Binding Sites of Calcitonin Gene-Related Peptide (CGRP): Abundant Occurrence in Visceral Organs

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Abstract—Calcitonin gene-related peptide (CGRP) is a novel peptide amide of 37 amino acid residues, which was first identified as the product of alternative processing of RNA transcripts of the calcitonin gene in humans and rats. Using \(^{125}\)I-human CGRP (hCGRP) as the binding ligand and hCGRP or salmon calcitonin (sCT) as the specific inhibitor of binding, it was examined how the receptor-like binding sites distribute among rat tissues including the nervous system, which is already known to contain binding sites in discrete regions. Some visceral organs (liver, spleen and lung) and possibly the bone marrow of Wistar male rats (8-10 weeks old) were found to be relatively rich in these binding sites. The following parameters were calculated by Scatchard analysis of binding data for the cerebellum, spleen and liver: \(K_d\) (nM) and \(B_{max}\) (fmol/mg protein) were 0.61 and 408, 1.08 and 858, and 0.89 and 356, respectively. In these three tissues, both hCGRP and sCT were able to completely suppress the specific binding; the IC50s (nM) of hCGRP for the cerebellum, spleen and liver were 2.57, 2.29 and 3.02, respectively, and the IC50s (\(\mu\)M) of sCT 2.69, 0.41 and 1.78, respectively. The results obtained herein strongly suggest the physiological function of CGRP in these visceral organs including bone marrow.

Calcitonin gene-related peptide (CGRP) is a novel peptide amide of 37 amino acid residues, biosynthesized in humans and rats by alternative processing of RNA transcripts of the calcitonin gene (1–7). According to Rosenfeld et al. (3), the processing proceeds in a tissue-specific manner to synthesize the precursor of CGRP for the neural tissues and the precursor of calcitonin (CT) in the thyroidal \("\)C\("\)-cells, respectively. The distribution pattern of the CGRP-like immuno-reactivity (3, 8–11) and its receptor-like binding sites (12–15) in the nervous system suggest a potential function of CGRP in the neural control of nociception, gustation and cardiovascular homeostasis.

Recently, we noticed that the receptor-like binding sites for CGRP abundantly occur in some non-neural visceral organs and possibly bone marrow. The sites in the viscera appear to be comparable to those of the central nervous system in terms of specificity,
affinity and density. The types or functions of the cells responsible for binding still remain unidentified, but it is highly possible that the sites mediate the physiological functions of CGRP which have never been conceptualized. In this report, some characteristics of the visceral binding sites so far clarified in our laboratory are presented.

Materials and Methods

Chemicals: Human CGRP (hCGRP) (16, 17), human β-endorphin (18), substance P (19), neurokinin A and B (20), porcine vasoactive intestinal peptide (21) and human parathyroid hormone (1-34) (22) were synthesized at Kyoto University. Salmon CT (sCT) was the product of Armour Pharmaceutical Co. (Kankakee, IL, U.S.A.). Rat atrial natriuretic factor was purchased from Peptide Institute, Inc. (Minoh, Osaka, Japan); katalcalcin from Peninsula Laboratories (Belmont, CA, U.S.A.); (2-[125I]-iodohistidyl10) hCGRP (2 Ci/μmol) from Amersham International plc (Buckinghamshire, England); bacitracin, bovine serum albumin (BSA) and polyethylenimine from Sigma (St. Louis, MO, U.S.A.).

Binding assays: Tissues were obtained from Wistar male rats (8-10 weeks old) killed by cervical dislocation and homogenized with a ten-fold volume of 5 mM Tris HCl buffer (pH 7.4). The homogenate was centrifuged at 50,000×g at 4°C for 30 min. The pellet was suspended in buffer and recentrifuged. This suspending and centrifuging procedure was repeated totally 3 times before use. If not used on the day of preparation, the pellet was suspended in the buffer containing 0.32 M sucrose and stored at -80°C; and on the day of use, it was washed twice. In either case, the final pellet was suspended in 50 mM Tris HCl buffer (pH 7.4). Unless otherwise stated, the assay mixture (0.40 ml) contained 1% BSA, 0.10% bacitracin, 0.025% lactose, 2 KIU/ml aprotinin, 125I-hCGRP (0.5 nM, ca. 5×10^4 dpm), 0 or 25 μM sCT (in some experiments replaced by 1.0 μM hCGRP) and ca. 0.2 mg membrane fraction protein, in 50 mM Tris HCl (pH 7.4). Binding was allowed to proceed at 4°C for 2 hr and stopped by dilution with a ten-fold volume of buffer followed by filtration through a glass-fiber disc (Whatman GF/B) precoated with polyethylenimine (23). The cold sCT- or hCGRP-suppressible binding was taken as the measure of the specific binding. Protein was quantified by the method of Lowry et al. (24) using BSA as the standard.

Results

Tissue distribution: The sCT-suppressive binding sites of 125I-hCGRP was found to distribute broadly among the tissues examined, except for the kidney (Fig. 1). In addition to the tissues which are known to respond to CGRP (25, 26) or to contain its specific binding sites (12-15), three visceral organs exhibited high levels of specific binding: spleen, liver and lung.

Tissues or cells which lack notable binding and not listed in Fig. 1 include a cancer cell line (A 431) from human epithelium, fibroblasts from Swiss 3T3 mice and rat blood cells. The alveolar macrophage is also considered to be free of binding sites, because repeated lavage of the lung with saline (27) failed to remove any binding sites from the lung.

Peptide specificity: As shown in Fig. 2...
and Table 1, both hCGRP and sCT were able to nullify the sCT-suppressible binding in the cerebellum, liver and spleen, although hCGRP was always a far more potent suppressor than sCT. The IC50 of each peptide did not differ depending on the tissue (e.g., no significant difference between the IC50s of sCT in the cerebellum and spleen). The following peptides failed to affect the specific binding of the cerebellum and spleen at the concentration of 1 μM; rat atrial natriuretic factor, porcine vasoactive intestinal polypeptide, human β-endorphin, substance P, neurokinin A and B, human parathyroid hormone (1–34) and katacalcin.

**Binding parameters:** The sCT-suppressible binding to spleen, liver and cerebellum proceeds in a saturable manner. Scatchard analysis (Fig. 3) of binding data revealed that the density of binding sites is the highest in the spleen, and the Kd does not differ among these tissues (Table 1). The Kd of the cerebellum estimated herein, 0.61 nM, corresponds with 0.96 nM, the value reported for the rat CGRP-specific binding sites of rat cerebellum slices (15).

**Discussion**

The results described above first demonstrate that the CGRP-specific binding sites abundantly occur not only in the nervous

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**Table 1.** Characteristics of CGRP binding in cerebellum, liver and spleen

| Tissues   | Binding parameters from Fig. 3 | IC50 from Fig. 2 |
|-----------|-------------------------------|------------------|
|           | Kd (nM) | Bmax (fmol/mg protein) | CGRP (nM) | sCT (μM) |
| Cerebellum| 0.61    | 408                   | 2.57      | 2.69     |
|           |         |                       | (1.12–5.89)*| (0.89–8.13)|
| Liver     | 0.89    | 356                   | 3.02      | 1.78     |
|           |         |                       | (1.86–4.60)| (0.38–8.32)|
| Spleen    | 1.08    | 858                   | 2.29      | 0.41     |
|           |         |                       | (1.41–3.72)| (0.08–2.09)|

* The 95% confidence limits of each IC50 were given in parentheses.
tissues but also in some peripheral organs: spleen, liver and lung. Scatchard analysis revealed that the affinity and density of the binding sites at least in the liver and spleen are comparable to those of the cerebellum sites. So far, two groups of investigators have analyzed the binding data of CGRP in the central nervous system. Henke et al. (14) characterized single classes of binding sites in the spinal cord and the hypothalamus of rats. The \( K_D \)s of these tissues were 0.94 and 0.61 nM, respectively. The \( B_{\text{max}} \)s can not be compared directly with our estimate because they calculated the parameters per mg wet tissue. Skofitsch and Jacobowitz (15) reported a \( K_D \) of 0.96 nM and a \( B_{\text{max}} \) of 76.4 fmol/mg protein for rat cerebellum. As compared with the \( B_{\text{max}} \) in this report, 408 fmol/mg protein, their \( B_{\text{max}} \) is lower possibly due to the direct use of sliced tissue for binding.

It has been observed that there is a mutual antagonism between CGRP and sCT in the binding studies, possibly reflecting structural homology between them (2-5). In the rat hypothalamus and spinal cord, sCT completely inhibited the saturable binding of CGRP if employed in high concentrations and vice versa (14). In rat pancreas, sCT was a weak inhibitor of the specific binding of rat CGRP to the acini (28). Goltzman and Mitchell (13) reported that sCT-specific binding to kidney was reversed by CGRP and the specific binding of CGRP to the spinal cord by sCT. We observed a similar antagonism of CGRP binding by sCT in the peripheral organs. Based on these observations, sCT was employed as a specific suppressor in the experiments in Figs. 1 and 3.

The next step of our study is to clarify the physiological significance of the binding sites and also to know whether the antagonism observed in the binding studies does actually reflect the real antagonism or the additive phenomenon in the actions of both peptides. For these purposes, first of all, we must develop a proper assay system specific for the action of each peptide. One of the prominent peripheral actions of CGRP is vasodilation, as first noted by Brain et al. (25) in skin and aorta. Immunochemical studies by Mulderry et al. (11) have disclosed that major arteries and veins are the richest sources of CGRP. Zaidi et al. (29) noted that vascular CGRP could be mobilized into the circulation. Taken together, these observations seem to suggest a functional role of CGRP in the vascular system, which may serve as a proper assay for the action of CGRP. Unfortunately, we have been unable to reproduce a constant relaxation response to CGRP in catecholamine-contracted rat aorta as reported by Brain et al. (25).

Spleen, liver and bone marrow are known to be endowed with latent or active erythropoietic potency (30). High to medium levels of binding sites for CGRP were found to exist in these tissues. So far, no appreciable binding was detectable in the membrane fraction of whole blood cells, indicating that the binding sites would not be present at least in mature blood cells.

One of the discrepancies between our results and those of the other workers is our failure to detect any binding sites in the kidney. Goltzman and Mitchell (13) observed the presence of the binding sites, though in a low level. They used a purified preparation of renal membrane. A possible explanation for this discrepancy would be that in their preparation, the binding sites were concentrated highly enough to become detectable.

In conclusion, the hCGRP-specific, receptor-like binding sites exist in some visceral organs of rats, although the function of the sites remains unidentified.

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