MET-ENKEPHALIN-ARG-GLY-LEU-LIKE IMMUNOREACTIVITY IN ADRENAL CHROMAFFIN CELLS AND CAROTID BODY CHIEF CELLS OF THE DOG AND MONKEY

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ABSTRACT

Met-Enkephalin-Arg⁶-Gly⁷-Leu⁸ (Met-Enk-Arg-Gly-Leu)-like immunoreactivity is shown in adrenal chromaffin cells and carotid body chief cells of the dog and monkey by immunohistochemistry using rabbit antiserum (R-0171) raised against synthetic Met-Enk-Arg-Gly-Leu conjugated with ascaris protein by the glutaraldehyde method. Immunoreactive sites were detected at the light microscopic level by the peroxidase antiperoxidase method and at the electron microscopic level by the protein A-colloidal gold method. Positive immunostaining was detected in both adrenal chromaffin cells and carotid body chief cells. The immunostaining was absorbed by preincubation of the antiserum with Met-Enk-Arg-Gly-Leu ($0.1 \times 2^{-6-7}$ g/l; dilution of the antiserum, 1: 4,000), whereas no absorption was observed with Leu- or Met-enkephalin (50 mg/l; dilution of the antiserum, 1: 4,000). Thus the present immunostaining using the R-0171 antiserum was specific to the C-terminal region of the Met-Enk-Arg-Gly-Leu molecule. In the electron microscopy, most of the immunoreactivity was demonstrated in the secretory granules. These results indicate that one or more Met-Enk-Arg-Gly-Leu-related peptides are contained in the carotid body as well as in the adrenal medulla.

Met-Enkephalin-Arg⁶-Gly⁷-Leu⁸ (Met-Enk-Arg-Gly-Leu) is an endogenous opioid peptide recently identified in bovine adrenal medulla (7, 14), and is contained together with Met-enkephalin (Met-Enk) and Leu-enkephalin (Leu-Enk) in the preproenkephalin A molecule deduced from the nucleotide sequence analysis of cDNA in bovine adrenal chromaffin cells (14).

As reviewed by Böck (1), adrenal chromaffin cells and carotid body chief cells are closely related to each other in ultrastructural, embryological, and histochemical aspects. We have now demonstrated Met-Enk-Arg-Gly-Leu-like immunoreactivity in the adrenal medulla and

carotid body of dog and monkey by Sternberger's peroxidase antiperoxidase (PAP) method for light microscopy (16, 22) and by protein A-colloidal gold method of Roth *et al.* (18, 19) for electron microscopy.

MATERIALS AND METHODS

Antiserum

The antiserum (R-0171) used in the present study was raised in a mixed-breed female rabbit using Met-Enk-Arg-Gly-Leu (synthesized by Yanaihara *et al.* (5)) conjugated to ascaris protein by the glutaraldehyde method. In the immunoas-

say, this antiserum showed no cross-reaction with α -endorphin, β -endorphin, Met-Enk, Leu-Enk, or Met-Enk-Arg-Gly-Leu-related peptides such as H-Arg-Gly-Leu-OH. At a high concentration (more than 2,000 fmol), H-Phe-Met-Arg-Gly-Leu-OH was slightly cross-reactive with the antiserum. Detailed procedures for the preparation of the antiserum, and the results of a characterization study for radio-immunoassay will be published elsewhere.

Animals

Adrenal medulla and carotid bodies from seven adult mongrel dogs and four young monkeys (*Macaca fasciata*) of both sexes were investigated.

Specimen Preparation for Light Microscopy

Animals anesthetized with Nembutal (50 mg/kg body weight) were perfused intracardially with Ringer's solution followed by Bouin's fluid. Adrenal glands (slices cut with a razor blade not exceeding 3 mm in thickness) and carotid bodies were removed and further fixed in Bouin's fluid for 2 h at room temperature. Fixed tissue pieces were dehydrated through a graded ethanol series and embedded in paraffin. Sections about 4 μ m thick were stained with the immunoperoxidase (PAP) method of Sternberger (16, 22). Counterstaining was performed in Mayer's hematoxylin.

The specificity of the immunohistochemical reaction was tested by the use of the R-0171 antiserum incubated with Met-Enk-Arg-Gly-Leu, Leu-Enk, or Met-Enk overnight at 4°C before application to the tissue section. Additional control experiments were performed by substitution of the following solutions for the R-0171 antiserum: 1) phosphate-buffered saline (PBS: 0.01 M, pH 7.2, 8 g/l NaCl) and 2) non-immune rabbit serum.

Specimen Preparation for Electron Microscopy

The fixative used for the perfusion fixation of the animals for electron microscope immunogold staining was a mixture of 4% paraformaldehyde plus 1% glutaraldehyde in phosphate buffer (0.067 M, pH 7.4). Pieces of fixed adrenal medulla and carotid body were embedded in Araldite after dehydration through a graded ethanol series. Thin sections which were cut on a Sorvall MT5000 microtome with a diamond knife and which showed gray to silver-gold interference colors were collected on uncoated

(150-mesh) nickel grids. The preparation of the colloidal gold particles and their linking to protein A (Pharmacia) was performed as described by Roth *et al.* (18, 19). The immunostaining was performed as follows: 1) Aqueous hydrogen peroxide (10%) for 5 min followed by a wash with PBS. Sometimes this step was omitted. 2) Bovine serum albumin (2%) diluted in PBS for 30 min at room temperature. 3) Rabbit Met-Enk-Arg-Gly-Leu antiserum (R-0171) diluted to optimal titer (1:500 to 1:5,000) in PBS. Subsequent wash with PBS. 4) Protein A-colloidal gold complex diluted to optimal titer in PBS for 30 min at room temperature. 5) After washing in PBS and water, the sections were counterstained in uranyl acetate followed by lead acetate and examined in a Hitachi H700 electron microscope.

Specificity controls of the electron microscopic immunohistochemistry were performed: 1) using non-immune rabbit serum in place of the R-0171 antiserum, and 2) using the R-0171 antiserum (dilution, 1:2,000) absorbed with Leu-Enk, Met-Enk, and Met-Enk-Arg-Gly-Leu (amount, 50 mg/l of the diluted antiserum).

RESULTS

Light Microscopy

Cellular localization Following incubation in the R-0171 antiserum, immunoreactivity was demonstrated in all chromaffin cells of the adrenal medulla in both dog and monkey (Figs. 1A and 3A). The intensity of immunocoloration varied from cell to cell. About half the chromaffin cells were intensely stained by the immunohistochemical treatment, and the rest showed moderate immunostaining (Figs. 1A and 3A). No coincidence was demonstrated between the distribution of intensely-stained chromaffin cells and the localization of noradrenaline-secreting (NA) and adrenaline-secreting (A) cells. Immunohistochemical reaction product was distributed throughout the cytoplasm of chromaffin cells, but the nucleus remained unstained.

In the carotid body, immunoreactive cells were dispersed throughout the organ (Fig. 2). They were frequently organized in clusters and surrounded by thin layers of connective tissue in between blood capillaries (Figs. 1B, 2 inset and 3B).

Specificity controls In both adrenal medulla and carotid body of the dog and monkey no positive immunostaining was obtained when the R-0171 antiserum was replaced by normal rabbit serum (dilution, 1:100). The preincubation of

Fig. 1 Immunoreactive adrenal chromaffin cells (A) and carotid body chief cells (B) in the monkey following incubation with Met-Enk-Arg-Gly-Leu antiserum (R-0171, dilution, 1: 4,000). In the adrenal medulla (A), strongly stained (asterisks) and moderately stained chromaffin cells (arrowheads) are intermingled. Blood vessels were indicated by C. $\times 900$

Fig. 2 Distribution of immunoreactive chief cells in the monkey carotid body. Chief cells with Met-Enk-Arg-Gly-Leu-like immunoreactivity are organized in clusters. Inset is a higher magnification of the enclosed area in Fig. 2 showing the relationship between blood vessels and chief cells. $\times 150$. Inset, $\times 420$

Fig. 3 Immunoreactive adrenal chromaffin cells (A) and carotid body chief cells (B) in the dog following incubation with Met-Enk-Arg-Gly-Leu antiserum (R-0171, dilution, 1: 4,000). Some adrenal chromaffin cells (asterisks) contain strong immunoreactivity, while others (arrowheads) show a moderate staining. $\times 900$

the R-0171 antiserum (dilution, 1: 4,000) with a graded amount of Met-Enk-Arg-Gly-Leu abolished completely the immunostaining when the concentration exceeded 0.1×2^{-6} g/l. Positive immunostaining reappeared when the concentration of Met-Enk-Arg-Gly-Leu became less than 0.1×2^{-7} g/l (Table 1).

Preincubation of the R-0171 antiserum with Leu-Enk and Met-Enk (50 mg/l) had no effect on the positive immunostaining (Table 1).

Electron Microscopy

Subcellular localization Colloidal gold particles, which indicate the localization of Met-Enk-Arg-Gly-Leu-like immunoreactivity, were seen in many of the secretory granules of both adrenal chromaffin cells and carotid body chief cells in the dog and monkey (Figs. 5–8). A small number of secretory granules were devoid of the immunoreactivity (Figs. 7 and 8). A few colloidal gold particles were accumulated in the secretory granules in the Golgi region of the cell (Fig. 6). A limited number of colloidal gold particles were present in the nucleus and in the cytoplasm containing granular endoplasmic reticulum (Fig. 5). Labeling of mitochondria was rare.

Specificity controls for protein A-colloidal gold method No specific labeling of adrenal chro-

maffin cells and carotid body chief cells was seen in either dog or monkey when the R-0171 serum was replaced by non-immune rabbit serum. Preincubation of the R-0171 antiserum with Met-Enk-Arg-Gly-Leu (50 mg/l of the antiserum diluted to 1: 2,000 with PBS) abolished the positive immunostaining (Figs. 6 and 7, insets). Preincubation of the R-0171 antiserum with Leu-Enk or Met-Enk (50 mg/l of the antiserum diluted to 1: 2,000 with PBS) showed no effect on the positive immunostaining.

DISCUSSION

Immunohistochemical localization of enkephalins in the adrenal chromaffin cells was reported by Schultzberg *et al.* (20, 21) in the cat, rat, and guinea pig and by Lundberg *et al.* (11) in man. This was later confirmed by Hervonen *et al.* (4) in the rat, by Linnoila *et al.* (10) in man, and by Peltto-Huikko *et al.* (17) in the golden hamster. In the carotid body, enkephalin-like immunoreactivity has been demonstrated in the cytoplasm of chief cells by Lundberg *et al.* (12), Wharton *et al.* (25), and Hansen *et al.* (3) in the cat and by Varndell *et al.* (23) in the cat and piglet.

In the present study we have clearly demonstrated the presence of Met-Enk-Arg-Gly-Leu-like immunoreactivity in the carotid body chief

Table 1 Absorption Test of Immunohistochemical Reaction to Met-Enk-Arg-Gly-Leu Antiserum (R-0171; dilution, 1:4,000 in PBS) in Adrenal Chromaffin Cells and Carotid Body Chief Cells of the Dog and Monkey

Animal	No peptide	Met-enkephalin					Leu-enkephalin					Met-enkephalin-Arg-Gly-Leu				
Cell type	(Control)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
		2	22	23	24	25	26	27	28	29	210	2	22	23	24	25
Monkey																
Adrenal chromaffin cells	≠	≠	≠	≠	≠	≠	≠	≠	≠	≠	≠	≠	≠	≠	≠	≠
Carotid body chief cells	≠	≠	≠	≠	≠	≠	≠	≠	≠	≠	≠	≠	≠	≠	≠	≠
Dog																
Adrenal chromaffin cells	≠	≠	≠	≠	≠	≠	≠	≠	≠	≠	≠	≠	≠	≠	≠	≠
Carotid body chief cells	≠	≠	≠	≠	≠	≠	≠	≠	≠	≠	≠	≠	≠	≠	≠	≠

The primary antiserum was incubated with Leu-Enk, Met-Enk or Met-Enk-Arg-Gly-Leu at 4°C overnight before application to the sections. The amount of each opioid peptide was decided by a doubling-dilution method ($\times 0.1$ g/l of the diluted antiserum). Symbols: — negative, + slightly positive, ≠ moderately positive, ≡ intensely positive

Met-Enk

Leu-Enk

Met-Enk-Arg-Gly-Leu

Fig. 4

Fig. 5 Ultrastructural localization of Met-Enk-Arg-Gly-Leu-like immunoreactivity in adrenal chromaffin cells of the dog. Colloidal gold particles are specifically localized on electron dense secretory granules. $\times 19,000$

cells and adrenal chromaffin cells. Electron microscopic immunohistochemistry using protein A-colloidal gold method revealed that the Met-Enk-Arg-Gly-Leu-like immunoreactivity is mainly localized in the secretory granules.

The antiserum used was raised against the octapeptide Met-Enk-Arg-Gly-Leu. The specificity control study showed that the positive immunostaining disappeared after preincubation of the antiserum with Met-Enk-Arg-Gly-Leu,

whereas no change was detectable after the preincubation with either Leu-Enk or Met-Enk. These results may indicate the high specificity of the Met-Enk-Arg-Gly-Leu antiserum (R-0171), which recognizes the C-terminal region of the Met-Enk-Arg-Gly-Leu molecule. A preliminary study by radioimmunoassay also showed that the R-0171 antiserum is C-terminal specific (unpublished).

Kohn (9) proposed that the adrenal medulla and the carotid body belong to the same category of paraganglia in spite of the fact that many chief cells show no chromaffin reaction in the carotid body. Later, Watzka (24) classified the adrenal medulla as a member of chromaffin paraganglia, whereas the carotid body represents as a member of non-chromaffin paraganglia. Fluorescence histochemical studies using Falck-Hillarp's method indicated that not only chromaffin paraganglia but also non-chromaffin paraganglia contain catecholamines (8, 15). Thus Böck has recently recalled Kohn's unifying theory that both adrenal chromaffin cells and carotid body chief cells are essentially the same kind of paraganglionic cells (1).

Fig. 4. Absorption test of the immunostaining with the Met-Enk-Arg-Gly-Leu antiserum (R-0171) in the adrenal chromaffin cells and carotid body chief cells in the monkey. Compare with Table 1. The experiment was performed using the R-0171 antiserum diluted to 1:4,000. The amount of peptide (indicated in the picture with numerals) was decided by a doubling dilution method. 'No peptide' means control without opioid peptide added. Immunostaining of adrenal chromaffin cells and carotid body chief cells with the R-0171 antiserum is absorbable with the immunogen, but shows no change after absorption with Leu-Enk or Met-Enk. Magnification of all the pictures: $\times 300$

Fig. 6 Higher magnification of an adrenal chromaffin cell in the dog, showing ultrastructural distribution of Met-Enk-Arg-Gly-Leu-like immunoreactivity. A few colloidal gold particles are seen on the stacked cisterns of Golgi complex (g). Secretory granules in the Golgi area (arrows) are moderately labeled. $\times 45,000$. Inset: Absorption test in adrenal chromaffin cell in the dog. The R-0171 antiserum (dilution, 1: 2,000) was incubated with immunogen (50 mg/l). $\times 36,000$

Paraganglia include a variety of tissues such as adrenal medulla, carotid body, subclavian bodies, aorticopulmonary bodies, tympano-jugular glomera, vagal and juxtavagal paraganglia, laryngeal paraganglia, Zuckerkandl's organ, retroperitoneal paraganglia, paraganglia related to pelvic viscera, peribronchial micro-paraganglia, and cardiac paraganglia (1). Since no significant differences in the cytological features were observed among these tissues, it could be assumed that these paraganglia contain opioid peptides.

Preproopiomelanocortin related to pituitary hormones such as β -lipotropin and ACTH (13) and preproenkephalin B related to hypothalamic opioid peptides such as β -neo-endorphin and dynorphin (6) contain no copy of Met-Enk-Arg-Gly-Leu (2). The R-0171 antiserum specific to Met-Enk-Arg-Gly-Leu may therefore be used as a marker for the identification of proenkephalin A-related peptides in a variety of paraganglia. This work was partly supported by a grant from the Ministry of Education, Science and Culture, Japan (No. 57010082).

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Fig. 7 Met-Enk-Arg-Gly-Leu-like immunoreactivity shown in some electron-dense secretory granules of the carotid body chief cell in the monkey. $\times 48,000$. Inset: Absorption test. The primary antiserum (R-0171; dilution, 1: 2,000) was preabsorbed to Met-Enk-Arg-Gly-Leu (50 mg/l). $\times 45,000$

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Fig. 8 Carotid body chief cell of the dog showing Met-Enk-Arg-Gly-Leu-like immunoreactivity localized mainly in the peripheral portion of secretory granules (A). $\times 92,000$. Ultrastructural localization of Met-Enk-Arg-Gly-Leu-like immunoreactivity in the secretory granules of adrenal chromaffin cell in the monkey (B). $\times 75,000$

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