Immunohistochemical Evidence Against the Coexistence of a Corticotropin-Releasing Factor and Oxytocin or Vasopressin in the Rat Paraventricular Nucleus

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Summary. A corticotropin-releasing factor (CRF), oxytocin and arginine-vasopressin were localized immunohistochemically in the paraventricular nucleus of colchicine-treated male rats. Small CRF-containing neurons were distributed in the parvocellular region. Some magnocellular neurons were also immuno-stained with anti-CRF serum, but after preabsorption of the antiserum with pure synthetic CRF, the staining of these magnocellular neurons was unaffected, which suggests that this CRF-positive reaction was non-specific. By comparing the distribution of CRF and oxytocin or vasopressin on serial 5 µm cryostat sections, it was revealed that CRF and oxytocin or CRF and vasopressin do not coexist in the same neuron.

Since the purification and determination of the amino acid sequence of CRF (Vale et al., 1981), some immunohistochemical studies on the localization of CRF neurons in the hypothalamus have been accomplished in rats (Merchenthaler et al., 1982; Antoni et al., 1983; Burlet et al., 1983; Kawata et al., 1983; Swanson et al., 1983; Tramu et al., 1983) and some other mammals (Kawata et al., 1982). In these reports, it has been debated whether or not CRF and arginine-vasopressin (AVP) or CRF and oxytocin (OXT) might exist in the same neurons. Burlet et al. (1983), Swanson et al. (1983), and Tramu et al. (1983) argued for the coexistence of CRF and one of these posterior pituitary hormones, while Antoni et al. (1983) and Merchenthaler et al. (1982) denied it. These findings were based on the results of comparisons between adjacent serial sections stained immunohistochemically with anti-CRF and anti-OXT (or anti-AVP) sera, respectively. Most of these earlier studies, however, used relatively thick (20-50 µm) sections, and therefore, a precise comparison between adjacent sections was somewhat difficult. In the present study we used thinner (5 µm) serial cryostat sections, so that a more accurate comparison between contiguous sections could be achieved, as the same cell bodies appeared on two or more serial sections which were immuno-stained with antibodies against two or more different hormones by this method.
MATERIALS AND METHODS

1. Tissue preparation
Adult male rats of the Wistar strain (200–300 g body weight) were used throughout the study. Colchicine (75 μg/rat) was infused into the lateral ventricle. One or two days after the colchicine treatment, the animals were killed under nembutal anesthesia by cardiac perfusion of Zamboni’s fixative. The brains were immediately dissected out and immersed in 30% sucrose dissolved in PBS for 2–4 days at 4°C. After freezing the tissue on dry ice, serial 5 μm sections were cut on a cryostat (DAMON/IEC Minotome) at −12° to −18°C. The sections were placed onto a slide glass coated with gelatine, melted by putting a finger on the other side of the slide, and stored in PBS until use.

2. Immunohistochemical staining
The sections were stained by an unlabeled antibody method (STERNBERGER et al., 1970) through the following steps: normal goat serum 1:20 for 2 hrs; primary antibody, either anti-CRF 1:500, anti-OXT 1:1,000 or anti-AVP 1:1,600 for 14–20 hrs; goat anti-rabbit IgG 1:200 for 1 hr; peroxidase-anti-peroxidase (PAP) complex 1:200 for 2 hrs; and 3, 3’-diamino-benzidine tetrahydrochloride (20 mg%), 0.005% H2O2 in 0.05 M Tris buffer (pH 7.4) for 30–60 min. All incubations were performed at room temperature. The sections were washed three times with PBS between each steps. After dehydration by ethanol, they were covered with a glass cover-slip with Entellan (Merck).

The anti-OXT and anti-AVP were gifts of Dr. ZIMMERMAN and Dr. NILAVER, Columbia University (New York), and anti-CRF was purchased from UCB-Bioproducts (Belgium).

3. Absorption test
To check the specificity of immunostaining with anti-CRF, 1 μg of ovine CRF was added to 1 ml of antiserum (diluted to 1:500) and incubated for 1 hr at 37°C prior to the staining.

4. Binding test of the antisera
In order to examine the specificity of the antisera, ovine CRF, OXT and AVP (purchased from Protein Research Foundation, Osaka) were labeled with 131I using chloramine T (GREENWOOD et al., 1963), and these radioiodinated peptides were purified by reverse phase high performance liquid chromatography. The binding of antisera to these peptides were measured as follows: the antisera were serially diluted to 1:200–1:204,800 with PBS which contained 0.05 M EDTA and 1% normal rabbit serum; the labelled antigens were then dissolved in PBS which contained 1% bovine serum albumin (BSA–PBS). The first incubation was carried out at 4°C for 2 days after mixing 100 μl of diluted antiserum, 100 μl of labelled peptide (ca. 20,000 cpm) and 100 μl of BSA–PBS. Then 200 μl of goat anti-rabbit gamma globulin dissolved in 0.05 M EDTA–PBS and 3.5% polyethylene glycol was added and mixed, and then were incubated at room temperature for 2 hrs. After centrifugation at 3,000 rpm for 15 min, the supernatant was removed by suction and the radioactivity of the precipitate was counted in an automatic gamma counter (Aloka ARC–300).
5. **Checking for coexistence**

Photomicrographs of the sections were taken with an Olympus BH-2 photomicroscope at a magnification of 50 times, and were enlarged into 8 x 10 inch prints (final magnification 350 x). The same neurons in the two consecutive sections immunostained with two different antibodies were compared, using blood vessels as landmarks.

**RESULTS**

1. **Results of the binding test of antisera**

As shown in Figure 1, each of anti-CRF, anti-OXT and anti-AVP binds only to its homologous antigen and does not bind to other antigens. Therefore, as far as these three peptides are concerned, there should be no cross reaction among the antisera used in this study.

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**Fig. 1.** Binding test of the antisera. Iodinated antigens bound to the antiserum (% of total) at various dilutions are plotted. **A.** anti-CRF. **B.** anti-oxytocin. **C.** anti-vasopressin. Each antiserum binds only to its homologous antigen and binding to the other antigens are negligible.
Fig. 2. a-c. Legend on the opposite page.
2. Immunohistochemical findings
Numerous cell bodies of small neurons stained with anti-CRF serum were observed in the dorsomedial region of the paraventricular nucleus (Fig. 2b, 3b). A number of magnocellular neurons in the ventrolateral region were also immunostained with anti-CRF, but the staining intensity of the latter was always much weaker than the former (Fig. 2b, 3b). Anti-OXT and anti-AVP antisera revealed many magnocellular neurons mostly in the ventrolateral region and a small number of neurons in the dorsomedial area (Fig. 2a, 3a). The anti-CRF-reactive neurons appearing in the parvocellular region did not react with anti-OXT or anti-AVP, as examined through the comparison of sections stained with anti-CRF to those reacted with either anti-OXT or anti-AVP (Fig. 2a, b, 3a, b). On the other hand, some of the weakly CRF-positive cells in the magnocellular region of the paraventricular nucleus were conspicuously stained with anti-OXT or anti-AVP, as observed in the contiguous sections (Fig. 2a, b, 3a, b). When the anti-CRF serum was preabsorbed with 1 μg/ml of CRF prior to the staining, the CRF-like immunoreactivity of the neurons in the parvocellular region was completely abolished, while the weak reaction to the anti-CRF of magnocellular neurons was unaffected (Fig. 2c, 3c). Therefore, the CRF-like immunoreaction of only the parvocellular neurons could be considered specific for CRF, but the weak reaction to anti-CRF of the magnocellular neurons, most of which also reacted to anti-OXT or anti-AVP, would
Fig. 3. a–c. Legend on the opposite page.
be more likely due to some non-specific reaction. It may be concluded that, as far as the specific reaction is concerned, there are no CRF-positive neurons which are also stained immunohistochemically with anti-OXT or anti-AVP.

DISCUSSION

Though a number of immunohistochemical studies have been published, there are only a few reports which unequivocally show the coexistence of CRF and OXT or AVP. Merchantaler et al. (1982) reported that nerve fibers immunoreacted with anti-CRF in the pathway from the paraventricular nucleus to the median eminence were different from those stained by anti-OXT, anti-AVP or anti-neurophysin, but there was no statement concerning the neuronal perikarya. Swanson et al. (1983) showed that about one fifth of the CRF-containing neurons were localized in the magnocellular region of paraventricular nucleus, where OXT-neurons are also distributed. But the sections they used were 30 μm thick, so that precise identification of cell bodies (10–15 μm in diameter) in contiguous sections was almost impossible. Burlet et al. (1983) reported
the coexistence of CRF and OXT in Long Evans and Brattleboro homozygous rats which had been given replacement with synthetic vasopressin. They used the double staining technique in which the antibodies applied for the first immunostaining were eluted before the second immunostaining. It seemed possible, however, that the elution was incomplete in some cases. This possibility holds also to the data by TRAMU et al. (1983) showing the coexistence of CRF and AVP, in which the same elution technique was employed. On the other hand, ANTONI et al. (1983) presented evidence that anti-CRF-reactive cells were different from those stained by anti-AVP, anti-neurophysin and anti-TRH. They used serial 5 μm paraffin sections, a thickness which might enable comparison between the two consecutive sections, though they did not present a comparison precise enough in their paper. The results of the present study are in agreement with those of ANTONI et al. (1983).

As shown in Figure 1, OXT or AVP does not bind to anti-CRF serum, which may react non-specifically to some magnocellular neurons. The above results might indicate that these non-specific reactions may be due neither to the cross reaction between anti-CRF serum and OXT or AVP, nor to the contamination of anti-OXT or anti-AVP antibody in the anti-CRF serum. When anti-CRF was replaced by normal rabbit serum in the course of immunohistochemistry, almost the same weak reaction occurred. Therefore, such a weak reaction might be caused by some unknown factor contained in the normal rabbit serum.

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