PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE **180**, 562-566 (1985)

SECRETION OF CALCITONIN GENE-RELATED PEPTIDE FROM BABY RAT THYROID GLANDS IN VITRO

CARY W. COOPER, SUSAN A. BOROSKY, AND TAI-CHAN PENG

Dept. of Pharmacology & Toxicology,
University of Texas Medical Branch,
Galveston, TX 77550

Dept. of Pharmacology,
University of North Carolina School of Medicine,
Chapel Hill, NC 27514

Abstract. Thyroid glands from 8-day-old rat pups were incubated in serum-free medium for 6 hr. Both calcitonin (CT) and calcitonin gene-related peptide (CGRP) released into medium were measured by radioimmunoassay. In 6 separate experiments CGRP was easily detected in medium in ng/ml concentrations. In 4 of the 6 experiments, where CT release was stimulated by high medium [Ca], the concentration of CGRP in medium showed a positive, significant correlation with the medium CT concentration ($r=0.41-0.69$, $p < .05-<.01$). The results are in concert with reports describing the presence of CGRP in the C-cell, and they further show that CGRP, as well as CT, can be secreted by C-cell.

Introduction. Calcitonin gene-related peptide (CGRP) is now recognized as an alternate product of the calcitonin (CT) gene (1,2). The peptide has been shown to be widely distributed in the body, especially in neuronal tissue (3-5), and a number of pharmacologic actions have been reported following central or parenteral administration of CGRP, including norepinephrine release (6), vasodilation (7), alteration in plasma calcium (8), inhibition of gastric acid release (9), inhibition of feeding (10), and suppression of growth hormone release (11). In this study we sought to extend earlier studies by us and others that showed CGRP was present in the thyroid C-cell by determining whether or not thyroid glands in vitro could secrete CGRP as well as CT.

Materials and Methods. Rat thyroid glands (from which parathyroid glands had been removed surgically) were taken from 8-day-old Sprague-Dawley rat pups (Texas Animal Specialties, Humble, TX). Glands were incubated individually in 2 ml serum-free tissue culture medium for 6 hr. Details of the procedure have been reported previously (12). The incubation medium consisted of minimal Eagle's medium containing Hank's balanced salt solution, 400 mg/dl glucose, and 1 mM Ca (GIBCO, Grand Island, NY) to which we added 20 mM HEPES, .003 mM l-proline, 2 mM l-glutamine, 1 mM l-glycine, 1 mM l-proline, 0.8 mM l-cysteine, .61 mM sodium acetate, and 1 mg/ml bovine serum albumin. Incubations were at 37°C under 95% O_2-5% CO_2. Test agents were were dissolved directly in medium; medium high in Ca (2.5 mM) was prepared by adding $CaCl_2$ to the medium. Ca concentrations were verified by analysis. In each experiment, treatment groups consisted of 6-12 flasks each. At the end of each experiment, medium was decanted and stored at -20°C until it was assayed for CT and CGRP. All samples from a single experiment were run in the same assay.

The radioimmunoassay for rat CT has been described previously (12) and

0037-9727/85 $1.50

was used with the following modification: synthetic human CT (Bachem, Torrance, CA) was used as unlabeled reference standard, and ^{125}I-human CT (Diagnostic Systems Labs, Webster, TX) was used as the labeled ligand.

The radioimmunoassay for CGRP was conducted using a guinea pig antiserum to synthetic rat CGRP developed in our laboratory. Buffer and other assay conditions were the same as those used in the assay for CT (12). Synthetic rat CGRP (Bachem) also was used as the unlabeled reference standard and for iodination with ^{125}I (chloramine T procedure). The lower limit of sensitivity of the assay was 50 pg/tube, and intra- and interassay variations were less than 10 and 15%, respectively. The antiserum was checked for specificity by using a variety of synthetic peptides at concentrations up to 1 µg/tube (human CT, porcine CT, salmon CT, human PTH 1-34, porcine glucagon, bovine and porcine insulins, somatostatin, substance P); none reacted.

Samples of media not exposed to tissue were run with each assay to check for nonspecific interference, but no effects were found. Assays were counted on a Beckman Gamma 5500 and were analyzed after logit transformation using the Beckman DP5500 and accompanying software. Values are shown as mean ±SEM. The significance of differences between mean values in each experiment were evaluated using the nonparametric Wilcoxon rank sum test (13). To determine whether a relationship between medium CT and medium CGRP existed, a correlation coefficient (r) was calculated, and its significance assessed (13).

Results. In all 6 experiments, both CT and CGRP were easily measured in the incubation medium, and were present in ng/ml amounts. In 4 of the 6 experiments, the pattern of response in terms of hormone release was similar for CT and CGRP. This is shown in Fig. 1 where a significant increase in medium CT and CGRP was observed when medium Ca was increased from 1 mM to 2.5 mM. Likewise, in Fig. 2 the results show that the secretory pattern for CT and CGRP was similar.

Fig. 3 shows the correlation between CT and CGRP for the individual samples in the same experiment represented in Fig. 1. The correlation coefficient (r) of 0.58 was highly significant. Table I summarizes the correlation calculations for the 4 experiments where a significant correlation was observed between levels of CT and CGRP in the medium.

Discussion. Numerous recent reports attest to the widespread distribution

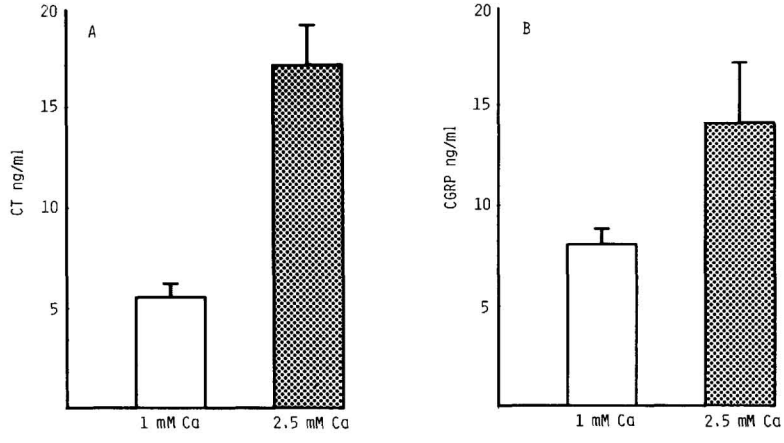

Fig. 1. Increase in concentrations of CT (A) and CGRP (B) in medium containing 2.5 mM Ca. The increase was significant ($p<.05$ vs 1 mM Ca) for both peptides. Values = mean ± SEM (N=12).

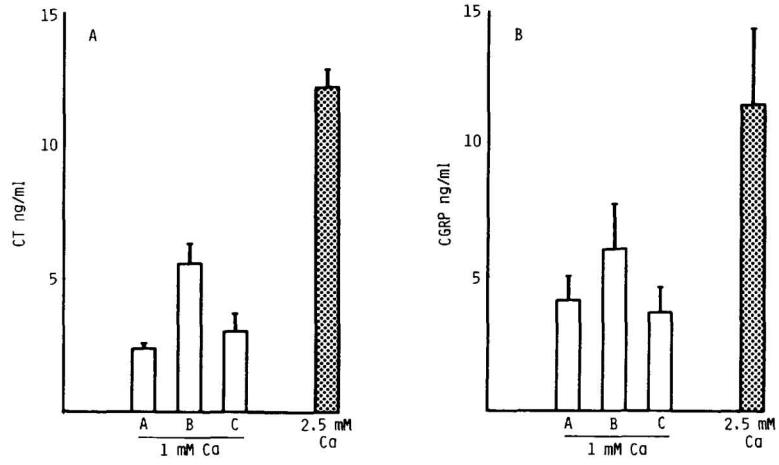

Fig. 2. Similar pattern of release into medium of both CT (A) and CGRP (B) in response to several test agents. Bars labeled A, B, and C in 1 mM Ca represent control, 10^{-4} M TMB-8 (Aldrich Chem. Co., Milwaukee, WI; a presumed inhibitor of mobilization of intracellular Ca stores (19)), and 10^{-5} M insulin, respectively. The increase produced by TMB-8 achieved statistical significance for CT ($p<.05$) but not CGRP. The increase with 2.5 mM Ca was significant ($p<.05$) for both peptides. Values = mean ± SEM (N=6).

of CGRP in the body, particularly in the nervous system (2-5). The fact that CGRP can be released from cultured neurons (14) and that neuronal binding sites for CGRP exist (15,16) supports the idea that CGRP may be an important peptide neuromodulator. In fact, CGRP appears to be a more frequently expressed product of the CT gene than CT itself.

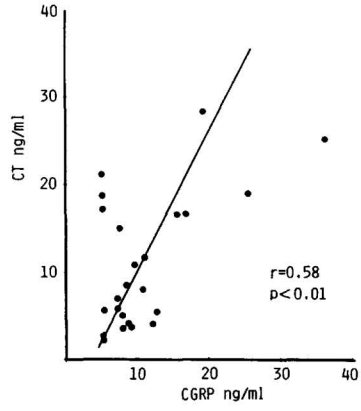

Fig. 3. Correlation of CT and CGRP in individual samples of medium from the same experiment represented in Figure 1.

Originally, Rosenfeld and coworkers suggested that CT gene in the thyroid gland did not express CGRP (1,2). However, that conclusion has been corrected by them in a more recent report which further indicated, by immunocytochemistry, a co-localization of CT and CGRP in the rat thyroid C-cell (17). This finding is in harmony both with an earlier report by Tschopp et al. (5) that CGRP could be found in the human thyroid and with our findings that CGRP easily could be detected in the young rat thyroid (18), albeit at a concentration (200-300 ng/gland), far lower than that of CT (3-5 µg/gland). Furthermore, in immunocytochemical studies not shown here, we have observed that CGRP, like CT, is localized in the

Table I. Summary of experiments where a positive correlation was found between the concentrations of CT and CGRP in incubation medium

Exp.	Correlation Coefficient (r)	No. pairs (N)	p
A	0.58	24	<.01
B	0.69	24	<.01
C	0.55	12	<.05
D	0.41	24	<.05

thyroid C-cell (18), a finding that agrees with the recent report of Sabate et al. (17).

Considerable recent attention has focused on the localization of CGRP in neurons and its possible action as a peptide neuromodulator. However, the fact that CGRP is present in the thyroid C-cell suggests that a possible endocrine role for the peptide cannot be ignored. Our current findings show that CGRP is released in vitro from the rat thyroid and, under some circumstances, its release may coincide with that of CT. Whether CT and CGRP influence the actions of one another, or whether their biological effects are completely independent remains to be determined. However, it is worth noting that CT and CGRP apparently can interact at the same receptor site (16) and that both peptides can affect the concentration of plasma calcium (8).

The biologic importance of CGRP originating from the thyroid C-cell, if any, remains to be determined. We cannot be certain that all of the CGRP released into the medium originated from the C-cell since it has been reported that immunoreactive CGRP can be found in nerve fibers surrounding thyroid vasculature (17). However, the fact that we could provoke release with high Ca and that release of CGRP could be correlated with CT release suggest that immunoreactive CGRP we measured in the medium most likely originated from the C-cell. If this is so, than as far as we know, this is the first demonstration of C-cell release of CGRP.

Acknowledgement. This work was supported by USPHS Grant AM-32060 from the NIAMDDK.

References

1. Amara SG, Jones V, Rosenfeld MG, Ong ES, Evans RM. Alternative RNA processing in calcitonin gene expression generates mRNAs encoding different polypeptide products. Nature 298:240-244, 1982.
2. Rosenfeld MG, Mermod J-J, Amara SG, Swanson LW, Sawchenko PE, Rivier J, Vale WW, Evans RM. Production of a novel neuropeptide encoded by the calcitonin gene via tissue-specific processing. Nature 304:129-135, 1983.
3. Wiesenfeld-Hallin Z, Hokfelt, T , Lundberg JM, Forssmann WG, Reinecke M, Tschopp FA, Fischer JA. Immunoreactive calcitonin gene-related peptide and substance P coexist in sensory neurons to the spinal cord and interact in spinal behavioral responses of the rat. Neurosci Letters 52:199-204, 1984.
4. Gibson SJ, Polak JM, Bloom SR, Sabate IM, Muldery PM, Ghatei MA, McGregor GP, Morrison JFB, Kelley JS, Evans RM, Rosenfeld MG. Calcitonin gene-related peptide immunoreactivity in the spinal cord of man and eight other species. J Neurosci 4:3101-3111, 1984.
5. Tschopp FA, Tobler PH, Fischer JA. Calcitonin gene-related peptide in the human thyroid, pituitary and brain. Mol Cell Endocrinol 36:53-57, 1984.
6. Fischer LA, Kikkawa DO, Rivier JE, Amara SG, Evans RM, Rosenfeld MG, Vale WW, Brown MR. Stimulation of noradrenergic sympathetic outflow by calcitonin gene-related peptide. Nature 305:534-536, 1983.
7. Brain SD, Williams TJ, Tippins JR, Morris HR, MacIntyre I. Calcitonin gene-related peptide is a potent vasodilator. Nature 313:54-56, 1985.
8. Tippins JR, Morris HR, Panito M, Etienne T, Bevis P, Girgis S, MacIntyre I, Azria H, Attinger M. The myotropic and plasma-calcium modulating effects of calcitonin gene-related peptide (CGRP). Neuropeptides 4:425-434, 1985.
9. Hughes JJ, Levine AS, Morley JE, Gosnell BA, Silvis SE. Intraventricular calcitonin gene-related peptide inhibits gastric acid secretion. Peptides 5:665-667, 1984.
10. Krahn DD. Gosnell BA, Levine AS, Morley JE. Effect of calcitonin gene-related peptide on food intake. Peptides 5:861-864, 1984.
11. Tannenbaum GS, Goltzman D. Calcitonin gene-related peptide mimics calcitonin actions in brain on growth hormone release and feeding. Endocrinology 116:2685-2687, 1985.

12. Cooper CW, Ramp WK, Becker DI, Ontjes DA. In vitro secretion of immunoreactive thyrocalcitonin. Endocrinology 101:304-311, 1977.
13. Snedecor GW, Cochran WG. Statistical Methods, ed. 6, Ames, Iowa State University Press, p. 128 & 172, 1967.
14. Mason RT, Peterfreund RA, Sawchenko PE, Corrigan AZ, Rivier JE, Vale WW. Release of the predicted calcitonin gene-related peptide from cultured rat trigeminal cells. Nature 308:653-655, 1984.
15. Tschopp FA, Henke H, Petermann JB, Tobler PH, Janzer R, Hokfelt T, Lundberg JM, Cuello C, Fischer JA. Calcitonin gene-related peptide and its binding sites in the human central nervous system and pituitary. Proc Natl Acad Sci (USA) 82:248-252, 1985.
16. Goltzman D, Mitchell J. Interaction of calcitonin and calcitonin gene-related peptide at receptor sites in target tissues. Science 227:1343-1345, 1985.
17. Sabate MI, Stolarsky LS, Polak JM, Bloom SR, Varndell IM, Ghatei MA, Evans RM, Rosenfeld MG. Regulation of neuroendocrine gene expression by alternative RNA processing: colocalization of calcitonin and calcitonin gene-related peptide in thyroid C-cells. J Biol Chem 260:2589-2592, 1985.
18. Cooper CW, Borosky SA, Peng T-C. Presence of calcitonin gene-related peptide in the rat thyroid and its secretion from baby rat thyroids in vitro. Abstracts Am Soc Bone Min Res A377, 1985.
19. Chiou CY, Malagodi MH. Studies on the action of a new Ca^{2+} antagonist, 8-(N-N-diethylamino) actyl 3,4,5-trimethoxybenzoate hydrochloride in smooth and skeletal muscles. Br J Pharmacol 53:279-285, 1975.

Received July 29, 1985.
P.S.E.B.M. 1985, Vol. 180.
Accepted September 30, 1985.