Communication

Coordinated Up-regulation of Choline Acetyltransferase and Vesicular Acetylcholine Transporter Gene Expression by the Retinoic Acid Receptor \(\alpha\), cAMP, and Leukemia Inhibitory Factor/Ciliary Neurotrophic Factor Signaling Pathways in a Murine Septal Cell Line*

(Received for publication, June 5, 1995, and in revised form, July 11, 1995)

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The proteins responsible for acetylcholine (ACh) synthesis (choline acetyltransferase, ChAT) and storage (vesicular ACh transporter, VACHT) are encoded by two closely linked genes in vertebrates, with the VACHT coding sequence contained within the first intron of the ChAT gene. This unusual genomic organization suggests that the transcription of these two genes is coordinately regulated. Using Northern analysis we studied the modulation of ChAT and VACHT expression in a murine septal cell line (SN56) by three groups of agents: retinoids, trophic factors belonging to the leukemia inhibitory factor (LIF/CNTF) family, and cAMP. All-trans-retinoic acid increased both ChAT and VACHT mRNA levels in SN56 cells up to 3.5-fold, and elevated intracellular ACh levels by 2.5-fold. This effect was mimicked by a retinoic acid receptor \(\alpha\) (RAR\(\alpha\)) agonist (Ro 40-6055) and prevented by a specific antagonist (Ro 41-5253), indicating that it was mediated by RAR\(\alpha\). ChAT- and VACHT-specific transcripts were also induced (up to 3-fold) by treatment with CNTF or LIF (20 ng/ml, 48 h), as well as by dibutyryl cAMP (1 mm). All these agents increased the ACh level in the cells (up to 2.5-fold). Dibutyryl cAMP had a greater effect on the level of VACHT mRNA (4-fold induction) than on the level of ChAT mRNA (2-fold induction), suggesting a quantitatively differential transcriptional regulation of the two genes by the cAMP pathway. The effects of the three groups of agents studied on ChAT and VACHT mRNA levels were additive, pointing to several independent mechanisms by which the cholinergic properties of septal neurons can be modulated.

Cholinergic neurotransmission depends on coexpression of proteins involved in the synthesis, storage, and release of acetylcholine (ACh). Collectively, these proteins make up the cholinergic phenotype of a variety of neuronal populations, including certain basal forebrain cells that may function in processes underlying memory (1, 2). Of the proteins contributing to the cholinergic phenotype the best studied so far has been the ACh-synthesizing enzyme, choline acetyltransferase (ChAT, EC 2.3.1.6) (3). ChAT activity and expression have been used as markers for cholinergic neurons and as indices for the actions of trophic factors on those neurons, and previous studies have shown that ChAT activity and/or expression can be up-regulated by a variety of extracellular signals, including cholinergic differentiation factor (4) shown to be identical to leukemia inhibitory factor (LIF) (5) and ciliary neurotrophic factor (CNTF) (6, 7). In addition, pharmacologic treatments that cause increases in cAMP concentrations (8), and retinoids (9, 10), have been used to increase ChAT activity in a variety of experimental systems (3). Studies of the ChAT gene have shown that differential promoter use and alternative RNA splicing contribute to the formation of several ChAT mRNA variants, which differ at the 5′ end (3). The promoter region of this gene is rich in putative regulatory nucleotide sequences (Fig. 1), including ones identical or homologous to cAMP response element (CRE), retinoic acid response element (RARE), and CNTF response element (CNTF-RE), but only a few have been demonstrated to serve as cis-acting regulatory elements in reporter gene assays (11, 12).

Recently, the gene for rat and human vesicular ACh transporter (VACHT), a protein catalyzing the uptake of ACh into secretory vesicles, was cloned (13–15). The entire VACHT coding sequence was shown to be contained within the first intron of the ChAT gene (Fig. 1), prompting Erickson et al. (14) to coin the term “cholinergic gene locus.” The mechanisms regulating ChAT expression are partially understood, while the regulation of VACHT expression has not yet been explored. However, the unusual (for mammals) organization of the ChAT and VACHT genes strongly suggests that they may share some transcriptional signals and that their expression may be regulated in a coordinated fashion by extracellular factors (16). Using the SN56 neuronal cell line derived from the basal forebrain (septum), we have previously shown that ChAT expression, activity, and intracellular ACh levels are increased by activation of the retinoic acid receptor \(\alpha\) (RAR\(\alpha\)) (17) and by elevations of intracellular cAMP concentrations (17). Consistent with the prediction that ChAT and VACHT expression are coordinately regulated, we report that mRNA levels for both ChAT and VACHT are increased by three groups of agents in SN56 cells: retinoids, growth factors of the CNTF/LIF family, and cAMP. Significantly, this up-regulation results in proportional increases in the steady-state levels of intracellular ACh. The effects of these agents are additive, pointing to several independent mechanisms by which the cholinergic properties of septal neurons can be modulated.

* This work was supported by Grant AG09525 from NIA, National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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EXPERIMENTAL PROCEDURES

Reagents—All-trans-retinoic acid (t-RA), dibutyryl cAMP (Bt2cAMP), and all molecular biology reagents (except as noted) were purchased from Sigma. The RARα-selective agonist Ro 40-6055 and the antagonist Ro 41-8013 were purchased from Arthur Lein and Michael Klaus of Hoffmann-La Roche. Manipulations involving retinoids were conducted under reduced light conditions. Recombinant mouse LIF and rat CNTF were purchased from R&D Systems. Oligonucleotides were custom synthesized by DNA International.

Cell Culture—Mouse septal neuron x neuroblastoma hybrid SN56 cells (18, 19) were maintained at 37°C in an atmosphere of 95% air, 5% CO2 in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, and 50 μg/ml gentamicin. The cells were subcultured by mechanically removing them from the dishes with sprints of fresh medium. Cells were grown to subconfluence in 35-mm culture dishes and then refed with fresh medium containing test compounds, e.g. retinoids or growth factors. The medium was then replaced every 24 h. 0.1% SDS at 55°C for ChAT probe and at 80°C for 1–2 days. Signal intensities were quantified directly from the blots with a PhosphorImager 400E and ImageQuant software (Molecular Dynamics).

RESULTS AND DISCUSSION

In order to compare the expression of VACHT and CHAT in SN56 cells, we prepared mouse cDNA probes for these genes. The CHAT probe was described previously (17), and the VACHT probe was obtained by PCR amplification of mouse brain cDNA, using primers based on the published rat VACHT gene sequence. In Northern analysis of RNA prepared from SN56 cells, we prepared mouse cDNA probes for these genes. The transcription start site at the 5’ end of exon R may be shared between certain CHAT and VACHT transcripts. Vertical arrows indicate initiation codons for VACHT and CHAT. Some of the relevant putative cis-acting regulatory elements are shown. Using the nomenclature proposed by Wu and Hersh (3) in which the +1 nucleotide within the gene sequence corresponds to the “A” of the ATG translation start codon of CHAT, the positions of these sequences are as follows: RARE-like at -274, -221, -126, -1385, -2629, and -3691; CNTF-RE-like at -377, -2721, and -3117; CRE-like at -3249. In the rat gene, the region upstream exon R contains a sequence homologous to a CRE (TGAGCTCA) and a CNTF-RE consensus sequence TTCCTGAAA.
effects of cAMP and t-RA on ChAT and VACHT mRNA by probing the same blots in succession with ChAT and VASH mRNA following a 48 h treatment of the cells with maximally effective concentrations of Bt2cAMP (1 μM) and t-RA (1 μM). Messenger RNA levels of ChAT and VACHT were increased by either agent (Fig. 2A). However, t-RA was more effective than Bt2cAMP in inducing ChAT, whereas Bt2cAMP was more effective than t-RA in inducing VACHT (Fig. 2A). The combination of Bt2cAMP and t-RA resulted in an additive increase of ChAT and VACHT mRNA levels, indicating that the two agents operate through two independent mechanisms. Cyclic AMP- and t-RA-evoked induction of ChAT and VACHT mRNA were accompanied by roughly proportional increase in intracellular ACH levels (Fig. 2B). A combined treatment with t-RA and Bt2cAMP resulted in an additive increase in ACH content. Thus the up-regulation of the expression of cholinergic genes is an effective mechanism for increasing the amounts of stored ACH in SN56 cells.

Using synthetic retinoids, one selective agonist (Ro 40-6055), and one antagonist (Ro 41-5253) of the RARα (28), we have previously shown that activation of this receptor increases ACH levels in SN56 cells (17). We have now used these compounds to determine whether they similarly affect the expression of ChAT and VACHT. Both ChAT and VACHT mRNA levels were markedly increased (3–4-fold) upon treatment of SN56 cells with 100 μM Ro 40-6055 for 48 h (Fig. 3). Moreover, a 100-fold molar excess (10 μM) of Ro 41-5253 abolished the Ro 40-6055-evoked increases in the abundance of both mRNAs. Treatment of cells with Ro 41-5253 alone (10 μM) slightly reduced the basal levels of ChAT and VACHT mRNA, indicating that retinoids, present in serum, may participate in maintaining the cholinergic phenotype of SN56 cells under our culture conditions (Fig. 3). These results constitute the first demonstration that retinoids modulate ChAT and VACHT expression, and that RARα mediates this process.

ChAT activity (29) and expression (30) can be increased in cultured primary septal neurons by treatments with nerve growth factor (NGF), and it would be of interest to examine the effects of NGF on VACHT mRNA levels in septal cells. However, SN56 cells do not respond to NGF (19). Another neurotrophin, LIF, is also of particular interest because it may be important in maintaining the cholinergic phenotype of certain neuronal populations after injury (31, 32). In order to determine whether the cholinergic phenotype of the septal cell line could also be up-regulated by LIF, we treated SN56 cells with this protein and with a functionally related neurotrophin, CNTF (33). Both trophic factors increased intracellular ACH content of SN56 cells (Fig. 4B) in a dose-dependent and saturable fashion (with a maximally effective concentration of 10 ng/ml; data not shown). Northern analysis of RNA from cells grown for 2 days in the presence of 20 ng/ml LIF or 20 ng/ml CNTF showed that these growth factors up-regulate both ChAT and VACHT expression, albeit to a lesser extent than do retinoids or Bt2cAMP (up to 2-fold induction, Fig. 4A). The effects of LIF and CNTF on ChAT and VACHT mRNA levels were nearly additive with those of t-RA (Fig. 4A). However, no additive with the effects of t-RA was observed when steady-state ACH levels were used as an index of the neurotrophin action (Fig. 4B). The reason for this lack of additive is presently unclear; however, this result could indicate that cells treated with t-RA and neurotrophins are characterized by disproportionately accelerated release of ACH. In a previous study we showed that ACH release can be enhanced by treating SN56 cells with Bt2cAMP (34). Whether the released ACH derives entirely from the vesicular pool of the transmitter is currently not known. It will be interesting to determine if the up-regulation of VACHT by the agents described here correlates with the amounts of ACH stored in secretory vesicles, and with the ability of SN56 cells to secrete ACH.

Taken together, these data suggest a coordinated up-regulation of ChAT and VACHT gene expression by cAMP, retinoid, and CNTF/LIF signaling pathways. However, subtle differences exist in the regulation of expression of these two genes, e.g. cAMP increases VACHT mRNA level more efficiently than that of ChAT (Fig. 2). In addition, the fact that these pathways
exert additive effects on ChAT and VAChT mRNA levels suggests that they are independent from each other.

The observation that these two closely linked genes, both essential components of the cholinergic phenotype, are expressed coordinately is consistent with the observations that the tissue distributions of the ChAT and VAChT transcripts are virtually identical (13–15). This coexpression of VACHT and ChAT suggests that both are regulated by the same tissue-specific transcriptional signals. It is worth noting that a region upstream of exon R (Fig. 1), which would be expected to direct the expression of both VACHT and ChAT in the appropriate tissues, has been shown to confer cholinergic tissue-specific expression of a reporter gene (12). Although we did not measure the rates of formation of the mRNA for VACHT and ChAT, the available data suggest that retinoids, cAMP, and LIF/CTNF directly stimulate transcription of these genes. Analysis of the ChAT/VACHT genomic sequence reveals numerous putative cis-acting regulatory sequences that may take part in this process (Fig. 1). There are six sequences with high homology to the RARE (35) in the N/M promoter region of the ChAT gene (17), i.e., positioned 5′ of the ChAT first coding exon and 3′ of the VACHT open reading frame (Fig. 1). It is possible that some of them confer the retinoic acid response to both ChAT and VACHT promoters. Interestingly, in the ChAT gene, the RARE present in inverted orientation in position −1242 to −1264 overlaps with a CRE-like sequence (−1242 to −1249).

Using DNA constructs containing a relatively long region of the ChAT gene (2.7 kb) linked to a reporter, Misawa et al. (11) showed that the region downstream from exon M is responsible for the induction of ChAT by cAMP, and suggested that this putative CRE conferred the effect.

The transcriptional effects of CTNF and LIF are known to be mediated by the members of the STAT family of proteins (36–39), which bind to a cis-acting CNTF-RE (consensus sequence TTCC(N2)AA(A) (36, 40). Two perfect matches of the CNTF-RE and two sequences homologous to CNTF-RE are present in the ChAT/VACHT locus (7) (Fig. 1). Additionally, recent studies demonstrated that, in addition to STATs, the CREBP transcription factors are necessary for CNTF/LIF inducibility of the gene encoding vasoactive intestinal peptide (41), a neuropeptide induced by CNTF and LIF in sympathetic neurons. There are four perfect matches of the CREBP consensus binding site (TT/G)NGNAA(T/G) clustered in the murine M promoter region, and the rat R promoter also contains four perfect matches of this sequence. Moreover, although the CRE-binding protein is generally credited for activating transcription via the CRE sites, it has been reported that CEBP may also bind to CRE and mediate the effects of cAMP on transcription (42-44). Thus, it is possible that modulation of ChAT/VACHT gene expression could be a result of complex cross-talk among STAT, CEBP, cAMP, and retinoic acid regulatory pathways.

The coordinated up-regulation of ChAT and VACHT mRNA levels indicates that common signaling pathways control both genes. A better understanding of the mechanisms that regulate transcription of those genes awaits the detailed characterization of their promoters, and determination of the functional activity of the putative cis-acting elements and of the transcription factors interacting with these elements. This information will be useful in understanding the mechanisms of diseases characterized by malfunction of cholinergic neurons, e.g., Alzheimer’s disease or amyotrophic lateral sclerosis, and may help in designing treatment strategies directed toward repair of those defects.

Acknowledgments—We are grateful to Drs. Levin and Kraus for providing the retinoids and to Dr. Bruce Wainer for the gift of SN56 cells.

Addendum—While this paper was under review, similar results were reported by Berrard et al. (45).