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Functional Expression of Receptors for Calcitonin Gene-Related Peptide, Calcitonin, and Vasoactive Intestinal Peptide in the Human Thymus and Thymomas from Myasthenia Gravis Patients

J.-C. Marie, A. Wakkach, A.-M. Coudray, E. Chastre, S. Berrih-Aknin, and C. Gespach

The molecular and functional expression of serpentine membrane receptors for vasoactive intestinal peptide (VIP), calcitonin gene-related peptide (CGRP), and calcitonin (CT) were characterized in human thymus and thymomas from myasthenia gravis (MG) patients and thymic epithelial cells either in primary culture (PTEC) or transformed by the simian virus 40 large T (SV40LT) oncogene (LT-TEC). Using RT-PCR combined with Southern analysis, we identified the PCR products corresponding to the receptor (-R) transcripts for VIP, CGRP, and CT in thymus from control subjects and MG patients with either hyperplasia or thymoma. Similar expressions of the VIP- and CGRP-R transcripts were observed in PTEC, whereas the CT-R message was not detected. In LT-TEC, the signals for VIP-R, CGRP-R, and CT-R transcripts were seen with a lower intensity than those in control and MG thymus. In agreement with our molecular analysis, 1) VIP was the most potent peptide among VIP-related peptides (VIP > PACAP > PHM > PHV) to stimulate cAMP production through specific type 1 VIP receptors in both PTEC and LT-TEC; 2) cAMP generation was induced by CGRP in PTEC and by CT in LT-TEC; 3) in frozen thymic sections and by flow cytometry, type 1 VIP-R, CGRP-R, and CT-R were localized in epithelial cells; and 4) in parallel, the transcription of the acetylcholine receptor α subunit (the main autoantigen in MG) was induced by CGRP and CT in PTEC and LT-TEC, respectively. Our data suggest that the neuroendocrine peptides VIP, CGRP, and CT may exert functional roles during MG and malignant transformation of the human thymus. The Journal of Immunology, 1999, 162: 2103–2112.

The thymus is an essential organ in the differentiation and maturation of lymphoid precursor cells into mature T lymphocytes (1–4). These precursors, derived from bone marrow stem cells, are subjected to positive and negative selections by thymic stromal cells, including dendritic cells, macrophages, and epithelial cells (5–7). In this context, thymic epithelial cells have been shown to protect thymocytes from apoptosis (8) and to promote the maturation of the double-positive (CD4+/CD8+) thymocytes into single-positive cells CD4+CD8−, CD4−CD8+ (9). In turn, thymocytes also modulate their environ-

ment through the release of cytokines and inhibition of stromal epithelial cell proliferation (10).

The cellular heterogeneity of the thymus and the cross-talk between the different cell types constitute the main limit in understanding the physiology and pathophysiology of the thymus. Pathological thymi, including thymic hyperplasia and thymoma, are frequently found in myasthenia gravis (MG), and MG patients are improved after thymectomy (11). Autosensitization to acetylcholine receptors (AChR), which is the main autoantigen implicated in MG, is thought to take place in the thymus (12). AChR α subunit (α-AChR) transcripts have been shown to be up-regulated by the neuropodptide calcitonin gene-related peptide (CGRP) in chicken myocytes in primary culture (13). Thus, such an effect occurring in the thymus would represent an important neuroendocrine control in MG and thymic neoplasia. CGRP is a 37-amino acid peptide generated by tissue-specific alternative processing of the calcitonin/CGRP gene transcript in central and peripheral neurons (14, 15). Several neuroendocrine peptides, such as vasoactive intestinal peptide (VIP), are also involved in the regulation of immune responses (16, 17). VIP is a 28-amino acid neuropeptide

5 Abbreviations used in this paper: MG, myasthenia gravis; AChR, acetylcholine receptors; α-AChR, α subunit of AChR; CGRP, calcitonin gene-related peptide; VIP, vasoactive intestinal polypeptide; PACAP-38, pituitary adenylate cyclase-activating polypeptide; CT, calcitonin; PTEC, thymic epithelial cells in primary culture; LT-TEC, thymic epithelial cells transformed by the simian virus 40 large T oncogene; PHM, peptide with NH2-terminal histidine and C-terminal methionine; PHV, peptide with NH2-terminal histidine and C-terminal valine; TGLP-1, truncated glucagon-like peptide-1; IAPP, islet amyloid polypeptide; pAbs, polyclonal Abs; RAMPS, receptor activity-modifying proteins; RCP, receptor component protein.
structurally related to glucagon, pituitary adenylate cyclase-activating peptide (PACAP), and secretin (18). These regulatory peptides exert their biological activities through activation of specific membrane receptors (19). These receptors have seven transmembrane domains and are positively coupled to adenylyl cyclase. Subtypes of VIP and CGRP/calcitonin (CT) receptors were identified on the basis of pharmacological and molecular analysis. VIP- and CGRP-immunoreactive fibers were identified in the thymus, and both neuropeptides were reported to inhibit IL-2 production and proliferation of thymocytes in vitro (20–22). Further, VIP has been shown to rescue the immature double-positive CD4+CD8+ thymocytes from the glucocorticoid-induced apoptosis (23, 24). Thus, it can be postulated that VIP exerts its protective effects against apoptosis in thymocytes through an indirect action on thymic epithelial cells.

In the current hypotheses that neuroendocrine peptides exert a regulatory role on the development of the thymus and its immune function, our aim was to characterize the molecular and functional expression of VIP and CGRP/CT receptors in human thymus under physiological and pathophysiologic conditions. We, therefore, investigated 1) the expression of the genes encoding VIP-R, CGRP-R, and the closely related CT-R in human thymus from controls and MG patients (hyperplasia and thymomas) as well as in thymic epithelial cells either in primary culture (PTEC) or transformed by the SV40LT oncogene (LT-TEC); 2) the functional expression and pharmacological properties of VIP-R, CGRP-R, and CT-R in PTEC and LT-TEC cultures regarding cAMP generation and regulation of the transcripts encoding the α-AChR.

### Materials and Methods

#### Materials

All peptides used, human PACAP-38, human peptides with NH2-terminal histidine and C-terminal methionine (PHM) or C-terminal valine (PHV), pancreatic glucagon, human truncated glucagon-like peptide-1 (TGLP-1), and islet amyloid polypeptide (IAPP), were of synthetic origin (Peninsula Laboratories, Merseyside, U.K.), except for VIP, which was isolated from pig upper intestine (Laboratory of Prof. V. Mutt, Karolinska Institute, Stockholm, Sweden). Adenosine 3’5’-cyclic phosphoric acid, 2′-O-succinyl-3′,2′-O-diotyrosine methyl ester (sp. act. 74 Tbam) was obtained from Amersham International (Orsay, France). All other chemicals used were of reagent grade.

#### Thymic tissues

Fresh samples of thymus were obtained from patients undergoing corrective cardiovascular surgery (age range, 2 mo to 27 yr) or from patients undergoing therapeutic total thymectomy for MG (age range, 15–50 yr) at Hôpital Marie Lannelongue (Le Plessis Robinson, France). A fragment of each specimen was flash-frozen in liquid nitrogen and then either stored at −80°C in RNase-free conditions and/or prepared for propagation in primary culture.

#### Cell culture

PTEC were established as previously described (25). Briefly, small fragments of thymic tissue (1 mm3) were washed in RPMI 1640 (Life Technologies, Cergy-Pontoise, France) and transferred into 75-cm2 culture dishes in culture medium supplemented with 20% horse serum (Boehringer Mannheim, Mannheim, Germany), 0.2% Ultroser (Life Technologies), 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin. Explant cultures were maintained at 37°C in a humidified atmosphere containing 5% CO2 for 8–12 days. Thereafter, the confluent monolayers were passaged using trypsin-EDTA treatment (Life Technologies).

#### Flow cytometric analysis

The epithelial nature of the thymic cell cultures was established by flow cytometry using a mix of MNF116 and CK-1 anti-keratin Abs (Dako, Trappes, France). The epithelial nature of the thymic cell cultures was established by flow cytometry using a mix of MNF116 and CK-1 anti-keratin Abs (Dako, Trappes, France). Most contaminant fibroblasts were eliminated by selective trypsinization. They represent <10% of the total cell population as assessed by FACS analysis using anti-collagen III Ab (ICN, Costa Mesa, CA). The thymic epithelial cell population (at least 90% enriched), obtained after selective resistant to trypsin, was then used for PTEC culture and SV40-LT transformation (see below).

To obtain evidence of VIP-R expression in PTEC and LT-TEC1 cells in culture, we used the rabbit polyclonal Ab (pAb) A directed against the first extracellular loop of this serpineptide receptor (26) at a dilution of 1/100 in PBS. Primary and SV40-LT-transformed thymic cells were then washed, incubated with goat anti-rabbit bound to tetramethylrhodamine isothiocyanate (Immunotech, Marseille, France), and washed twice in PBS.

To our knowledge, there is no available CGRP-R or CT-R Ab. We therefore biotinylated the CGRP and CT peptides according to the immunoprobe biotinylation kit, as described by the manufacturer (Sigma, Saint-Quentin Fallavier, France). Biotinylated CGRP or CT was incubated for 60 min with PTEC and LT-TEC1 cells, then unbound probe was eliminated by three washes, and fluorescence was revealed by avidin-phycocerythrin (Immunotech). After three additional washes, thymic cells were analyzed by flow cytometry.

### Histochemistry on frozen thymic sections

The expression of the type 1 VIP-R in normal human thymus was investigated by immunohistochemistry, using rabbit pAbs A and B directed against the first extracellular and the intracellular loops, respectively, of the transmembrane receptor (26) at a dilution of 1/100 in PBS. Briefly, thymic sections were fixed with 4% paraformaldehyde for 10 min, incubated for 60 min with the primary type 1 VIP-R pAbs, washed three times and then revealed by goat anti-rabbit bound to tetramethylrhodamine isothiocyanate, as described above. Double labeling with anti-keratin Abs (a mix of mAbs CK1 and MNF116) revealed by goat anti-mouse Iggs coupled to fluorescein (Silenius, Eurobio, Les Ulis, France) was performed to visualize the epithelial network in the thymus. Controls were performed by omitting the primary Abs.

The expression of CGRP-R and CT-R was analyzed on normal human thymic sections using the corresponding biotinylated peptides as described above. Briefly, histochemistry was performed by incubating parafomaldehyde-fixed thymic sections with the probes overnight in the presence of the protease inhibitors aprotinin, pepstatin, and PMSF (Sigma, France). The sections were then incubated with streptavidin coupled to Texas Red (Amersham). Double staining with anti-keratin Abs was performed as described above. Controls were performed by omitting the biotinylated peptides.

### Transformation of thymic epithelial cells by the SV40LT oncogene

Thymic epithelial cells (4 × 106 cells) were harvested by trypsinization; washed in a solution containing 10 mM Na2HPO4/NaH2PO4, 250 mM sucrose, and 1 mM MgCl2 (pH 7.45); and incubated for 10 min at 4°C in the same buffer in the presence of the pMK16 plasmid (10 μg/ml) recombined with the origin-defective mutant of the SV40 (27). Cells were transiently permeabilized by eight square electric pulses generated by an electropulsator (100 μs, 1350 V/cm, 1 Hz; Bioblock, Rungis, France) as previously described (28, 29). After 3 wk in culture, four independent proliferative clones of epithelial cells were isolated using cloning rings and amplified. The resulting SV40LT-transformed cell lines were designated LT-TEC1 to LT-TEC4 and were cryopreserved. The expression of the SV40LT oncogene in LT-TEC was checked by Northern blot (26, 29) and immunofluorescence, using a mAb against the viral antigen (dilution, 1/10; Pharmingen, San Diego, CA) and revealed with an anti-mouse IgG coupled to fluorescein (Eurobio).

### Expression of VIP-R, CGRP-R, and CT-R genes by RT-PCR and Southern blot

Total RNA was isolated by guanidinium isothiocyanate extraction and cesium chloride density gradient ultracentrifugation. RNA samples (5 μg) were reverse transcribed for 60 min at 37°C, using 200 U of Moloney murine leukemia virus reverse transcriptase (Life Technologies). The cDNAs (0.5–2 μg) were diluted in 25 μl of 20 mM Tris-HCl buffer (pH 8.5) containing 16 mM (NH4)2SO4, 2.5 mM MgCl2, 150 μg/ml BSA, 12.5 mM of each primer, 100 μM each of deoxynucleoside triphosphate, and 1.25 U of Bio-Taq polymerase (Bioprobe Systems, Montrouls sous Bois, France).
The amplification of the CGRP-R and CT-R cDNA (30, 31), respectively, consisted of 30 and 40 cycles of denaturation for 1 min at 92°C, annealing for 30 s at 53 and 58°C, and a 1-min extension at 72°C in an automated thermal cycler (PHC-3, Techne, Osti, Paris, France). The reaction was initiated by a 5-min incubation at 94°C and was ended after a 7-min extension at 72°C. For Southern analysis, PCR products were resolved on a 1.5% agarose gel stained with ethidium bromide, transferred to Hybond N membranes by alkali blotting, and hybridized overnight with the CGRP-R internal probe or the cDNA of the CT-R gene labeled with [α-32P]dCTP (Mega prime, Amersham, Aylesbury, U.K.). Membranes were washed twice at room temperature in 2× SSC (20× SSC is 3 M NaCl and 0.3 M sodium citrate, pH 7.0) 0.1% SDS, followed by a 45-min incubation at 55°C in 0.1× SSC/0.1% SDS. Amplification of the type VIP-R cDNA (19, 32) consisted of 30 cycles of 1 min at 94°C, annealing for 1 min at 56°C, and a 1-min extension at 72°C in the presence of 0.5 μCi of [α-32P]dATP. PCR products were resolved by agarose gel electrophoresis, transferred to nylon membranes, and subjected to autoradiography. Autoradiography was performed for 3–8 h at ~70°C, using Kodak Biomax MR films (Eastman Kodak, Rochester, NY) and a Chronex Quanta III intensifying screen (NEN, Boston, MA).

The sequences of the sense and antisense oligonucleotides for the amplification of the receptor transcripts were 5′-GACATCCAGACAAAGCAGA-3′ and 5′-CAATGCGAAGGTGCGACTG-3′ for the CGRP-R, 5′-GGGTCCCTATCATCTGTGC-3′ and 5′-GAGATAATACCATTTGCGG-3′ for the CT-R, 5′-GGGCTCGTGGCGTCTGAAGG-3′, and 5′-GACAGAGGACTGCGGTGGT-3′ for the VIP-R, and 5′-TGCTCGTCCACTCAGGA-3′ and 5′-GTCGAGGATCTCTCCTTTAT for insulin (33). The expected sizes of the PCR products were 707 bp (CGRP-R), 529 bp (CT-R), 754 bp (VIP-R), and 446 bp (insulin). The corresponding internal probes for the Southern analysis were 5′-TCACCTCTACCTGCAAGGC-3′ for the CGRP-R, 5′-GAGGATTATGTTCTGCTCAG-3′ for the VIP-R, and 5′-TTCTGCTGACGTTCTTGGAT-3′ for insulin.

To evaluate the integrity and the relative amounts of RNA samples, a 574-bp sequence of the GAPDH mRNA was amplified using the sense primer 5′-TCTCCTGCACCACTGTC-3′ and the antisense primer 5′-CCTGTTCACTACCCATTT-3′.

**Competitive RT-PCR for quantification of the α-AChR mRNA**

The standard α-AChR mRNA was constructed by site-directed mutagenesis, introducing a new Bal3 restriction site (34). Immediately before RT, the standard RNA was diluted to 0.05 × 10^18 M. Two micrograms of total RNA together with a known amount of standard RNA was reverse transcribed in a 50-μl reaction mixture containing the downstream primer GGAACGATACGTGCAGCGAGC (50 pmol). The PCR conditions were as previously described (12), and a trace amount of 32P-labeled 5′ primer (GGAATCCTACGATGTGCTGGCG) was added to the reaction mixture (2–3 × 10^3 cpm/tube). The corresponding PCR product was 431 bp. After amplification, the products were digested with Bal3I and separated by electrophoresis with a 1.5% agarose gel containing ethidium bromide. Then, the standard control was revealed as two bands of 279 and 152 bp. The bands were excised, and the amount of radioactivity was determined by scintillation counting and is expressed as counts per minute.

To analyze the regulation of the AChR α-chain transcripts by CGRP and CT, PTEC and LT-TEC1 were distributed and incubated overnight in six-well plates (5 × 10^5/well). Then, each peptide (10–50 nM) was added to the culture for a 24-h period. Treated and control cultures were harvested, and RNA was extracted.

**Cellular cAMP production**

The cAMP production in primary and SV40LT-transformed thymic epithelial cells was measured after incubation in the presence or the absence (control) of the neuroendocrine peptides investigated in the present study as previously described (35). Briefly, the subconfluent cells in culture (10–25 × 10^4 cells/well) were preincubated for 10 min at 20°C in 0.9 ml of Krebs-Ringer phosphate buffer (pH 7.5) containing 1 mM isobutylmethylxanthine as a phosphodiesterase inhibitor, and 2% BSA (w/v). Thereafter, 0.1 ml of the same buffer containing the peptides at different concentrations was added, and the cultured cells were incubated for an additional 60 min. The cAMP production was then quantitated by RIA (28). For each experiment, the mean cell number per well was determined after trypanoscopy of four separate wells. Data are expressed as picomoles of cAMP produced per 10^6 cells.

**Results**

**Establishment of SV40LT-transformed thymic epithelial cells**

When seeded for primary culture, most normal thymic explants were rapidly surrounded by epithelial cell monolayers. As shown in Fig. 1, primary cultures of PTEC obtained from human thymus are highly enriched in epithelial cells as assessed by flow cytometry (>90%). The percentage of fibroblasts detected by the anti-collagen III Ab was consistently <10%, and the percentage of macrophages detected by the HLA-DR Ab was <2%.

Most contaminant fibroblasts were then removed by a series of scratchings and selective trypsinizations. The resulting packed polygonal epithelial cells containing 90–95% epithelial cells were subjected to electronmicroscopy in the presence of the pMK16 vector combined with the origin-defective (ori-) SV40 genome. Fast growing colonies were selected and amplified. The resulting transfected cells were designated LT-TEC1 to LT-TEC4. Primary and SV40LT-transformed thymic epithelial cells in culture exhibited similar morphological appearances by phase-contrast microscopy (Fig. 2, a and b). The epithelial nature of PTEC and LT-TEC cells was confirmed by the expression of cytokeratins, using their respective corresponding cytospin slide preparations (Fig. 2, c and d), distinguishing them from bone marrow-derived stromal components.

The large T oncogene was functionally inserted in SV40LT-transformed thymic cells, as shown by Northern blot and immunofluorescence analysis (Fig. 3). The transcript of SV40LT was identified as a main band of 2.5 kb in the LT-TEC1 and LT-TEC2 cell lines and simian kidney fibroblasts COS-7, which were used as a positive control (36). No hybridization was detected in nontransformed primary thymic epithelial cells. Intense nuclear staining of the LT Ag was observed in Lab-Tek (Corning Costar, Brumath, France) chamber preparations from LT-TEC1 cell cultures, using indirect immunofluorescence revelation (Fig. 3).

**Expression of the CGRP-R/CT-R and VIP-R genes in human thymus and thymic epithelial cells by RT-PCR and Southern blot**

To characterize the expression of the genes encoding the receptors for CGRP, calcitonin, and VIP, RNA samples from human thymus and derived epithelial cells in culture were examined by RT-PCR and Southern blot (Fig. 4). The PCR products corresponding to the CGRP-R transcripts were widely observed among the samples tested (707 bp), including four different resections of control thymus, thymi from three patients with MG, and thymi from two...
patients with thymoma. Again, the CGRP-R transcript was clearly detected in two different primary cultures of PTEC, whereas the signal was weaker in their SV40LT-transformed counterparts LT-TEC (lanes 1–3) or in human colonic cancer cells Caco-2 and HT29 (lanes 1 and 2, respectively).

As seen in control thymus, hyperplasia, and thymoma from MG patients, two bands of CT-R transcripts (529 and 578 bp) were also detected in LT-TEC and human colonic cells, but were absent in PTEC1 and PTEC2. In contrast, the signal for VIP-R transcript (754 bp) was seen in all preparations from human thymus, including control and MG thymomas, thymic epithelial cells in culture, and Caco-2 cells, with quite similar intensities (Fig. 4).

**Histochemical localization of type 1 VIP-R, CGRP-R, and CT-R in human thymus**

The expression and cellular localization of type 1 VIP-R were also characterized by immunohistochemistry in normal human thymus. As shown in Fig. 5, type 1 VIP-R-positive cells were mostly epithelial cells, as assessed by double labeling with the type 1 VIP-R pAbs (A and B) and anti-keratin Abs. The arrows indicate the double-stained cells. The staining was observed in both the medulla and the cortex areas of the thymus. In the medulla, nonepithelial cells such as thymocytes were also labeled.

Expression of CGRP-R and CT-R revealed on thymic sections by the biotinylated peptides is shown in Fig. 5. In both cases, many epithelial cells were double stained with anti-keratin Abs. Other cell types also harbor these neuroendocrine peptide receptors.
Flow cytometric analysis of the neuroendocrine receptors for VIP, CGRP, and CT in derived thymic epithelial cells in culture

PTEC and LT-TEC1 cells were incubated either with the rabbit pAb directed against the extracellular domain of the type 1 VIP-R (pAb A) or with the biotinylated peptide CGRP. FACS analyses indicated the high percentage of keratin-positive cells in PTEC (92%) and LT-TEC1 cultures (73%), as shown in Fig. 6. Most interestingly, PTEC, but not LT-TEC cells, did express CGRP-R, while similar percentages of thymic cells positive for type 1 VIP-R were observed in PTEC and LT-TEC1 cells (respectively, 60 and 47%). Using anti-type I VIP-R Abs, we detected two cell populations in PTEC and LT-TEC1: one brightly stained in 35 and 10% of cells, and the second one displaying a dim expression in 25 and 37% of cells, respectively (Fig. 6). Double labeling of PTEC with anti-keratin Abs showed that gated type I VIP-R bright cells were 100% keratin positive (mean fluorescence intensity, 1000), while the gated type I VIP-R dim cells were 84% keratin positive (mean fluorescence intensity, 236; data not shown).

FIGURE 5. Histochemical localization of the type 1 VIP-R, CGRP-R, and CT-R in the normal human thymus. Frozen sections from control human thymus were double stained with the type 1 VIP-R polyclonal Abs (pool of the pAbs A and B, see Materials and Methods) and the CK1 anti-cytokeratin Ab. The VIP-R Abs stain epithelial cells in both the cortex and the medulla as well as most thymocytes in the medulla. Controls obtained by omitting the primary VIP-R Abs were negative. Staining of thymic sections performed with biotinylated CGRP or CT and revealed by streptavidin coupled to Texas Red detected numerous epithelial cells (keratin positive) displaying these neuropeptide receptors. The arrows indicate the double-stained epithelial cells. Controls obtained by omitting the biotinylated peptides CGRP and CT were negative.
the human thymus and derived primary epithelial cells PTEC in culture.

Effects of CGRP, CT, VIP, and their related peptides on cAMP production in human thymic epithelial cells in culture

Since the transcripts encoding CGRP-R, CT-R, and VIP-R are clearly identified in human thymus, we investigated the effects of these peptides and their naturally occurring analogues on cellular cAMP production in PTEC and SV40LT-transformed LT-TEC thymic epithelial cells. The rationale behind such an approach was to evaluate the functional status of the CGRP-R, CT-R, and VIP-R, which, upon coupling with trimeric GTP-binding proteins, are known to increase adenylyl cyclase activity (37).

VIP-related peptides. As shown in Fig. 7, VIP was the most potent effector among the VIP-related peptides tested in stimulating cAMP production in both PTEC and LT-TEC1 cell lines. A dose-dependent effect by VIP was observed in primary and SV40LT-transformed thymic epithelial cells, with respective EC50 values of 0.22 ± 0.02 and 0.06 ± 0.01 nM VIP. The maximal effective concentration of VIP (10^-9 M) raised basal cAMP levels by approximately 2-fold (from 5.4 ± 0.6 to 9.0 ± 0.4 pmol cAMP/10^6 PTEC cells) and 13-fold (from 3.5 ± 0.4 to 44.8 ± 4.2 pmol cAMP/10^6 LT-TEC1 cells; n = 4). In contrast, the following natural VIP analogues were much less potent than VIP, according to their respective relative potencies: VIP > PACAP > PHM, PHV. Secretin and the other VIP-related peptides, pancreatic glucagon and TGLP-1 (18), were also ineffective in the same biochemical assay. These pharmacological profiles demonstrate the presence of a high affinity, VIP-preferring receptor (type 1) in both primary and SV40LT-transformed human thymic epithelial cells.

CGRP-related peptides. As shown in Fig. 8, human CGRP and the potent agonist of CGRP-R, chicken CGRP (37), dose dependently stimulated cAMP production in PTEC. The CGRP-related peptides, human and salmon CT, were ineffective, as were human and rat islet amyloid polypeptide IAPP (data not shown). Similar results based on receptor binding assays were obtained in 293 cells expressing the recombinant human CGRP type 1 receptor and in

FIGURE 6. FACS analysis of the PTEC and LT-TEC1 cell lines showing CGRP-R and type 1 VIP-R and the percentage of keratin-positive cells. PTEC and LT-TEC1 were incubated either with the polyclonal anti-VIP-R Ab A and revealed with goat anti-rabbit bound to FITC or with biotinylated CGRP and revealed with avidin-phycocerythrin. The labeling with anti-keratin Ab shows the high percentage of epithelial cells in PTEC and LT-TEC1 cultures (92 and 73%, respectively). The unshaded areas correspond to the control data obtained by omitting the first layer (either the primary Ab or the biotinylated peptides).

FIGURE 7. Comparative effects of VIP and related peptides on cAMP production in PTEC and LT-TEC1 cultures. PTEC and LT-TEC1 were incubated with increasing concentrations of VIP, PACAP, PHM, PHV, and secretin. The experiment was performed as described in Materials and Methods. The data are expressed as picomoles of cAMP produced per million cells and are the mean ± SEM values from four experiments performed in triplicate.

FIGURE 8. Comparative effects of human CGRP, CT, and related peptides on cAMP production in PTEC and LT-TEC1 cultures. PTEC and LT-TEC1 were incubated with increasing concentrations of human CGRP (hCGRP), chicken CGRP (cCGRP), human CT (hCT), and salmon CT (sCT). The data are expressed as picomoles of cAMP produced per million cells and are the mean ± SEM values from four experiments performed in triplicate.
the human neuroblastoma SK-N-MC cell line (30). Chicken CGRP was about 6 times more efficient than the corresponding human peptide in stimulating cAMP production in PTEC according to their respective EC50 values (37 ± 5 and 193 ± 21 pM). Both peptides at 10 nM exerted similar maximal stimulations, i.e. a 10-fold increase over control cAMP levels. These data confirm our molecular detection of CGRP type 1 receptors by RT-PCR and flow cytometry in PTEC (Figs. 4 and 6) and the absence of CT-R by RT-PCR in PTEC.

In contrast to the above results, transformation of PTEC by the SV40LT Ag resulted in the molecular expression of CT-R detected by RT-PCR (Fig. 4) and their functional coupling to cAMP production in the LT-TEC1 cell line (Fig. 8). Salmon CT, a potent agonist of CT-R, was more efficient than human CT to stimulate cAMP generation with respective EC50 values of 98 ± 15 pM salmon CT and 227 ± 28 pM human CT. Human and salmon CT displayed maximal stimulation within the 1–10 nM concentration range, corresponding to a 2- to 1.5-fold increase over control values, respectively. Human CGRP was ineffective in stimulating cAMP production over control cAMP levels in LT-TEC1, and this observation was consistent with the absence of CGRP binding to LT-TEC1 cells (Fig. 6). Chicken CGRP at 100 nM exerted a 2-fold increase in cAMP levels in this system (3.5 ± 0.4 pmol/million cells), according to the interaction of this peptide with CT-R. Similar results were obtained in two other SV40-LT-transformed thymic epithelial cell lines, LT-TEC2 and LT-TEC3.

Regulation by CGRP and CT of the gene encoding α-AChR in PTEC and LT-TEC1

We previously demonstrated that the AChR is expressed in PTEC (12). Since the α-AChR mRNA and number of surface AChR are known to be up-regulated by CGRP in cultured chicken myotubes (13), we addressed the question of whether PTEC and LT-TEC are sensitive to the actions of these neuropeptides for AChR gene expression. We used quantitative RT-PCR technology for quantification of the α-AChR (Fig. 9A) as previously described (12). Using this method, Fig. 9B demonstrates that human CGRP (10⁻⁹ M), but not human CT at the same concentration, up-regulated α-AChR gene expression threefold in PTEC. An inverse situation was observed in LT-TEC1, since α-AChR gene expression increased threefold in the presence of human CT, whereas CGRP was ineffective despite expression of the CGRP-R gene by RT-PCR in LT-TEC1–3 (Fig. 4). On the other hand, we observed that VIP (10⁻⁹ M), was unable to increase the accumulation of the transcripts encoding the α-AChR in both PTEC and LT-TEC1 (data not shown) despite the presence of functional VIP-R coupled to cAMP generation.

Discussion

In the present study we demonstrate the expression of the genes encoding CGRP-R, CT-R, and VIP-R in normal and pathological human thymi as well as in thymic epithelial cells either in primary
CGRP is also distributed in cells and nerve fibers in hemopoietic rat thymocyte subsets (CD4+). Also, VIP gene expression was detected in capsular regions as well as in the connective tissue trabeculae separating the lobules (40). VIP-positive nerve fibers are distributed in the capsular and subcapsular regions as well as in the connective tissue trabeculae separating the lobules (40). Also, VIP gene expression was detected in rat thymocyte subsets (CD4+, CD8+). CGRP is also distributed in cells and nerve fibers in hemopoietic and lymphoid organs, including bone marrow, lymph node spleen, and thymus (41, 42). Unlike VIP, which is located in the deep thymic cortex, CGRP was recently identified in a subpopulation of thymic epithelial cells, in nerve fibers at the cortico-medullary junction, and in perivascular and paravascular plexus supplying arteries, veins, and the microvasculature (43, 44). Thus, the presence of CGRP- and VIP-immunoreactive cells and nerve fibers in the thymus suggests a possible autocrine/paracrine release and function of these neuroendocrine peptides in the thymic microenvironment (8, 23, 45). In the case of CGRP, it has also been shown to regulate several immune and inflammatory responses in vitro, including inhibition of mitogen-stimulated proliferation of T cells, inhibition of Ag presentation, and modulation of B cell differentiation (46). More recently, CGRP was shown to enhance the apoptosis of thymocytes (47) as opposed to the protection exerted by VIP. It can be questioned whether these neuropeptides would also affect programmed cell death of thymic epithelial cells, which have now been shown to contain specific VIP and CGRP receptors. Whether these interactions could be related to cell death of thymic lymphocytes and epithelial cells and thus to the physiological involution of the thymus observed after puberty remains to be investigated.

Since the VIP-R present in PTEC and LT-TEC are not coupled with the induction of the gene encoding the α-AChR, one can speculate that this transcriptional up-regulation induced by CGRP and CT is not dependent on the elevation of cAMP, but is probably activated by another signaling pathway. Accordingly, the cAMP-elevating agent forskolin was shown to down-regulate the transcript levels of the AChR α- and e-chains in PTEC and the TE671 rhabdomyosarcoma cell line (12, 48). Unlike VIP type 1 receptors, the activation of either CT-R or CGRP-R results in coupling to several G proteins, such as Gα1 and Gαq/11, which activate several downstream signaling pathways, such as adenyl cyclase and the phospholipase C/protein kinase C/inositol triphosphate/calcium cascade (49–51). At least two subtypes, CGRP1 and CGRP2 receptors, have been identified, and the existence of multiple human CT receptor isoforms is also suggested in ovarian, breast cancer, and giant cell tumor of the bone (30, 31, 51–57). In the rat, the C1b isoform contains a 37-amino acid insert in the putative first extracellular loop that confers altered ligand binding characteristics, but does not modify their ability to generate multiple second messengers (54, 55). In contrast, the 16-amino acid insertion in the first intracellular loop in the human CT receptor abolishes stimulation of the phospholipase C signal transduction pathway while allowing stimulation of the cAMP pathway (52, 53, 56). These data are in agreement with the observation that the CT-R can exert opposite biological effects via selective transduction pathways during the cell cycle, with the receptor coupling through a Gq protein during G2 phase and through Gq/11 proteins during S phase (49, 50).

Another level of regulation related to the cAMP/signaling responses and expression of the AChR α-chain evoked by CGRP-R/CT-R in human thymus emerges from the interaction of these membrane receptors with endogenous proteins. In this connection, the CGRP-R family has been recently shown to interact with new single transmembrane domain proteins called RAMPs, i.e., receptor activity-modifying proteins, that regulate the ligand specificity, transport, glycosylation, and presentation of these serpentine receptors at the cell surface (58). Furthermore, a novel accessory factor, designated receptor component protein (RCP), is required for conferring endogenous CGRP receptor activity in Xenopus oocytes (59). This intracellular membrane-associated protein RCP is also required for CGRP-R function in NIH-3T3 cells. Thus, differential expression of RAMPs and RCP-like proteins may be involved in differential binding and signaling by the neuroendocrine peptides CGRP/CT during MG or neoplastic transformation of the thymus.
human thymus; the expression of these receptor-regulating factors remains to be investigated in PTEC and LT-TEC cell lines. In this connection, accumulating evidence suggests that elevated expression of the peptides CGRP and CT is associated with positive or negative mitogenic responses in several human tumors, such as breast, renal, lung, and gastric carcinoma (60), as well as in the thymus CD4+ T cell population (21).

In conclusion, this study clearly demonstrates the functional expression of CGRP-R, CT-R, and type 1 VIP-R in human thymus and thymic epithelial cells at various stages of the neoplastic transformation associated with MG. Thus, CGRP-R and CT-R might be involved in MG via induction of the AChR Ag in both epithelial and myoid thymic cells. Furthermore, thymic epithelial cells, thymocytes, and mesenchymal cells are known to affect each other’s functions, including growth, differentiation, and apoptosis. Therefore, we now propose that these neuroendocrine peptides, by acting on thymic epithelial cells, can be considered novel modulators of the cross-talk between these cell lineages.

Note added in proof. A recent paper by Throsby et al. (61) indicates that both dendritic cells and macrophages are the sites of preproinsulin synthesis in the murine thymus. This report is consistent with our findings related to the absence of insulin transcripts by RT-PCR/Southern blot in our preparations of primary PTEC and LT-TEC, while this message was clearly identified in all human thymus included in Fig. 4.

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