mRNA stimulated by nicotine raises the possibility that up to 20% of the response may result from an increase in influx of Na+ or from stimulation of second messenger systems.

Nicotine's effects on PNMT message expression are predominantly transcriptional in nature. Transcription run-on studies to compare PNMT expression after 1 hr of treatment with nicotine revealed approximately fourfold increases relative to saline-treated controls. Additional studies (Evinger et al., 1992a,

and unpublished observations) also show nicotine-stimulated expression of PNMT promoter reporter gene fusion constructs transfected into and transiently expressed in bovine chromaffin cells.

Implications

Understanding the mechanism through which nicotine and muscarine influence PNMT expression will necessarily involve investigation into the signal transducing mechanisms associated with each receptor type. Investigations by other workers have provided important information that narrows the candidates for consideration in many cases. Specifically, nicotinic stimulation opens voltage-gated calcium channels while the m4 receptor gene transfected and expressed in cell lines produces a receptor that inhibits adenylate cyclase via a pertussis toxin–sensitive G-protein (reviewed in Hulme et al., 1990). Additionally, increases of phosphoinositide turnover in bovine chromaffin cells treated with muscarine (Eberhard and Holz, 1987) further raise the possibility that the m4 subtype may also be coupled to phosphoinositide hydrolysis or that there is cross-talk between second messenger systems initiated by m4 receptor stimulation.

Our studies confirm reports from other laboratories that chromaffin cells possess functional second messenger systems that appear to regulate gene expression in other model systems. Specifically, cAMP (Pocotte et al., 1986; Anderson et al., 1992) and cGMP (Yanagihara et al., 1979; Derome et al., 1981) production and phosphoinositide turnover (Eberhard and Holz, 1987; Plevin and Boarder, 1988; Regunathan et al., 1991b) can be enhanced by ACh or carbachol treatment. In particular, we have demonstrated that muscarine blocks forskolin-stimulated cAMP in the manner and to the extent seen in other systems. The fact that 4-DAMP abrogates these effects is supportive of the fact that this system is coupled to the m4 muscarinic receptor subtype. Notably, the relative stimulation for some of these second messenger effects is less than in other systems. However, the important point is that these systems are present and show the ability to respond to these stimuli.

Moreover, we have shown in previous studies that specific second messenger intermediates can influence PNMT mRNA levels, either directly or possibly secondarily. PNMT mRNA levels respond to treatment with 8-bromo-cAMP and forskolin (10 μM), although this gene does not possess any motifs resembling the published cAMP-responsive elements (CREs) observed in tyrosine hydroxylase (Lewis et al., 1987) and several other genes (reviewed in Montminy et al., 1990). 8-Bromo-cGMP evokes a modest (approximately two- to fivefold) increase in PNMT mRNA. Moreover, angiotensin, a stimulus of phosphoinositide metabolism in chromaffin cells, increases levels of PNMT mRNA (Stachowiak et al., 1990b). In other studies, potassium-stimulated PNMT mRNA accumulation can be blocked by treatment of the cells with the calmodulin inhibitor calmidazolium, thereby implying that the Ca^{2+}/calmodulin kinase system can also be coupled to PNMT gene expression (Evinger et al., 1992b). Future studies are directed toward resolving the exact mechanisms responsible for these effects.

In summary, these studies establish for the first time the ability of cholinergic stimuli to regulate PNMT gene expression through two separate mechanisms. It is significant that a single transmitter's ability to regulate this gene invokes two independent pathways and distinct mechanisms. Does this constitute a functional redundancy, possibly as a backup mechanism to maintain epinephrine reserves for metabolic, cardiovascular, and hormonal responses? The ability to stimulate epinephrine production by enhanced gene expression would present an advantage in situations of chronic stress or pathological states, for example, hypertension.

Alternatively, activation of nicotinic and muscarinic receptors may independently regulate unrelated intracellular events. Conceivably, one receptor may respond preferentially to certain forms of stimulation. The significance of the present study is the demonstration that these nicotinic and muscarinic pathways do exist and each is coupled to PNMT gene expression. Whether this information is specifically encoded within the PNMT gene or whether it converges at a point along intracellular pathways would represent an advance in our fundamental knowledge of how neural cells, and specifically neural genes, process multiple inputs.

References

Aguilar JS, Ballesta JJ, Teig JA, Palmero M, Viniegra S, Criado M (1992) Muscarinic receptor subtypes in bovine adrenal medulla. Neurochem Res 17:1235–1239.

Anderson K, Robinson PJ, Marley PD (1992) Cholinoceptor regulation of cyclic AMP levels in bovine adrenal medullary cells. Br J Pharmacol 106:360–366.

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K, eds (1991) Preparation of cytoplasmic RNA from tissue culture cells. In: Current protocols in molecular biology. New York: Wiley.

Baetge EE, Suh Y-H, Joh TH (1986) Complete nucleotide and deduced amino acid sequence of bovine phenylethanolamine *N*-methyltransferase: partial amino acid homology with rat tyrosine hydroxylase. Proc Natl Acad Sci USA 83:5454–5458.

Barron BA, Hexum TD (1986) Muscarinic receptor modulation of release of [Met^5]enkephalin immunoreactive material and catecholamines from the bovine adrenal gland. Br J Pharmacol 89:713–718.

Carmichael SW (1986) Stimulus–secretion coupling: receptors. In: The adrenal medulla, Vol 4. Cambridge: Cambridge UP.

Carmichael SW, Stoddard SL (1989) Stimulus-secretion coupling: receptors. In: The adrenal medulla: 1986–1988. Caldwell, NJ: Telford.

Carroll JM, Evinger MJ, Goodman HM, Joh TH (1991) Differential and coordinate regulation of TH and PNMT mRNAs in chromaffin cell cultures by second messenger system activation and steroid treatment. J Mol Neurosci 3:75–83.

Derome G, Tseng R, Mercier P, Lemaire I, Lemaire P (1981) Possible muscarinic regulation of catecholamine secretion mediated by cyclic GMP in isolated bovine adrenal chromaffin cells. Biochem Pharmacol 30:855–860.

Duarte CB, Rosario LM, Sena CM, Carvalho AP (1993) A toxin fraction (FTX) from the funnel-web spider poison inhibits dihydropyridine-insensitive Ca^{2+} channels coupled to catecholamine release in bovine adrenal chromaffin cells. J Neurochem 60:908–913.

Eberhard DA, Holz RW (1987) Cholinergic stimulation of inositol phosphate formation in bovine adrenal chromaffin cells: distinct nicotinic and muscarinic mechanisms. J Neurochem 49:1634–1643.

Ernsberger P, Arneric AP, Arango V, Reis DJ (1988) Quantitative distribution of muscarinic receptors and choline acetyltransferase in rat medulla: examination of transmitter–receptor mismatch. Brain Res 452:336–344.

Evinger MJ, Joh TH (1989) Strain-specific differences in transcription of the gene for the epinephrine synthesizing enzyme phenylethanolamine *N*-methyltransferase. Mol Brain Res 5:141–147.

Evinger MJ, Carroll JM, Hyman SE, Goodman HM, Joh TH (1987) Differential regulation of the mRNAs for the catecholamine enzymes tyrosine hydroxylase and PNMT. Soc Neurosci Abstr 13:1086.

Evinger MJ, Ernsberger P, Reynolds BN, Reis DJ (1988) Neural modulation of phenylethanolamine *N*-methyltransferase mRNA in bovine chromaffin cells. Soc Neurosci Abstr 15:205.4.

Evinger M, Joh T, Reis D (1990) Transcriptional regulation of phenylethanolamine *N*-methyltransferase gene expression. In: Catecholamine genes (Joh TH, ed), pp 137–146. New York: Liss.

Evinger MJ, Hemmick LM, Regunathan S, Reis DJ, Ross ME (1992a)

Nicotine and muscarine stimulate expression of PNMT promoter constructs transfected into primary chromaffin cells. Soc Neurosci Abstr 18:254.3.

Evinger MJ, Towle AC, Park DH, Lee P, Joh TH (1992b) Glucocorticoids stimulate transcription of the rat PNMT gene *in vivo* and *in vitro*. Cell Mol Neurobiol 12:193–215.

Fernando JCR, Abdallah E-SAM, Evinger M, Forray C, El-Fakahany EE (1991) The presence of an M4 subtype muscarinic receptor in bovine adrenal medulla revealed by mRNA and receptor binding analyses. Eur J Pharmacol 207:297–303.

Fossom LH, Carson CD, Tank AW (1991) Stimulation of tyrosine hydroxylase gene transcription rate by nicotine in rat adrenal medulla. Mol Pharmacol 40:193–202.

Greenberg ME, Ziff EB, Greene LA (1986) Stimulation of neuronal acetylcholine receptors induces rapid gene transcription. Science 234: 80–83.

Haxhiu-Poskurica B, Ernsberger P, Haxhiu MA, Miller MJ, Cattarossi L, Martin RJ (1993) Development of cholinergic innervation and muscarinic receptor subtypes in piglet trachea. Am J Physiol 264: L606–L614.

Higgins LS, Berg K (1987) Immunological identification of a nicotinic acetylcholine receptor on bovine chromaffin cells. J Neurosci 7:1792–1798.

Holz RW (1988) Control of exocytosis from adrenal chromaffin cells. Cell Mol Neurobiol 8:259–268.

Hulme EC, Birdsall NJM, Buckley NJ (1990) Muscarinic receptor subtypes. Annu Rev Pharmacol Toxicol 30:633–673.

Jan C-R, Titeler M, Schneider AS (1990) Identification of omega-conotoxin binding sites on adrenal medullary membranes: possibility of multiple calcium channels in chromaffin cells. J Neurochem 54: 355–358.

Kilbourne EJ, Nankova BB, Lewis EJ, McMahon A, Osaka H, Sabban DB, Sabban EL (1992) Regulated expression of the tyrosine hydroxylase gene by membrane depolarization. J Biol Chem 267:7563–7569.

Kley N, Loeffler J-P, Pittius CW, Hollt V (1987) Involvement of ion channels in the induction of proenkephalin A gene expression by nicotine and cAMP in bovine chromaffin cells. J Biol Chem 262: 4083–4089.

Lazareno S, Buckley NJ, Roberts FF (1990) Characterization of muscarinic m4 binding sites in rabbit lung, chicken heart, and NG108-15 cells. Mol Pharmacol 38:805–815.

Lewis EJ, Harrington CA, Chikaraishi DM (1987) Transcriptional regulation of the tyrosine hydroxylase gene by glucocorticoid and cyclic AMP. Proc Natl Acad Sci USA 84:3550–3554.

Marley PD (1988) Desensitization of the nicotinic secretory response of adrenal chromaffin cells. Trends Pharmacol Sci 9:102–107.

McPherson GA (1985) Analysis of radioligand binding experiments: a collection of computer programs for the IBM PC. J Pharmacol Methods 14:213–228.

Michel AD, Delmendo R, Stefanich E, Whiting RL (1989) Binding characteristics of the muscarinic receptor of the NG108-15 cell line. Naunyn Schmiedebergs Arch Pharmacol 340:62–67.

Montminy MR, Gonzalez GA, Yamamoto KK (1990) Regulation of cAMP-inducible genes by CREB. Trends Neurosci 13:184–188.

Munson PJ, Rodbard D (1980) LIGAND. A versatile computerized approach for characterization of ligand binding systems. Anal Biochem 107:220–239.

Peterson GL (1977) A simplification of the protein assay method of Lowry et al., which is more generally applicable. Anal Biochem 83: 346–356.

Plevin R, Boarder MR (1988) Stimulation of formation of inositol phosphates in primary cultures of bovine adrenal chromaffin cells by angiotensin II, histamine, bradykinin, and carbachol. J Neurochem 51:634–641.

Pocotte SL, Holz RW, Ueda T (1986) Cholinergic receptor–mediated phosphorylation and activation of tyrosine hydroxylase in cultured bovine adrenal chromaffin cells. J Neurochem 46:610–622.

Regunathan S, Evinger MJ, Meeley MP, Reis DJ (1991a) Effect of clonidine and other agents that interact with imidazole receptors on second messenger systems and calcium influx in bovine adrenal chromaffin cells. Biochem Pharmacol 42:2011–2018.

Regunathan S, Meeley MP, Reis DJ (1991b) Clonidine displacing substance from bovine brain binds to imidazoline receptors and releases catecholamines in adrenal chromaffin cells. Mol Pharmacol 40: 884–888.

Regunathan S, Reis DJ, Wahlestedt C (1992) Specific binding of inositol hexakisphosphate (phytic acid) to adrenal chromaffin cell membranes and effects on calcium-dependent catecholamine release. Biochem Pharmacol 43:1331–1336.

Ross ME, Evinger MJ, Hyman SE, Carroll JM, Mucke L, Comb M, Reis DJ, Joh TH, Goodman HM (1990) Identification of a functional glucocorticoid response element in the phenylethanolamine *N*-methyltransferase (PNMT) promoter using fusion genes introduced into chromaffin cells in primary culture. J Neurosci 10:520–530.

Schalling M, Dagerlind A, Brene S, Hallman H, Djurkfeldt M, Persson H, Terenius L, Goldstein M, Schlesinger D, Hokfelt T (1988) Coexistence and gene expression of phenylethanolamine *N*-methyltransferase, tyrosine hydroxylase, and neuropeptide Y in the rat and bovine adrenal gland: effects of reserpine. Proc Natl Acad Sci USA 85:8306–8310.

Stachowiak MK, Hong JS, Viveros OH (1990a) Coordinate and differential regulation of phenylethanolamine *N*-methyltransferase, tyrosine hydroxylase, and proenkephalin mRNAs by neural and hormonal mechanisms in cultured bovine adrenal medullary cells. Brain Res 510:227–238.

Stachowiak MK, Jiang H-K, Poisner AM, Tuominen RK, Hong J-S (1990b) Short and long term regulation of catecholamine biosynthetic enzymes by angiotensin in cultured adrenal medullary cells: molecular mechanism and nature of second messenger systems. J Biol Chem 265:4694–4702.

Wakade AR, Wakade TD, Maholtra RK (1988) Restoration of catecholamine content of previously depleted adrenal medulla *in vitro*: importance of synthesis in maintaining the catecholamine stores. J Neurochem 51:820–829.

Wan DC-C, Marley PD, Livett BG (1991) Coordinate and differential regulation of proenkephalin A and PNMT mRNA expression in cultured bovine adrenal chromaffin cells: responses to secretory stimuli. Mol Brain Res 9:103–111.

Wilson SP (1987) Purification of adrenal chromaffin cells on Renografin gradients. J Neurosci Methods 19:163–171.

Yamanaka K, Kigoshi S, Muramatsu I (1986) Muscarinic receptor subtypes in bovine adrenal medulla. Biochem Pharmacol 35:3151–3157.

Yanagihara N, Isosaki M, Ohuchi T, Oka M (1979) Muscarinic receptor-mediated increase in cyclic GMP level in isolated bovine adrenal medullary cells. FEBS Lett 105:296–298.