Immunocytochemical Demonstration of the Co-storage of Noradrenaline with Met-Enkephalin-Arg⁶-Phe⁷ and Met-Enkephalin-Arg⁶-Gly⁷-Leu⁸ in the Carotid Body Chief Cells of the Dog

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Summary. Cellular and ultrastructural localization of catecholamines in the dog carotid body was investigated by peroxidase-antiperoxidase method for light microscopy and by protein A-colloidal gold technique for electron microscopy, and compared with that of preproenkephalin A-related opioid peptides, i.e., Met-enkephalin-Arg⁶-Phe⁷ (Met-Enk-Arg-Phe) and Met-enkephalin-Arg⁶-Gly⁷-Leu⁸ (Met-Enk-Arg-Gly-Leu). Immunocytochemistry at the light microscopic level using consecutive semi-thin sections of Araldite-embedded tissue showed that some 30% of the chief cells contained immunoreactive noradrenaline together with Met-Enk-Arg-Phe and Met-Enk-Arg-Gly-Leu. A considerable number of chief cells exhibited immunoreactivities representing one or two of these three kinds of substances. A few chief cells showed none of the specific immunoreactivities. Immunoreactive adrenaline was not demonstrated in any cells of the dog carotid body. At the electron microscopic level, co-existence of noradrenaline with Met-Enk-Arg-Phe or Met-Enk-Arg-Gly-Leu in the same secretory granules was shown using the double labeling protein A-colloidal gold technique. Possible interactions of catecholamines and opioid peptides deserve consideration for the understanding of the chief cell's role in the carotid body chemoreceptor.

Carotid body chief cells are involved in the arterial chemoreceptor function, whereas adrenal chromaffin cells act as endocrine cells. However, similarities in the staining properties and ultrastructure of these two kinds of cells are unequivocal (VERNA, 1979; McDONALD, 1981; BÖCK, 1982).

In the bovine adrenal medulla, the primary structure of preproenkephalin A was determined by nucleotide sequence analysis of cDNA (NODA et al., 1982). This enkephalin precursor contains Met-enkephalin-Arg⁶-Phe⁷ (Met-Enk-Arg-Phe) and Met-enkephalin-Arg⁶-Gly⁷-Leu⁸ (Met-Enk-Arg-Gly-Leu) together with Leu-enkephalin (Leu-Enk) and Met-enkephalin (Met-Enk). Recent immunocytochemical studies in the adrenal medulla of some mammals have shown that specific immunoreactivities representing Met-Enk-Arg-Phe and Met-Enk-Arg-Gly-Leu were co-stored with adrenaline

and/or noradrenaline within chromaffin cells (KOBAYASHI et al., 1983a; KOBAYASHI and UCHIDA, 1983). In the present study, we investigated the distribution of immunocytochemically demonstrable Met-Enk-Arg-Phe and Met-Enk-Arg-Gly-Leu as well as adrenaline and noradrenaline in the carotid body of the dog. Co-storage of catecholamines and preproenkephalin A-related opioid peptides in the same chief cells or in the same secretory granules was examined with particular attention. If these substances do in fact coexist in the carotid body, their effect on the chemoreceptor function must be reinvestigated because of their possible interactions.

MATERIALS AND METHODS

Antisera: The antisera used during the present study are given in Table 1. Data on their working dilution, carrier and coupler used in preparation of the immunogen, and the source of antiserum are also explained in Table 1.

The antisera to Met-Enk-Arg-Phe and Met-Enk-Arg-Gly-Leu were characterized for radioimmunoassay as reported previously (AP3-311, IKEDA et al., 1982; R-0171, see KOBAYASHI et al., 1983b). The anti-Met-Enk-Arg-Phe serum (AP3-311) showed no significant cross reactivity with α -neoendorphin, β -neoendorphin, β -endorphin, dynorphin, ACTH, Leu-Enk, Met-Enk or Met-Enk-Arg-Gly-Leu (IKEDA et al., 1982). The anti-Met-Enk-Arg-Gly-Leu serum (R-0171) showed no significant cross reactivity with α -endorphin, β -endorphin, Leu-Enk, Met-Enk or H-Arg-Gly-Leu-OH (see KOBAYASHI et al., 1983b).

Specimen preparation: Six mongrel dogs of both sexes weighing 2–7 kg were anesthetized with an intraperitoneal injection of Nembutal (50 mg/kg body weight). They were perfused via the left ventricle of the heart with Ringer's solution for 2 min followed by either 4% paraformaldehyde in a phosphate buffer (0.1 M, pH 7.4) or 4% paraformaldehyde plus 1% glutaraldehyde in the phosphate buffer for 5–7 min. The perfusion-fixed carotid body and adrenal medulla were removed and cut into small blocks (less than 1 mm) or thin slices (less than 2 mm) with razor blades. The tissue pieces were further fixed in the same paraformaldehyde solution or paraformaldehyde-glutaraldehyde mixture for 30–120 min. They were then embedded in Araldite after dehydration through a graded ethanol series. Semi-thin sections (0.7 μ m in thickness) cut on a Sorvall MT5000 ultramicrotome with a diamond knife were immunostained by Sternberger's peroxidase-antiperoxidase method (see STERNBERGER, 1979), whereas

Table 1. Anti-catecholamine and anti-opioid peptide sera used in the present study

Antiserum to	Code	Working dilution	Immunized animal	Carrier and coupler	Source
Noradrenaline	NAP1-18	1 : 4,000	Sheep	Bovine serum albumin by formaldehyde	VERHOFSTAD et al., 1980, 1983
Adrenaline	AP16-2	1 : 1,000	Rabbit	Bovine serum albumin by formaldehyde	VERHOFSTAD et al., 1980, 1983
Met-Enk-Arg-Phe	AP3-311	1 : 1,000	Rabbit	Bovine thyroglobulin by carbodiimide	IKEDA et al., 1982
Met-Enk-Arg-Gly-Leu	R-0171	1 : 500 1 : 1,000	Rabbit	Ascaris protein by glutaraldehyde	YANAIHARA et al., (see KOBAYASHI et al., 1983b)

thin sections (gray to silver interference colors) collected on uncoated nickel grids (200 mesh) were used for electron microscopic immunocytochemistry by protein A-colloidal gold method (ROTH, BENDAYAN and ORCI, 1978; ROTH, 1982). Double-labeling immunostaining for visualization of two antigens in the same thin section was performed by the method described by BENDAYAN (1982).

An additional 4 mongrel dogs of both sexes weighing 2–7 kg were perfused with 4 percent paraformaldehyde in a phosphate buffer. The carotid body and adrenal gland were removed and embedded in paraffin (m.p. 57–60°C). Tissue sections of 3 μ m in thickness mounted on a glass slide were immunostained by the peroxidase-antiperoxidase method as described in our previous paper (KOBAYASHI et al., 1983a).

Specificity test: The specificity of the immunocytochemical reactions was tested by the use of antisera preincubated with 1–2 mg of corresponding catecholamine or 20–100 μ g of corresponding opioid peptide per ml diluted antiserum (dilution, 1:500–1:4,000) overnight at 4°C before application to the tissue section. Control experiments were also performed by substituting the antisera with a phosphate buffered saline (PBS: 0.01 M, pH 7.2, 0.14 M NaCl), non-immune rabbit and sheep sera, or antisera preincubated not with the target substance but with related substances.

RESULTS

Both the immunocytochemistry using paraffin-embedded sections and that using Araldite-embedded sections were performed with essentially identical results. The latter system, however, provided clearer photomicrographs; hence all the light microscopic pictures cited in this paper were from Araldite-embedded materials. For electron microscopic immunocytochemistry by the protein A-colloidal gold method, only Araldite-embedded tissues were used.

1. Immunostaining of catecholamines

Strong immunoreaction was demonstrated with the anti-noradrenaline serum (NAP1-18) in some 30% of the carotid body chief cells (Fig. 1E), whereas no immunopositive cells were demonstrated with the anti-adrenaline serum (AP16-2) (Fig. 2A).

In the adrenal medulla, two groups of chromaffin cells were distinguishable (Fig. 2B). The first group was the A cells which comprised some 70% of the total chromaffin cells, and were immunostained with the anti-adrenaline serum in tissues fixed in 4% paraformaldehyde. However, this specific immunoreactivity was not seen in specimens fixed in the paraformaldehyde-glutaraldehyde mixture. Most A cells showed a weak but distinct noradrenaline-like immunoreactivity besides adrenaline-like immunoreactivity. The intensity of the noradrenaline-like immunoreaction in a few A cells was striking. The second group was the NA cells, occurring in 30% of the adrenal chromaffin cells (Fig. 2B). They were localized in cellular islands among the A cells, and were intensely immunostained with the anti-noradrenaline serum, but not with the anti-adrenaline serum (Fig. 2B).

2. Immunostaining of opioid peptides

The majority of the carotid body chief cells were immunostained with both anti-Met-Enk-Arg-Phe (AP3-311: Fig. 1A, Fig. 3) and Met-Enk-Arg-Gly-Leu sera (R-0171: Fig. 1C). The intensity of the immunocytochemical reaction varied greatly, and some of these cells were negative in reaction (Fig. 1A, C, Fig. 3). The distribution of opioid

Fig. 1. Legend on the opposite page.

Fig. 2. Demonstration of immunoreactive adrenaline in the carotid body (**A**) and adrenal medulla (**B**) of the dog. Semi-thin ($0.7\ \mu\text{m}$ thick) sections of tissues fixed in 4% paraformaldehyde were immunostained using the AP16-2 antiserum (1:1,000). In **A**, staining in the nuclei (non-specific) of vascular endothelial cells (arrows), sustentacular (arrowheads) and chief cells (asterisks) is stronger than that in the cytoplasm of chief cells. In **B**, A cells (**A**) in the adrenal medulla are intensely immunostained, whereas NA cells (**NA**) exhibit no adrenaline-like immunoreactivity. **A**, **B**: $\times 940$

peptide immunoreactive cells showed no correlation with that of noradrenaline immunoreactive cells. However, careful examination of consecutive sections of the carotid body immunostained with different antisera revealed that a large proportion of noradrenaline immunoreactive cells were also positively immunostained to anti-Met-Enk-Arg-Phe and anti Met-Enk-Arg-Gly-Leu sera and vice versa (Fig. 1A, C, E).

In the adrenal medulla, many A and NA cells were immunostained with both anti-Met-Enk-Arg-Phe and anti-Met-Enk-Arg-Gly-Leu sera. However, the distribution of intensely-immunostained cells coincided with neither that of the A cells nor that of the NA cells.

Fig. 1. Six consecutive sections ($0.7\ \mu\text{m}$ in thickness) of the dog carotid body fixed in paraformaldehyde-glutaraldehyde mixture illustrating the co-existence of Met-Enk-Arg-Phe (**A**), Met-Enk-Arg-Gly-Leu (**C**) and noradrenaline-like (**E**) immunoreactivities in chief cells. The same numerals in the various pictures indicate the same cells cut at consecutive levels. A chief cell labeled 1 is immuno-positive to Met-Enk-Arg-Phe (**A**) and noradrenaline (**E**), but negative to Met-Enk-Arg-Gly-Leu (**C**). Chief cells labeled 2 and 3 are positively immunostained with all the three antisera. A chief cell labeled 4 is immuno-positive to Met-Enk-Arg-Phe (**A**) and Met-Enk-Arg-Gly-Leu (**C**), but negative to noradrenaline (**E**). **B**, **D** and **F** illustrate results of an absorption test being immunostained with the anti-Met-Enk-Arg-Phe serum (AP3-311, 1:1,000) preincubated with Met-Enk-Arg-Phe ($100\ \mu\text{g}/\text{ml}$ diluted antiserum), anti-Met-Enk-Arg-Gly-Leu serum (R-0171, 1:1,000) preincubated with Met-Enk-Arg-Gly-Leu ($20\ \mu\text{g}/\text{ml}$ diluted antiserum) and the anti-noradrenaline serum (NAP1-18, 1:4,000) preincubated with noradrenaline ($1\ \text{mg}/\text{ml}$ diluted anti-serum), respectively. Immunostaining with the anti-Met-Enk-Arg-Phe, anti-Met-Enk-Arg-Gly-Leu and anti-noradrenaline sera is eliminated by pre-incubation of the antisera with each target substance. **A**–**F**: $\times 2,300$

Fig. 3. A portion of dog carotid body showing Met-Enk-Arg-Phe-like immunoreactivity in many of chief cells. Asterisks indicate intensely immunoreactive cells, whereas an arrowhead indicates moderately reactive cells. Chief cells, indicated by arrows, are devoid of the Met-Enk-Arg-Phe-like immunoreactivity. The nucleus (*n*) is identified as a rounded area surrounded by dark granular cytoplasm. Sustentacular cells (*s*) and blood vessels (*v*) show no immunoreactivity. Immunocytochemical staining was performed on a 0.7 μ m thick section of the tissue fixed in paraformaldehyde-glutaraldehyde mixture using the AP3-311 antiserum (1:1,000). $\times 1,400$

3. Ultrastructural localization of catecholamines and opioid peptides

The secretory granules of some 20% of the carotid body chief cells exhibited intense noradrenaline as well as Met-Enk-Arg-Phe and Met-Enk-Arg-Gly-Leu-like immunoreactivities (Fig. 4, 5). Co-existence of immunoreactive noradrenaline with Met-Enk-Arg-Phe-like or Met-Enk-Arg-Gly-Leu-like immunoreactivity was directly demonstrated in a single electron micrograph using the double labeling protein A-colloidal gold technique (Fig. 5A, B). Likewise, in the adrenal medulla, immunoreactivities representing both adrenaline/noradrenaline and Met-Enk-Arg-Phe/Met-Enk-Arg-Gly-Leu were localized to the secretory granules of chromaffin cells, when present.

4. Specificity controls

Immunostaining of the carotid body chief cells as well as adrenal chromaffin cells by peroxidase-antiperoxidase method and by protein A-colloidal gold method was not obtainable when the anti-catecholamine and anti-opioid peptide sera were replaced by non-immune rabbit or sheep serum, PBS, or antisera preincubated with target substances (Fig. 1B, D, F) (anti-adrenaline serum, AP16-2, diluted to 1:1,000, preincubated with 1 mg/ml adrenaline; anti-noradrenaline serum, NAP 1-18, diluted to 1:4,000, preincubated with 1 mg/ml noradrenaline; anti-Met-Enk-Arg-Phe serum, AP3-311, diluted to 1:1,000, preincubated with 100 μ g/ml Met-Enk-Arg-Phe; and anti-Met-Enk-Arg-Gly-Leu serum, R-0171, diluted to 1:1,000, preincubated with 20 μ g/ml Met-Enk-Arg-Gly-Leu). The preincubation of anti-adrenaline serum, AP16-2 (dilution, 1:500-

Fig. 4. Ultrastructural localization of noradrenaline-like immunoreactivity. Colloidal gold particles (diameter, 6 nm) representing immunoreactive noradrenaline are localized to secretory granules in a carotid body chief cell of the dog. Rounded gray structures (*M*) are mitochondria showing no immunoreactivity. Immunostaining was performed using the NAP1-18 anti-serum (1:4,000). $\times 93,000$

1:1,000), with noradrenaline (1 mg/ml), anti-noradrenaline serum, NAP1-18 (1:4,000), with adrenaline (2 mg/ml), anti-Met-Enk-Arg-Phe serum, AP3-311 (1:1,000), with Leu-Enk, Met-Enk or Met-Enk-Arg-Gly-Leu (100 μ g/ml) and anti-Met-Enk-Arg-Gly-Leu serum, R-0171 (1:1,000), with Leu-Enk, Met-Enk or Met-Enk-Arg-Phe (100 μ g/ml) had no demonstrable effects on the specific immunostaining in both light and electron microscopic immunocytochemistry.

DISCUSSION

One of the advantages of the protein A-colloidal gold method may be that it allows the simultaneous localization of two kinds of antigenic sites at the electron microscopic level in the same tissue sections (BENDAYAN, 1982). The co-storage of opioid peptides with catecholamines in the carotid body chief cells has been proposed, based on the singular immunoreactions of opioid peptides (LUNDBERG et al., 1979; WHARTON et al., 1980; VARDELL et al., 1982; HANSEN et al., 1982; KOBAYASHI et al., 1983b). However, morphological demonstration of the different opioid peptides and/or catecholamines in the same cell organelles has not yet been reported in the carotid body. In the present report, we have immunocytochemically demonstrated for the first time the co-existence of noradrenaline with Met-Enk-Arg-Phe or Met-Enk-Arg-Gly-Leu in the same secretory granules of carotid body chief cells. Furthermore, using consecutive semi-thin sections different catecholamines and opioid peptides were evidenced to occur in the same cells.

The results of the present study showed that many chief cells of the carotid body, as adrenal chromaffin cells, store in their secretory granules specific immunoreactivities for noradrenaline as well as Met-Enk-Arg-Phe and/or Met-Enk-Arg-Gly-Leu. Some

Fig. 5. Electron micrographs of the dog carotid body chief cell showing storage of noradrenaline-like immunoreactivity and Met-Enk-Arg-Phe or Met-Enk-Arg-Gly-Leu-like immunoreactivity in the same secretory granules. In **A**, small gold particles (diameter, 6 nm) represent noradrenaline-like immunoreactivity (immunostained with the NAP1-18 antiserum diluted to 1:4,000), whereas large gold particles (diameter, 12 nm) indicate Met-Enk-Arg-Phe-like immunoreactivity (immunostained with the AP3-311 antiserum diluted to 1:1,000). Mitochondria (*M*) and granular endoplasmic reticulum (*ER*) exhibit minimal or no immunoreactivity. In **B**, small gold particles (diameter, 6 nm) display noradrenaline-like immunoreactivity (immunostained with the NAP1-18 antiserum diluted to 1:4,000), whereas large gold particles (diameter, 12 nm) show Met-Enk-Arg-Gly-Leu-like immunoreactivity (immunostained with the R-0171 antiserum diluted to 1:500). *N* indicates nucleus. **A:** $\times 100,000$, **B:** $\times 60,000$

chief cells showed one or two kinds of immunoreactivities out of the three, while a few chief cells were devoid of these immunoreactivities. For interpretation of these results, it must be pointed out that the absence of positive immunostaining with antisera against noradrenaline as well as Met-Enk-Arg-Phe and Met-Enk-Arg-Gly-Leu in a portion of the chief cells does not necessarily mean the non-existence of the target substances. In adrenal chromaffin cells a marked variation in the intensity of immunoreaction to anti-opioid peptide sera has been reported (SCHULTZBERG et al., 1978; HÖKFELT et al., 1980; KOBAYASHI et al., 1983a, b). The present study also revealed that the intensity of immunoreaction of each adrenal chromaffin cell to the anti-Met-Enk-Arg-Phe and Met-Enk-Arg-Gly-Leu sera was different. One explanation in the adrenal medulla may be that—in some cells—the antigenic sites are not exposed during the immunocytochemical procedures, or that the antigenicity of opioid peptides is modified during fixation and specimen preparation so that they are not stained with the specific antisera (KOBAYASHI et al., 1983a). Preproenkephalin A is a 263 amino-acid protein containing Met-Enk-Arg-Phe at the carboxyl terminus and Met-Enk-Arg-Gly-Leu in the middle portion of the molecule (NODA et al., 1982). In the adrenal medulla, preproenkephalin A-related opioid peptides coexist in secretory granules with chromogranins, adenine nucleotides, catecholamines and small molecules such as calcium ions (WINKLER and CARMICHAEL, 1982). Therefore, the masking and modulation of the antigenicity of Met-Enk-Arg-Phe

and Met-Enk-Arg-Gly-Leu in the adrenal medulla are a likely phenomenon. Further studies on the carotid body are needed to clarify whether non-immunoreactive chief cells represent a subpopulation lacking some of the specific substances.

The reason why no adrenaline-like immunoreactivity could be demonstrated in the A cells of the adrenal medulla fixed in paraformaldehyde-glutaraldehyde mixture is unknown. However, it seems likely that a trace of glutaraldehyde in the fixative impairs the antigenicity of adrenaline in the secretory granules of chromaffin cells. In the present study, no adrenaline immunoreactive cells were demonstrated in the carotid body. Simple technical reasons can be excluded when explaining this result, as a distinct immunocytochemical reaction was observed in adrenal A cells when fixed in paraformaldehyde. Now that it appears that carotid body chief cells contain no adrenaline-like immunoreactivity, the earlier postulation of one of the present authors needs an amendment. KOBAYASHI (1968), based on his light and electron microscopic studies, proposed that "chromaffin" and "non-chromaffin" cells in the dog carotid body correspond to noradrenaline- and adrenaline-storing cells respectively. "Non-chromaffin" cells, though similar in ultrastructure to adrenal A cells (KOBAYASHI, 1968), store catecholamine other than adrenaline. The present study showed that a percentage of the "non-chromaffin" cells contained noradrenaline in place of adrenaline.

There is little doubt that the release of catecholamines increases during the electrical stimulation of the carotid body, however their effects on the chemoreceptor function of this organ are debated (see McDONALD, 1981). As for the functional significance of opioid peptides in the carotid body, studies are now commencing (MCQUEEN and RIBEIRO, 1981; POKORSKI and LAHIRI, 1981). Why are catecholamines and opioid peptides stored together in the carotid body? Their possible interactions should be considered. It may be possible that they interact with each other to favor or impair the effects of the other substances, but experimental verification is needed for this hypothesis.

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