Induction and Expression of β-Calcitonin Gene-Related Peptide in Rat T Lymphocytes and Its Significance

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Induction and Expression of \( \beta \)-Calcitonin Gene-Related Peptide in Rat T Lymphocytes and Its Significance

Liyu Xing, Jingxuan Guo, and Xian Wang

Our previous data have shown that rat lymphocytes can synthesize calcitonin gene-related peptide (CGRP), a neuropeptide. In this study the type, characteristics, and functional role of lymphocyte-derived CGRP were investigated. The results showed that treatment with Con A (4 \( \mu \)g/ml) and recombinant human IL-2 (rhIL-2; 750 U/ml) for 3–5 days induced CGRP synthesis and secretion by lymphocytes from both thymus and mesenteric lymph nodes in a time-dependent manner. Stimulation of these cells with Con A (1–8 \( \mu \)g/ml) or rhIL-2 (94–1500 U/ml) for 5 days induced a significant increase in CGRP secretion in a concentration-dependent manner. The maximal secretion of CGRP with Con A by thymocytes was elevated from 104 \( \pm \) 11 to 381 \( \pm \) 44 pg/10\(^6\) cells, and that by mesenteric lymph node lymphocytes was elevated from 83 \( \pm \) 10 to 349 \( \pm \) 25 pg/10\(^6\) cells, respectively. The maximal CGRP secretion with rhIL-2 by thymocytes was elevated from 116 \( \pm \) 3 to 607 \( \pm \) 23 pg/10\(^6\) cells, and that by mesenteric lymph node lymphocytes was elevated from 117 \( \pm \) 9 to 704 \( \pm \) 37 pg/10\(^6\) cells, respectively. The nucleotide sequencing study showed that lymphoid cells expressed \( \beta \)-CGRP mRNA only. The levels of \( \beta \)-CGRP mRNA in mitogen-stimulated lymphocytes of both sources were also increased. However, LPS had no such effect on either source of cells. hCGRP\(_{8-37}\) (2.0 \( \mu \)M), a \( \beta \)-CGRP receptor antagonist, enhanced Con A-induced proliferation and IL-2 release of thymocytes by 41.3 and 35.8\% over those induced by Con A alone, respectively. The data suggest that T lymphocyte mitogens can induce the production of endogenous \( \beta \)-CGRP from T lymphocytes, which may partially inhibit the proliferation and IL-2 release of rat T lymphocyte under immune challenges.

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uring the last 10 years numerous studies have been performed to identify the mechanisms responsible for the bidirectional communication between neuroendocrine and immune systems. A large body of information obtained from both in vitro and in vivo studies indicates that shared ligands and receptors are used as a common chemical language within and between the immune and neuroendocrine systems. Corticotropin was the first de novo synthesized hormone found in the immune system (1, 2). Since then, >20 kinds of peptide hormones and neurotransmitters have been found to be synthesized in immunocytes, and the receptors of these substances have also been determined in immunocytes.

Calcitonin gene-related peptide (CGRP)\(^3\) is a 37-aa neuropeptide that is encoded by the calcitonin gene and is one of the first neuropeptides to be discovered by molecular biologic approaches (3). According to the differences in encoding gene and amino acid residues, CGRP is divided into two types, \( \alpha \)- and \( \beta \)-CGRP. The human calcitonin/\( \beta \)-CGRP gene is located in the q12-pter region of chromosome 11, whereas the human CT/\( \alpha \)-CGRP gene is assigned to the p13-p15 region of chromosome 11 (4). Human \( \alpha \)-CGRP differs from \( \beta \)-CGRP in 3 of the 37 aa (5). Rat \( \alpha \)-CGRP differs from \( \beta \)-CGRP in 1 aa (6). CGRP distributes widely in the central and peripheral nervous systems, particularly in the C fibers of sensory nerves and in the sensory neuron B cells. In vivo and in vitro studies demonstrate that CGRP plays an important biological role in cardiovascular, respiratory, and digestive systems. CGRP has been a particularly interesting candidate for a neuroendocrine immune modulator. It has been shown that CGRP also has extensive function in immunomodulation. It is localized in nerve endings in virtually every blood vessel, including blood vessels in lymph nodes (7) and thymus (8). In our previous work we proved that there is CGRP-like immunoreactivity (CGRP-LI), coeluted with synthetic rat \( \alpha \)-CGRP and sensory neuron-derived CGRP by reverse phase HPLC in the extracts of rat thymocytes and mesenteric lymph node lymphocytes observed by RIA. There is CGRP mRNA expression in lymphocytes demonstrated by RT-PCR (9). CGRP-LI has been localized also in rat thymocytes and mesenteric lymph node lymphocytes, which do not adhere to nylon fibers, by immunocytochemical methods (10). These data suggest that CGRP may be synthesized and secreted by T lymphocytes of both thymus and lymph node in the rat. Moreover, a CGRP binding site has been described in lymphoid tissue, such as those binding sites on mouse T cells (11), in canine mesenteric lymph nodes (7), and on rat thymocytes (12). The presence of CGRP and its binding sites in these microenvironments allows it access to cellular components, which mediate inflammatory and immune reactions.

The type of CGRP expressed in lymphoid cells was not identified in previous studies because the anti-CGRP Ab and CGRP primers for RT-PCR used previously could not distinguish \( \alpha \)- and \( \beta \)-CGRP. In this study the characteristics of synthesis and release of CGRP in lymphocytes were investigated by stimulating cultured lymphocytes with different concentrations of Con A, recombinant human IL-2 (rhIL-2), and LPS. The type of CGRP synthesized in lymphoid cells was identified by RT-PCR using \( \beta \)-CGRP-specific primers and nucleotide sequencing. Furthermore, the functional...
roles of lymphocyte-derived CGRP in Con A-induced thymocyte proliferation and IL-2 production were observed using the CGRP<sub>1</sub> receptor antagonist, hCGRP<sub>8-37</sub>.

**Materials and Methods**

**Animals**

Adult male Wistar rats were supplied by experimental animal center of Beijing Medical University (Beijing, China). Rats, weighing 200–250 g, were maintained in a constant temperature room with fixed light/dark intervals of 12 h. They were fed food and water ad libitum.

**Cell preparation**

Rats were anesthetized with urethane. Thymus and mesenteric lymph nodes were removed. Single-cell suspensions of thymus and lymph nodes were prepared by tapping organs on a 20-μm pore size nylon mesh gauze. After centrifugation (20 min, 1000 × g) over Ficoll-Hypaque density gradients (density, 1.079), the cell layer at the interface was resuspended in RPMI 1640 containing 10% heat-inactivated FBS and washed three times, and then the cell suspensions were separated into wall-adhesive cells (lymphocytes) and wall-adhesive cells (macrophages and other kinds of cells) by incubating 10 ml of the suspensions in petri dishes (10-cm diameter) for 1 h at 37°C. Lymphocytes were collected and pelleted at 800 × g for 10 min. Cell viability was monitored by trypan blue exclusion. Only cell preparations with a 95% viability or greater were used.

**CGRP release experiment**

The rat thymocytes and mesenteric lymph node lymphocytes were plated in petri dishes (10-cm diameter) at 4 × 10<sup>5</sup> cells/dish with RPMI 1640 containing 5% FBS. Dose-response curves were generated for Con A and rhIL-2. Con A was added at concentrations of 1, 2, 4, and 8 μg/ml. Reombinant rIL-2 was added at concentrations of 94, 188, 375, 750, and 1500 U/ml. The final total volume per dish was 20 ml. The dishes were incubated for 5 days at 37°C in humidified incubator containing 5% CO₂. They were fed food and water ad libitum.

**CGRP release experiment**

The cultures were centrifuged at 1000 × g for 20 min. The supernatants obtained were added with trifluoroacetic acid to a final concentration of 0.1% and loaded on a reverse phase C<sub>18</sub> column. After washing with 20 ml of 0.1 trifluoroacetic acid, CGRP was eluted with 3 ml of 60% acetonitrile in 0.1% trifluoroacetic acid. The cells were resuspended with 2 ml of 0.2 N hot acetic acid and bathed in 95–100°C water for 10 min to be extracted. After centrifugation at 3000 × g for 20 min, the supernatants were harvested. Samples were evaporated to dryness using a centrifugal vacuum evaporator-concentrator.

**RNA preparation**

Clean instruments were used for each tissue dissection to eliminate cross-tissue contamination. Total RNA of rat dorsal root ganglia (DRG), thymus lymphocytes, and mesenteric lymph node lymphocytes was isolated from fresh tissues or cultures by acid guanidinium thiocyanate-phenol-chloroform extraction. Briefly, the tissue or cells were homogenized in guanidinium thiocyanate followed by acid phenol/chloroform extraction and precipitated with isopropanol. Total RNA was digested with RQ1 RNase-free DNase to eliminate genomic DNA contamination. The purified RNA was confirmed by visualization of the 28S and 18S ribosomal RNA bands after electrophoresis of RNA through 1% agarose-formaldehyde ethidium bromide gel.

**Oligonucleotide primers**

The oligonucleotide primers for different fragments of α- and β-CGRP were synthesized on a DNA synthesizer (model 391, Applied Biosystems,

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**Table I. Nucleotide sequence of synthetic CGRP oligonucleotides used for PCR**

<table>
<thead>
<tr>
<th>Primer Type</th>
<th>Sequence (5′-3′)</th>
<th>Orientation</th>
<th>Nucleotide Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonspecific CGRP primer</td>
<td>TCCTGCAAACACCACCCACCTG</td>
<td>S</td>
<td>261 – 281</td>
</tr>
<tr>
<td></td>
<td>GGTGGGCACAAAAATGTTGCTT</td>
<td>AS</td>
<td>333 – 352</td>
</tr>
<tr>
<td>α-CGRP specific primer</td>
<td>TTTTTCGATCATGGGCTTTC</td>
<td>S</td>
<td>(–) 5–10</td>
</tr>
<tr>
<td></td>
<td>AGCCCAAAAGTGACACCATT</td>
<td>AS</td>
<td>708 – 728</td>
</tr>
<tr>
<td>β-CGRP specific primer</td>
<td>GAGGCAGTGATATCAGTGC</td>
<td>AS</td>
<td>727 – 745</td>
</tr>
</tbody>
</table>

* Nucleotide position data are from Amara and colleagues (6).

**RIA of CGRP**

The RIA technique for the measurement of CGRP was similar to the technique used in our previous studies of CGRP release in vivo. Briefly, the samples were reconstituted in RIA buffer (0.1 M phosphate buffer containing 0.1% BSA, 0.01% NaN₃, 50 mM NaCl, and 0.1% Triton X-100, pH 7.4). Typically, when samples were spiked with a known amount of synthetic rat CGRP before purification and drying, there was 80–90% recovery as determined in the RIA. Therefore, no adjustment for percent recovery was used in the calculations of CGRP levels in the samples.

**Materials and Methods**

**Animals**

Adult male Wistar rats were supplied by experimental animal center of Beijing Medical University (Beijing, China). Rats, weighing 200–250 g, were maintained in a constant temperature room with fixed light/dark intervals of 12 h. They were fed food and water ad libitum.

**Cell preparation**

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**CGRP release experiment**

The rat thymocytes and mesenteric lymph node lymphocytes were plated in petri dishes (10-cm diameter) at 4 × 10<sup>5</sup> cells/dish with RPMI 1640 containing 5% FBS. Dose-response curves were generated for Con A and rhIL-2. Con A was added at concentrations of 1, 2, 4, and 8 μg/ml. Recombinant rIL-2 was added at concentrations of 94, 188, 375, 750, and 1500 U/ml. The final total volume per dish was 20 ml. The dishes were incubated for 5 days at 37°C in humidified incubator containing 5% CO₂.

**Cell preparation**

Rats were anesthetized with urethane. Thymus and mesenteric lymph nodes were removed. Single-cell suspensions of thymus and lymph nodes was isolated from fresh tissues or cultures by acid guanidinium thiocyanate-phenol-chloroform extraction. Briefly, the tissue or cells were homogenized in guanidinium thiocyanate followed by acid phenol/chloroform extraction and precipitated with isopropanol. Total RNA was digested with RQ1 RNase-free DNase to eliminate genomic DNA contamination. The purified RNA was confirmed by visualization of the 28S and 18S ribosomal RNA bands after electrophoresis of RNA through 1% agarose-formaldehyde ethidium bromide gel.

**Oligonucleotide primers**

The oligonucleotide primers for different fragments of α- and β-CGRP were synthesized on a DNA synthesizer (model 391, Applied Biosystems,

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**Table II. Changes of intracellular CGRP-LI levels in rat thymocytes and lymphocytes stimulated with 4 μg/ml Con A for 1–5 days (pg/10⁶ cells, mean ± SEM)**

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>Thymocytes</th>
<th>Lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Stimulated</td>
</tr>
<tr>
<td>1</td>
<td>74 ± 3</td>
<td>79 ± 4</td>
</tr>
<tr>
<td>2</td>
<td>76 ± 5</td>
<td>77 ± 10</td>
</tr>
<tr>
<td>3</td>
<td>71 ± 5</td>
<td>115 ± 10⁸</td>
</tr>
<tr>
<td>4</td>
<td>69 ± 4</td>
<td>123 ± 19⁷</td>
</tr>
<tr>
<td>5</td>
<td>78 ± 4</td>
<td>167 ± 13⁷</td>
</tr>
</tbody>
</table>

* Compared with each time point control p < 0.01 (paired t test).
Table III. Changes of intracellular CGRP-LI levels in lymphocytes from thymus and mesenteric lymph nodes stimulated with 750 U/ml rhIL-2 for 1–5 days (pg/10⁶ cells, mean ± SEM)

<table>
<thead>
<tr>
<th>Time (day)</th>
<th>n</th>
<th>Control</th>
<th>Stimulated</th>
<th>Control</th>
<th>Stimulated</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>77 ± 5</td>
<td>77 ± 6</td>
<td>67 ± 3</td>
<td>77 ± 10</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>74 ± 7</td>
<td>74 ± 2</td>
<td>63 ± 2</td>
<td>73 ± 2</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>78 ± 6</td>
<td>100 ± 4a</td>
<td>68 ± 2</td>
<td>97 ± 5b</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>73 ± 4</td>
<td>115 ± 7a</td>
<td>65 ± 6</td>
<td>131 ± 8o</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>74 ± 5</td>
<td>133 ± 8a</td>
<td>67 ± 4</td>
<td>150 ± 4o</td>
</tr>
</tbody>
</table>

* Compared with each time point control p < 0.01 (paired t test).

Foster City, CA). The oligonucleotides were constructed from the cDNA sequences of cloned rat CGRP cDNA (Table I). The sequences of the β-actin primers were as follows: 5’-GAGACCTTCAACACCCAGCC-3’ (coding sense) corresponding to bases 2158–2187, and 5’-TCGGGGCA TCAGAACCCGCTCA-3’ (coding antisense) corresponding to bases 2550–2579. The predicted size of the amplified β-actin cDNA products was 422 bp.

Reverse transcription

The RT reaction was conducted by heating the mixed solution of 2.0 μg of total RNA and 1 μg of oligo(dT)₁₅–₁₇ for 10 min at 65°C and quenching on ice. Then, 200 U of Moloney murine leukemia virus (M-MLV) reverse transcriptase, deionized distilled diethylpyrocarbonate water, and reaction buffer solution were added for a total reaction volume of 25 μl, containing 50 mmol/l Tris-HCl, 75 mmol/l KCl, 3 mmol/l MgCl₂, 10 mmol/l DTT, 1.0 mmol/l of each dNTP, 10 U of RNasin (RNase inhibitor; pH 8.3), and 160 μmol/l of each dNTP, 10 U of Rnas (RNase inhibitor; pH 8.3), and RNase-free Dnase. This mixture was incubated at 37°C for 1 h. The reaction was terminated by heating at 95°C for 5 min and was quenched on ice.

PCR amplification

Negative control reactions without template or M-MLV RT were routinely included in PCR amplification with both primers in parallel. The PCR amplification profits consisted of denaturation at 94°C for 1 min, primer annealing at 62°C for 1 min, and extension at 72°C for 2 min. PCR amplifications for β-actin were conducted using 25 cycles, whereas for CGRP were various numbers of cycle were used. In each PCR, 0.1 μCi of [α-32P]dCTP was added to a total volume of 30 μl. The PCR buffer contained 50 mmol/l Tris-HCl, 10 mmol/l KCl, 2.0 mmol/l MgCl₂, 1% gelatin, 1% Triton X-100, 20 pmol of primers, 100 μmol/l dNTP, and 1 U of DNA polymerase. The PCR products were purified after PAGE. The radiation intensities of the bands were quantitated by a multipurpose scintillation counter (model LS 6500, Beckman, Palo Alto, CA), which expressed the intensities as counts per minute of 32P.

Direct sequencing of PCR-amplified products

Sequencing analysis is an important and essential independent check of PCR product identity and lack of cross-contamination in PCR assays. The RT-PCR products of β-CGRP cDNA were purified by low melting temperature agarose electrophoresis. Sequencing analysis was performed at Beijing SBS Biotechnology Corp. (Beijing, China). One-step sequencing reactions using 5’-end-labeled primers were conducted, and the products were analyzed on sequencer (model 373, Applied Biosystems). Every β-CGRP product was sequenced bidirectionally with both sense and antisense primers.

T cell proliferation assay

The rat thymocytes were plated in 96-well flat-bottom tissue culture plates at 5 × 10⁴ cells/well with serum-free RPMI 1640 medium. Con A was added at concentration of 1.0 μg/ml. Human CGRP_LI was added from 10⁻⁶ to 2 × 10⁻⁶ M. The final total volume per well was 0.2 ml. The plates were incubated for 72 h at 37°C in humidified incubator containing 5% CO₂. Triplicate wells were then pulsed with 1 μCi of [³H]thymidine/well and incubated for an additional 6 h. Cultures were harvested onto glass-fiber filter paper. After drying under 80°C for 2 h, the glass-fiber filter paper was placed in vials with scintillation cocktail and counted on a scintillation counter (Beckman). The results were expressed as the mean ± SEM percent change in tritiated thymidine up-take from Con A-stimulated cells alone.

ELISA for IL-2

The amount of IL-2 present in supernatants from stimulated thymocytes in the presence or the absence of hCGRP₁₋₃₇ was determined using a rat IL-2 ELISA kit (BioSource, Camarillo, CA). The kit was specific for rat IL-2, and the assay was performed according to the manufacturer’s instructions.

Reagents

Human CGRP₁₋₃₇ were purchased from Bachem (Torrance, CA). Rabbit antiserum to hCGRP II was purchased from Peninsula Laboratories (Belmont, CA). This Ab cross-reacts 100% with rat α-CGRP and β-CGRP and shows <0.01% cross-reactivity with human and rat amylin and 0% cross-reactivity with calcitonin, somatostatin, substance P, and vasoactive intestinal polypeptide (data from Peninsula Laboratories). The rat IL-2 ELISA kit was purchased from BioSource. RPMI 1640 was purchased from Life Technologies (Grand Island, NY). M-MLV transcriptase, RQ1 RNase-free DNase, RNasin, HaelIII oligo(dT), Taq polymerase, and pGEM 7zf(+).

Table IV. Changes of intracellular CGRP-LI levels in rat thymocytes and lymph node lymphocytes stimulated with increasing concentration of Con A for 5 days (pg/10⁶ cells, mean ± SEM)

<table>
<thead>
<tr>
<th>Con A (μg/ml)</th>
<th>n</th>
<th>Thymocytes</th>
<th>Lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>10</td>
<td>76 ± 2</td>
<td>65 ± 4</td>
</tr>
<tr>
<td>1.0</td>
<td>10</td>
<td>125 ± 9a</td>
<td>112 ± 2a</td>
</tr>
<tr>
<td>2.0</td>
<td>10</td>
<td>161 ± 8a</td>
<td>136 ± 15a</td>
</tr>
<tr>
<td>4.0</td>
<td>10</td>
<td>156 ± 8a</td>
<td>147 ± 6a</td>
</tr>
<tr>
<td>8.0</td>
<td>10</td>
<td>176 ± 3a</td>
<td>146 ± 12a</td>
</tr>
</tbody>
</table>

* Compared with control p < 0.05 (Student-Newman-Keuls test).
Table V. Changes of intracellular CGRP-LI levels in rat thymocytes and lymph node lymphocytes stimulated with increasing concentrations of rhIL-2 for 5 days (pg/10^8 cells, mean ± SEM)

<table>
<thead>
<tr>
<th>RhIL-2 (U/ml)</th>
<th>n</th>
<th>Thymocytes</th>
<th>Lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>5</td>
<td>73 ± 7</td>
<td>69 ± 7</td>
</tr>
<tr>
<td>94.0</td>
<td>5</td>
<td>103 ± 10</td>
<td>145 ± 13b</td>
</tr>
<tr>
<td>188.0</td>
<td>5</td>
<td>121 ± 8b</td>
<td>159 ± 10b</td>
</tr>
<tr>
<td>375.0</td>
<td>5</td>
<td>131 ± 5b</td>
<td>163 ± 18b</td>
</tr>
<tr>
<td>750.0</td>
<td>5</td>
<td>128 ± 7b</td>
<td>159 ± 11b</td>
</tr>
<tr>
<td>1500.0</td>
<td>5</td>
<td>141 ± 13b</td>
<td>163 ± 15b</td>
</tr>
</tbody>
</table>

* Compared with control p < 0.05 (Student-Newman-Keuls test).

Results

Con A- or IL-2-induced synthesis of β-CGRP by lymphoid cells

To test whether stimulation of Con A, a T cell mitogen, or IL-2, a T lymphocyte growth factor (13) could induce synthesis of CGRP by lymphoid cells in vitro, rat thymocytes and lymph node lymphocytes cultured in RPMI 1640 containing 5% FBS were stimulated with a single concentration of Con A or IL-2 for 1–5 days or with increasing concentrations of Con A for 5 days.

Stimulation of thymocytes and lymph node lymphocytes with Con A (4 μg/ml) for 1–5 days resulted in a significant increase in CGRP synthesis (intracellular CGRP) in a time-dependent manner. After culture with Con A for 3 days, CGRP synthesis in thymocytes and lymph node lymphocytes was increased significantly. After culture with Con A for 5 days, CGRP synthesis was increased further (Table II). Treatment of thymocytes and lymph node lymphocytes with rhIL-2 (750 U/ml) also significantly promoted CGRP synthesis by both cells in a time-dependent manner. Under stimulation with rhIL-2 for 3 days, intracellular CGRP levels in thymocytes and lymphocytes were increased significantly, and after stimulation for 5 days, intracellular CGRP levels in both cells were increased further (Table III).

Stimulation of thymocytes and lymph node lymphocytes with Con A (1–8 μg/ml) for 5 days induced a significant increase in CGRP synthesis in a concentration-dependent manner. Con A (1.0 μg/ml) induced a significant increase in intracellular CGRP in thymocytes and lymph node lymphocytes (Table IV). As shown in Table V, rhIL-2 (94 and 188 U/ml) significantly induced an increase in intracellular CGRP levels in lymph node lymphocytes and thymocytes, respectively.

Characteristics of Con A- or IL-2-induced CGRP secretion by lymphoid cells

Stimulation of thymocytes and lymph node lymphocytes with Con A (4 μg/ml) for 1–5 days resulted in a significant increase in CGRP secretion (extracellular CGRP) in a time-dependent manner. The amounts of CGRP secreted in culture medium by thymocytes and lymph node lymphocytes were increased in a parallel manner under stimulation with Con A (Fig. 1). Thus, CGRP secreted by thymocytes was increased from 84 ± 9 to 291 ± 14 pg/10^8 cells after 3 days and to 465 ± 38 pg/10^8 cells after 5 days of stimulation (Fig. 1A). The amount of CGRP secreted by lymph node lymphocytes was increased from 77 ± 10 to 165 ± 14 pg/10^8 cells after 3 days and to 325 ± 24 pg/10^8 cells after 5 days of stimulation (Fig. 1B).

Treatment of thymocytes and lymph node lymphocytes with rhIL-2 (750 U/ml) also significantly promoted CGRP secretion by both cells in a time-dependent manner. Under stimulation with rhIL-2 for 3 days, CGRP secreted in culture medium by thymocytes and lymph node lymphocytes was increased from 78 ± 6 to 288 ± 16 pg/10^8 cells and from 80 ± 6 to 220 ± 14 pg/10^8 cells, respectively (Fig. 2). After stimulation for 5 days, the amounts of CGRP secreted in medium were increased to 459 ± 40 and 699 ± 36 pg/10^8 cells, respectively (Fig. 2).

Stimulation of thymocytes and lymph node lymphocytes with Con A (1–8 μg/ml) for 5 days induced a significant increase in CGRP secretion in a concentration-dependent manner. Con A (1.0 μg/ml) induced a significant increase in extracellular CGRP in thymocytes and lymph node lymphocytes (Fig. 3A). Thus, CGRP secreted by thymocytes was increased from 38 ± 13 to 93 ± 14 pg/10^8 cells after 3 days and to 125 ± 21 pg/10^8 cells after 5 days of stimulation (Fig. 3A). The amount of CGRP secreted by lymph node lymphocytes was increased from 15 ± 3 to 32 ± 7 pg/10^8 cells after 3 days and to 46 ± 8 pg/10^8 cells after 5 days of stimulation (Fig. 3B).
controls.

278

6

the rat produces CGRP under mitogen induction. The data presented above indicated that the T, but not the B, lymphocyte of the lymphoid cells, which could not distinguish

...moted CGRP secretion, which was elevated from 116 

6  

12 pg/10^6 cells. The maximal CGRP secretions by both cells were 390 

6  

23 and 704 

6  

days. The supernatants were harvested and purified on a reverse phase C_18 column. CGRP-LI was assessed by CGRP RIA. The data are the mean ± SEM for each treatment group (n = 5). * p < 0.01 compared with controls.

FIGURE 4. Concentration curves of rhIL-2-induced CGRP secretion by rat thymocytes (A) and mesenteric lymph node lymphocytes (B). Cells were cultured with or without the indicated concentration of rhIL-2 for 5 days. The supernatants were harvested and purified on a reverse phase C_18 column. CGRP-LI was assessed by CGRP RIA. The data are the mean ± SEM for each treatment group (n = 5). * p < 0.01 compared with controls.

FIGURE 5. Amplification of mRNA from normal thymocytes, mesenteric lymph node lymphocytes, and DRG (positive control) in the rat by RT-PCR. The PCR products were separated by PAGE and visualized with ethidium bromide. A, RT-PCR production of β-CGRP mRNA. m, marker. b, β-CGRP sense paired with antisense primer. The predicted size was 90 bp. c, β-CGRP sense primer paired with CGRP antisense primer. The predicted size was 361 bp.

β-CGRP mRNA, but not α-CGRP mRNA, expression in rat lymphoid cells

Because we have reported that CGRP mRNA is expressed in rat lymphoid cells (9), we were interested in which type of CGRP mRNA was expressed in rat lymphoid cells. To detect the transcript and at the same time to ensure sufficient materials for sequencing, we used RT-PCR. Thymocyte and lymph node lymphocyte mRNA from rats were used as the template. RT and cDNA amplification were performed by using PCR primers for α-CGRP, β-CGRP, and fragment coding mature CGRP. The RT-PCR products were visualized with ethidium bromide after PAGE (7.75%; Fig. 5). The sizes of the amplified fragments were determined by a ladder marker obtained from pGEM 7zf(+) vectors digested by HaeIII. Using the primers for the fragment coding mature CGRP, a 90-bp cDNA fragment was amplified from both DRG, a sensory neuron, and lymphoid cells, which could not distinguish α- and β-CGRP (Fig. 5A). Using β-CGRP-specific sense primer combined with CGRP antisense primer, and β-CGRP-specific antisense primer combined with CGRP sense primer, two cDNA fragments with predicted sizes were amplified from both DRG and lymphoid cell mRNA (Fig. 5A). Using α-specific primers combined with CGRP primers, two predicted cDNA fragments were amplified only from DRG mRNA, not from lymphoid cell mRNA (Fig. 5B). In addition, the PCR amplifications of β-CGRP cDNA from thymocyte and lymph node lymphocyte RNA were verified by nucleotide sequencing (Fig. 6). Lymphocyte-derived CGRP cDNA sequence was different from α-CGRP cDNA by 45% and was identical with published nerve-derived β-CGRP cDNA sequence (6). The results strongly confirmed that freshly isolated rat thymocytes and lymph node lymphocytes expressed β-CGRP mRNA, whereas they lacked the expression of α-CGRP mRNA.

Four negative control reactions were performed to eliminate the possibility of faulty positive results. First, a mock RT-PCR was performed without addition of reverse transcriptase as a test for the presence of contaminating genomic DNA. As a second control, oligonucleotide primers alone were used in the PCR without giving rise to any detectable product. The other two negative control reactions of RT-PCR, without oligo(dT) and RNA, respectively, were also negative (data not shown).

β-CGRP mRNA expression under stimulation

It was shown, as indicated above, that Con A and IL-2 promoted the synthesis and secretion of CGRP by rat thymocytes and lymph node lymphocytes. To determine whether mitogens altered β-CGRP mRNA levels, total RNA was extracted from thymocytes and lymph node lymphocytes cultured in either medium alone or medium supplemented with Con A or rhIL-2, respectively, for 3 days. RT and cDNA amplifications were performed using

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Lympocyte-derived CGRP cDNA sequence.

It was also reported that CGRP inhibited IL-2 production of murine T lymphocytes (16). We investigated whether lymphocyte-derived β-CGRP had any effect on IL-2 production by rat thymocytes. Rat thymocytes were stimulated with Con A (2 μg/ml) for 72 h. Treatment with hCGRP<sub>8-37</sub> significantly increased IL-2 production by thymocytes induced by Con A. This was significantly different from that stimulated with Con A alone (Fig. 8B). This result suggests that in addition to suppressing thymocyte proliferation, endogenous lymphocyte β-CGRP suppresses IL-2 production from thymocytes induced by Con A.

Discussion

In this study we demonstrated for the first time that rat lymphocytes expressed β-CGRP only, and that activation of rat thymocytes and mesenteric lymph node lymphocytes by Con A and rhIL-2 in vitro induced an increase in β-CGRP synthesis and secretion in a time- and concentration-dependent manner through altering the level of β-CGRP mRNA. By using the CGRP<sub>1</sub> receptor antagonist, hCGRP<sub>8-37</sub>, we also proved that lymphocyte-derived β-CGRP could suppress proliferation and IL-2 production in rat thymocytes induced by Con A. The data obtained in this study confirmed and expanded those from our previous reports (9, 10). Morphological studies show that α- and β-CGRP usually co-distributes throughout the central and peripheral nervous system and nonnervous tissue. However, only α-CGRP mRNA is detected in rat heart (17), and only β-CGRP is expressed in enteric neurons (18) and rat alveolar type II epithelial cells (19). To determine the sequence of CGRP mRNA expressed by rat lymphoid cells, PCR products amplified with β-CGRP-specific primers were sequenced bidirectionally. The sequences in rat thymocytes and lymphocytes were identical with the reported β-CGRP cDNA sequence in the nervous system. The RT-PCR method has high sensitivity and is capable of detecting low numbers of transcripts from cells. We could not find α-CGRP mRNA in rat lymphoid cells unstimulated or stimulated by Con A and IL-2, suggesting that rat lymphoid cells do not express α-CGRP. It was unlikely that in addition to blocking the CGRP effect, hCGRP<sub>8-37</sub> initiated proliferation by binding to an unknown receptor, because in cultures without Con A, hCGRP<sub>8-37</sub> did not induce proliferation. This result indicates that lymphocyte-derived endogenous β-CGRP could suppress lymphoproliferation induced by T lymphocyte mitogen.

β-CGRP-specific sense primer CGRP antisense primer and β-actin-specific primers. The PCR was run for 25 cycles for β-actin and 30 cycles for β-CGRP. The predicted β-CGRP and β-actin cDNA fragments were 361 and 422 bp, respectively. The thymocytes and lymph node lymphocytes were cultured for 3 days with Con A (4 μg/ml) and rhIL-2 (750 U/ml), respectively. Faint bands of 361 bp were also observed in unstimulated thymocyte and lymph node lymphocyte samples, representing constitutive expression of β-CGRP mRNA (Fig. 7A). This was consistent with the results reported previously (9). Distinct bands of 361 bp were observed in mitogen-stimulated thymocyte and lymph node lymphocyte samples. By comparing the amounts of PCR products of β-actin (Fig. 7B), it was shown that the level of CGRP mRNA expressed in the stimulated thymocytes and lymph node lymphocytes was higher than that in the unstimulated cells (Fig. 7C). This result was consistent with that obtained in the CGRP secretion tests. However, no α-CGRP cDNA was amplified from unstimulated or stimulated lymphoid cells using α-CGRP-specific sense primer combined with CGRP antisense primer in a parallel test, suggesting that rat lymphoid cells do not express α-CGRP even stimulated by Con A or IL-2 (data not shown).

Effect of lymphocyte-derived CGRP on rat thymocyte proliferation and IL-2 production

It was reported that CGRP could suppress lymphoproliferation induced by Con A (14, 15). This study proved that T lymphocyte mitogens could promote β-CGRP synthesis and secretion by lymphoid cells. A question arose as to whether lymphocyte-derived β-CGRP was involved in the regulation of lymphoproliferation. Freshly isolated thymocytes were stimulated with Con A (1 μg/ml) for 72 h in RPMI 1640 without FBS. DNA synthesis was assayed by [3H]thymidine incorporation. We tested the effect of hCGRP<sub>8-37</sub>, a selective antagonist of CGRP<sub>1</sub> receptor, on the thymocyte proliferation induced by Con A. It was shown in Fig. 8A that when Con A-stimulated cultures were treated with hCGRP<sub>8-37</sub>, proliferation of thymocytes was significantly enhanced over that in cultures treated with Con A alone. DNA synthesis was significantly augmented by hCGRP<sub>8-37</sub> (0.1–2 μM). It was unlikely that in addition to blocking the CGRP effect, hCGRP<sub>8-37</sub> initiated proliferation by binding to an unknown receptor, because in cultures without Con A, hCGRP<sub>8-37</sub> did not induce proliferation. This result indicates that lymphocyte-derived endogenous β-CGRP could suppress lymphoproliferation induced by T lymphocyte mitogen.

FIGURE 6. Sequence of lymphocyte-derived CGRP. It was identical with nerve-derived β-CGRP cDNA sequence (6). The underlining indicates the differences between α- and β-CGRP cDNA. The bracketing indicates which cDNA sequence.

It was shown in Fig. 8 that α-CGRP mRNA in rat lymphoid cells unstimulated and stimulated lymphoid cells using α-CGRP-specific primers in a parallel test, suggesting that rat lymphoid cells do not express α-CGRP even stimulated by Con A or IL-2 (data not shown).
induced lymphocyte proliferation and promoted IL-2 production simultaneously. Mitogen-activated T lymphocytes produce IL-2, which is the molecule responsible for the stimulation of thymocyte proliferation (20). Furthermore, it was shown in this study that IL-2 stimulated CGRP synthesis and secretion by lymphocytes in a time- and concentration-dependent manner. All these data provide direct evidence for the reports that CGRP-LI is increased significantly in nasal lavage fluids obtained from allergic patients (21) and in peritoneal fluids obtained from IL-2-activated killer cell therapy of peritoneal carcinomatosis patients (22), which indirectly suggests that IL-2 may induce CGRP secretion from lymphocytes. On the contrary, LPS, a B lymphocyte mitogen, has no effect on the production of CGRP by lymphocytes in this study. All these data infer that the majority of CGRP-producing lymphocytes are T lymphocytes.

Most of the peptide hormones and neurotransmitters produced by immunocytes are well-characterized regulators of immune function. A question arose as to what was the biological significance of lymphocyte-derived β-CGRP. The data presented here showed that CGRP inhibited Con A-induced rat thymocyte proliferation, and the CRGPR receptor antagonist, hCGRP8-37, could augment the proliferative effect of Con A. These results are consistent with the observation by Bulloch et al. (23) that hCGRP8-37 can promote mouse thymocyte proliferation induced by Con A. We proved in this study that hCGRP8-37 could enhance the IL-2 production by rat thymocytes induced by Con A. A plausible model to explain the relationship between IL-2 and CGRP might be that as a negative feedback effect, endogenous β-CGRP suppresses T lymphocyte proliferation by inhibiting IL-2 production. Most experiments investigating the immunological effect of CGRP using α-CGRP indicate that CGRP has extensive functions in immunomodulation. CGRP inhibits the proliferation of T lymphocytes (14, 15) and the production of cytokines, such as IL-2, IFN-γ, and TNF-α in T lymphocytes (16), and also inhibits the killing function of NK cells and IL-2-activated lymphocytes (24, 25). CGRP may be involved in thymocyte development by inducing apoptosis of thymocytes via CGRP-specific receptors (26). These reports highlight a potential important role for CGRP in inflammation and immunoreaction. Our observations showed that lymphocyte-derived β-CGRP could also inhibit lymphoproliferation and IL-2 production induced by Con A in the same fashion as α-CGRP. These data suggest that lymphocyte-derived CGRP may possess biological functions similar to those of nerve-derived CGRP and may respond mainly to immune challenge.

Comparison of the peptide sequences of α-CGRP and β-CGRP reveals a striking degree of sequence homology; β-CGRP in the rat

**FIGURE 7.** Amount of RT-PCR products for β-CGRP mRNA from stimulated thymocytes, mesenteric lymph node lymphocytes, and DRG (positive control). A, RT-PCR products of β-CGRP. B, RT-PCR products of β-actin. The PCR products were separated by PAGE and visualized with ethidium bromide. m, Marker; n, negative control of PCR. a, Control; b, stimulated by Con A; c, stimulated by IL-2; d, positive control. C, Quantification of RT-PCR products. The quantification was based on incorporation of [3H]dCTP in the specific PCR bands and was expressed as the ratio of β-CGRP/β-actin mRNA. This figure was representative of three experiments.

**FIGURE 8.** Effect of hCGRP8-37 on Con A-stimulated DNA synthesis and IL-2 production in rat thymocytes. A, Cells were cultured with or without Con A (1.0 μg/ml) with the indicated concentrations of hCGRP8-37 for a total of 72 h. DNA synthesis was assayed by measuring [3H]thymidine incorporation over the final 6 h. The data are the mean ± SEM percentage of the value with Con A stimulation alone for each treatment group (n = 5). *, p < 0.05 compared with the group stimulated with Con A alone. B, Thymocytes of the rat were cultured with Con A (2 μg/ml) and with or without hCGRP8-37 (1 μM) for 72 h. The supernatants were harvested, and IL-2 was assayed by ELISA. The data are the mean ± SEM for each treatment group (n = 5). *, p < 0.05 compared with controls; #, p < 0.05 compared with Con A-stimulated group.
differ from α-CGRP only by the substitution of a lysine residue for glutamate at position 35 in the sequence of 37 aa. However, studies indicate that α-CGRP and β-CGRP are regulated independently. The synthetic glucocorticoid, dexamethasone, causes an increase in α-CGRP mRNA in rat medullary thyroid carcinoma cells. In contrast, the level of β-CGRP mRNA does not significantly change following dexamethasone treatment (27). In response to peripheral axotomy, α-CGRP mRNA levels, but not β-CGRP mRNA levels, are increased in neurons of the rat (28, 29). Local administration of fibroblast growth factors into axotomized motoneurons is able to entirely abolish the up-regulation of α-CGRP mRNA (30). The differences in encoding gene, gene regulation, distribution, and function of these two CGRPs point to the possibility that β-CGRP, especially of lymphoid cell origin, has important and as yet undiscovered roles different from those of α-CGRP. The roles of lymphocyte-derived β-CGRP need to be further investigated.

Singaram et al. (31) have demonstrated the localization of CGRP in human esophageal Langerhans cells. They found that immunoreactivity for CGRP is weak in Langerhans cells of normal specimens, but it becomes abundant in those cells of specimens from esophagitis, inferring an increased amount of CGRP in activated Langerhans. Tokman et al. (32) reported that IL-2 has a key role in a toxicogenic strain of Staphylococcus-coccus caused hypotension. The hypotension can be recovered by blocking the effect of IL-2. IL-2 is increasingly used to treat patients with cancers or virus infection. It is well known that IL-2 causes hypotension during treatment. Ogibene et al. (33) reported that IL-2 administration causes reversible hemodynamic changes similar to those induced by CGRP (34). CGRP-LI is significantly increased in peritoneal fluid of patients receiving IL-2 plus lymphokine-activated killer cell therapy (22). Combined with our results, it is suggested that lymphocyte-derived CGRP may be partially involved in the hemodynamic changes caused by IL-2.

In conclusion, the results suggest that synthesis and secretion of endogenous β-CGRP from lymphocytes can be induced by T lymphocyte mitogens, and lymphocyte-derived β-CGRP may act as a negative modulator in the feedback loop under immune challenge in an autocrine or paracrine manner. This study provides new important information about the immunomodulating effects of endogenous CGRP from lymphocytes in physiologic and pathologic conditions.

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References