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Co-expression of corticotropin-releasing factor and vasopressin immunoreactivity in parvocellular neurosecretory neurons of the adrenalectomized rat

(co-localization/hypothalamus/immunohistochemistry/neurohypophysial peptide/paraventricular nucleus)

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ABSTRACT **To clarify the anatomical organization that allows for the synergy of vasopressin and oxytocin with corticotropin-releasing factor (CRF) in promoting adrenocorticotropic hormone secretion from the anterior pituitary, immunohistochemical double staining methods were used to compare the distribution of these peptides in the hypothalamic paraventricular nucleus of normal, colchicine-treated, and adrenalectomized male rats. In untreated animals, a few CRF-stained cells were found in the parvocellular division of the paraventricular nucleus, while brightly stained oxytocin- and vasopressin-immunoreactive cells were centered in the magnocellular division. In animals treated with colchicine, an inhibitor of axonal transport, large numbers of CRF-stained cells were found in the parvocellular division of the nucleus, and 1–2% of these also stained with antivasopressin. As reported previously, a substantial number of oxytocin-stained cells, centered in a discrete anterior part of the magnocellular division, also expressed CRF immunoreactivity. In contrast, after adrenalectomy, CRF immunostaining of cells in the parvocellular division was enhanced selectively and >70% of these cells also stained positively for vasopressin. The distribution of oxytocin-stained cells was not influenced by adrenalectomy. The unusual localization of vasopressin immunoreactivity in parvocellular neurosecretory neurons in the adrenalectomized rat suggests that a single population of cells can produce CRF and vasopressin, both of which are potent promoters of adrenocorticotropic hormone secretion. These findings indicate that there is a state-dependent plasticity in the expression of biologically active peptides by individual neuroendocrine neurons.**

The isolation and characterization of a peptide that has potent corticotropin-releasing activity (1) has effectively ended a longstanding controversy as to whether the posterior pituitary hormone vasopressin might serve an additional role as the principal corticotropin-releasing factor (CRF) of the hypothalamus (2). Nevertheless, it is clear that both vasopressin and the related nonapeptide, oxytocin, can potentiate the effects of CRF on the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary (3–5). The anatomical organization of neurons that might allow for this kind of synergy is unclear.

Physiological (6) and anatomical (7–10) studies indicate that neurons delivering CRF to hypophysial portal vessels in the median eminence are concentrated in a discrete zone of the parvocellular division of the paraventricular nucleus of the hypothalamus (PVH). In contrast, most oxytocinergic and vasopressinergic neurons in the PVH of the normal rat are concentrated in an anatomically distinct magnocellular division that projects to the posterior pituitary (11, 12), though a few cells of each type are scattered throughout the parvocellular division (12). A vasopressinergic projection to the hypophysiotropic zone of the median eminence has been described, and it is known from ablation studies to arise in the PVH (13). The cells of origin of this pathway have not been identified.

We have recently used a sequential double immunohistochemical staining method to compare directly the distribution of cells stained for CRF *and* oxytocin or vasopressin immunoreactivity in the PVH of colchicine-treated male rats. The results indicated that a moderate number of neurons concentrated in a discrete, anterior, part of the magnocellular division of the PVH jointly expressed CRF and oxytocin immunoreactivity (14, 15), while very few neurons centered in the parvocellular division of the nucleus stained positively for both CRF and oxytocin or vasopressin (14). While the expression of two peptides in individual parvocellular neurosecretory neurons does suggest a substrate that allows for interactions in the control of ACTH secretion, the fact that co-localization was found in so few neurons has led us to examine further these relationships under different experimental conditions.

The present report describes the results of a repetition of these co-localization experiments in the adrenalectomized rat. Several groups have reported that chronic adrenalectomy markedly enhances CRF immunostaining of cells in the PVH (7–9), presumably as a consequence of removing circulating adrenal steroids that provide feedback inhibition of CRF production and release. It has been shown that vasopressinergic and, to a lesser extent, oxytocinergic projections to the median eminence also show enhanced immunoreactivity after adrenalectomy (16). In view of the well-documented activational effects of steroid hormones, which include modulation of specific neurotransmitter systems by acting at the level of the genome (17), it is possible that adrenalectomy might effect qualitative, as well as quantitative, changes in the production of specific peptides. The results suggest that vasopressin and CRF can be produced by a discrete and sizeable population of parvocellular neurosecretory neurons, and that the expression of vasopressin in this population follows the withdrawal of adrenal steroids.

METHODS

Tissue Preparation. Adult male albino rats of the Sprague–Dawley strain (Zivic-Miller, Pittsburgh, PA) were used in all experiments. One group (n = 6) was not pretreated in any way. A second group (n = 8) received single injections of colchicine (50 μg in 25 μl of saline) into a lateral cerebral ventricle 48–72 hr prior to sacrifice. A third group (n = 7) was bilaterally adrenalectomized 7–60 days before perfusion. Effectiveness of adrenalectomy was confirmed at autopsy. The animals were perfused with ice-cold 4% parafor-

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Abbreviations: ACTH, adrenocorticotropic hormone; CRF, corticotropin-releasing factor; PVH, paraventricular nucleus.

maldehyde in a two-step procedure in which the pH of the perfusate is varied (7). Up to five one-in-five series of 20-μm-thick frozen sections through the PVH were saved. One was stained with thionin for reference purposes; the remainder were prepared for immunohistochemical localization of cells that cross-react with antisera against rat or ovine CRF, oxytocin, and vasopressin, using a conventional indirect immunofluorescence method (18). The area of the PVH in each section was photographed, and the distribution of cells stained with each antiserum was plotted onto projection drawings made from the thionin-stained series.

Immunological reagents were then eluted from the tissue by using a modification (13) of the method of Tramu *et al.* (19). The effectiveness of this procedure was tested by reincubating the sections in fluorescein-conjugated secondary antiserum. The presence of any immunoreactivity in tissue after this step was taken as evidence that the elution procedure had been ineffective, and the material was either reprocessed or discarded. Sections in which the antibodies were effectively removed were then stained for the presence of a second neuropeptide using the protocol outlined above, except that incubations were done on slide-mounted sections.

The material was then rephotographed, and comparisons of the distributions of two antigens were made by superimposing tracings made of all clearly stained neurons in one set of photographic enlargements onto the other. In this way, comparisons of the distributions of neurons in the PVH stained for oxytocin and/or CRF, and vasopressin and/or CRF were made in each intact, colchicine-treated, and adrenalectomized rat. To control for possible diminution of immunoreactivity as a consequence of the elution procedure, the sequence in which the primary antisera were applied to tissue was varied in different experiments.

Antisera and Controls. Antisera against oxytocin and vasopressin were the gifts of F. Vandesande and K. Dierickx and were cross-adsorbed in the solid phase against the heterologous antigen using the method of Swaab and Pool (20). Specific staining with each was blocked by preabsorption of the serum with the respective synthetic immunogen (1 mg/ml) overnight at 4°C.

Three antisera against CRF were used in each experiment. Sera designated C24 and C30 are directed against an ovine CRF–human α-globulin conjugate and have been found in radioimmunoassay to recognize the NH_2- and COOH-termi-

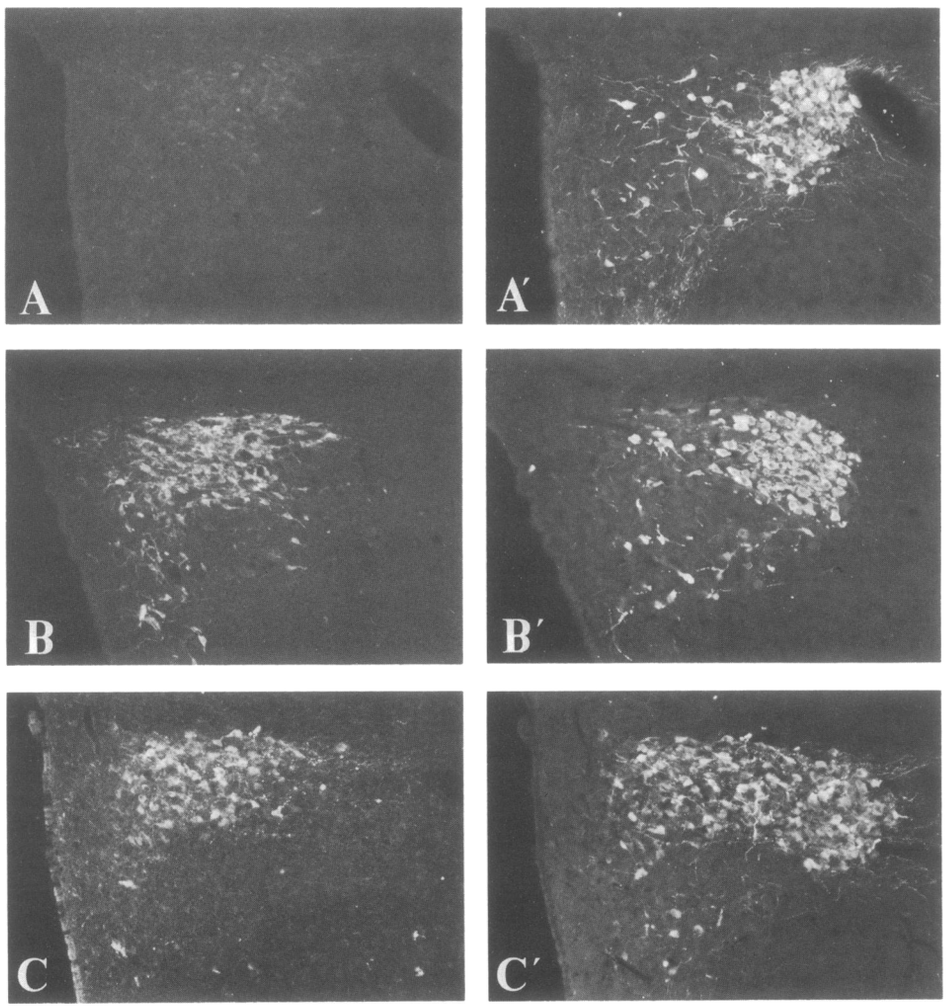

FIG. 1. Distribution of CRF- and vasopressin-immunoreactive neurons in the PVH under three conditions. (*A*, *A′*) Fluorescence photomicrographs of the PVH of the normal (untreated) rat showing cells stained sequentially with antisera against CRF (*A*) and vasopressin (*A′*). Few CRF-positive cells are apparent, while in the same section many neurons centered in the compact magnocellular division of the nucleus are stained with anti-vasopressin. (*B*, *B′*) PVH of colchicine-treated rat showing CRF (*B*) and vasopressin (*B′*) immunoreactivity in a single tissue section. Colchicine treatment enhances CRF staining in a discrete subset of neurons in the parvocellular division of the PVH, while the distribution of vasopressin-stained neurons is similar to that seen in the normal rat. (*C*, *C′*) PVH of adrenalectomized rat, showing CRF (*C*)- and vasopressin (*C′*)-immunoreactive neurons. Adrenalectomy enhances CRF immunostaining of cells in the parvocellular division of the PVH; vasopressin-immunoreactive neurons are again seen in the magnocellular division of the nucleus and now are also seen in the same region of the parvocellular division in which CRF-stained neurons were found. (×103.)

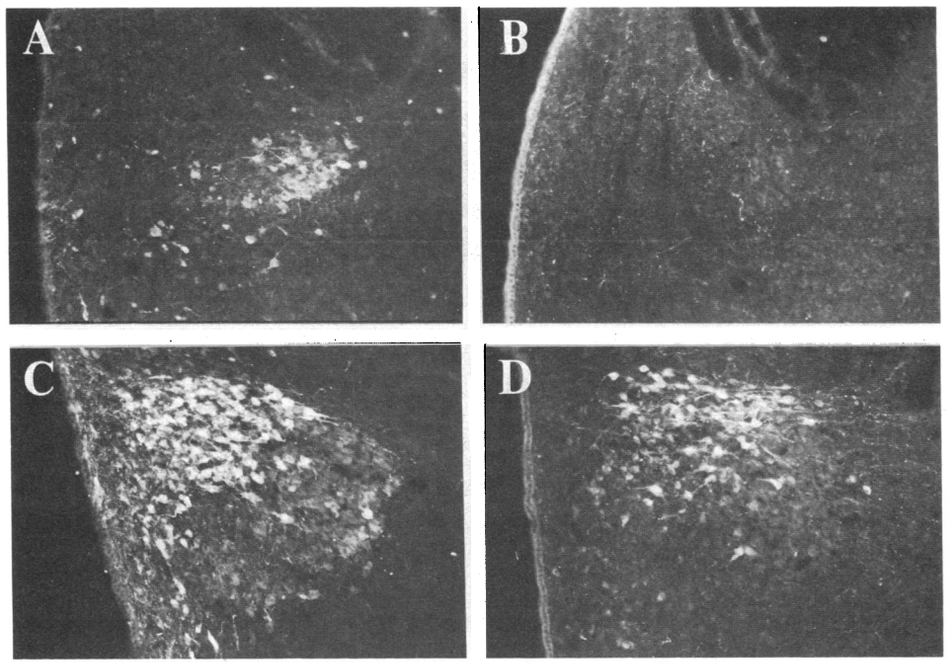

FIG. 2. Fluorescence photomicrographs of CRF-immunoreactive cells in frontal sections through rostral (*A* and *B*) and midcaudal (*C* and *D*) levels of the PVH taken from colchicine treated (*A* and *C*) and adrenalectomized (*B* and *D*) male rats. Colchicine treatment nonspecifically enhances CRF immunostaining of cells in the anterior magnocellular part of the nucleus (*A*), many of which have also been shown to stain positively for oxytocin (14, 15) and in the medial parvocellular part of the nucleus (*C*), the probable source of projections to the external lamina of the median eminence. Adrenalectomy selectively enhances CRF immunoreactivity in the medial parvocellular part of the nucleus (*D*) but does not increase staining in the anterior magnocellular part (*B*). (×103.)

nal portions, respectively, of ovine CRF. The third serum (C70) was raised against the recently characterized rat CRF peptide (21) and was similarly conjugated for immunization. All anti-CRF sera were first absorbed with the carrier protein at 10 mg/ml. Specific staining with each was eliminated by prior absorption with their respective synthetic immunogen at 10 mg/ml. As we have reported elsewhere (14) the number and distribution of cells in the PVH that were stained using anti-rat CRF was similar to that identified by anti-ovine CRF. We thus refer below to CRF immunoreactivity without reference to species. As an additional control, all antisera used in co-localization experiments were differentially cross-absorbed in the liquid phase against the heterologous antigen using concentrations of peptide (10–20 mg/ml) that completely blocked specific staining in the PVH when added to the homologous antiserum.

RESULTS

Normal Rats. As we (7) and others (8–10) have reported previously, few CRF-immunoreactive cells were found in the PVH of untreated rats (Fig. 1*A*). These neurons were weakly stained and were concentrated in the parvocellular division of the nucleus. In contrast, characteristic dense clusters of oxytocin- and vasopressin-stained cells in the magnocellular division and scattered cells of variable size in the parvocellular division (see ref. 22 for parcellation) of the PVH were obvious (Fig. 1*A'*). Given the paucity of CRF-stained cells in normal animals, no evidence was obtained for co-localization of CRF with either oxytocin or vasopressin in the PVH.

Colchicine-Treated Rats. Our findings in colchicine-treated rats confirmed the results of a previous analysis (14) and will

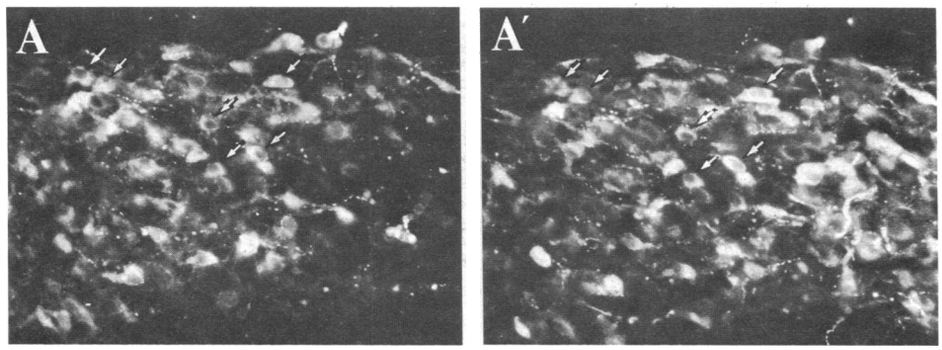

FIG. 3. Higher power photomicrographs of the PVH of an adrenalectomized rat showing the distribution of CRF- and vasopressin-immunoreactive neurons in a single tissue section. (*A*) CRF-positive neurons centered in the parvocellular division of the PVH. (*A'*) Vasopressin-stained neurons occupying portions of both the parvocellular (left) and magnocellular (right) divisions of the nucleus. In the adrenalectomized rat, many parvocellular neurons (some obvious examples shown by arrows) can be seen to possess both CRF and vasopressin immunoreactivity, while those in the magnocellular division stain positively only for the presence of vasopressin. Such extensive co-localization was never seen in normal or colchicine-treated rats. (×190.)

be outlined here only briefly. Prior treatment with this drug dramatically enhanced the number and intensity of CRF-stained cells in the PVH. The majority were found in the anterior and dorsal medial parts of parvocellular division (Fig. 1*B*), though a substantial number were seen in the anterior part of the magnocellular division. The number and distribution of oxytocin- and vasopressin-stained (Fig. 1*B'*) neurons was not perceptibly different in treated and untreated rats, though, consistent with the disruptive effects of colchicine on axonal transport, staining of cell bodies was somewhat more intense in pretreated animals. Co-localization studies indicated that CRF-stained neurons in the anterior magnocellular part of the PVH were almost invariably oxytocin immunoreactive. Only a few neurons in the parvocellular division were found to stain positively for CRF and oxytocin or vasopressin. Thus the number of cells found to express CRF and vasopressin immunoreactivity (20–40 per brain) was estimated to comprise only 1–2% of the total number of CRF-stained neurons in the parvocellular division of the PVH.

Adrenalectomized Rats. Results obtained in rats perfused 7–60 days after adrenalectomy confirmed that immunostaining for CRF is markedly enhanced in a subset of some 800–1000 parvocellular neurons in the PVH. Cells stained for CRF in the adrenalectomized rat were found almost exclusively in the medial part of the parvocellular division of the nucleus (Fig. 1*C*), where their distribution was virtually identical to that seen after treatment with colchicine. In contrast to the results obtained with colchicine-treated animals, however, CRF-stained neurons were never found in the anterior magnocellular part of the PVH in adrenalectomized rats (Fig. 2).

The appearance and distribution of oxytocin-immunoreactive neurons in both major divisions of the PVH was not discernibly influenced by adrenalectomy. Similarly, vasopressinergic neurons in the magnocellular division of the PVH were brightly stained as expected. In addition, however, a large population of vasopressin-stained neurons was evident in the parvocellular division of the adrenalectomized rat (1*C'*), whose topography appeared to be identical with that of CRF-stained cells (Fig. 1*C*). Double staining experiments indicated that at least 70% of all CRF-stained cells in the parvocellular division of the adrenalectomized rat (or 500–600 per brain) also contained immunoreactive vasopressin (Fig. 3). This localization of vasopressin was not evident when vasopressin antiserum that had been absorbed with excess (1 mg/ml) synthetic vasopressin was used.

DISCUSSION

The results suggest that vasopressin and CRF can be expressed together in a rather large subpopulation of parvocellular neurons in the PVH and that the expression of vasopressin in this population is dependent on the withdrawal of adrenal steroids. Based on the correspondence of their distribution with that of neurons that are known from retrograde transport studies to project to the median eminence (23, 24), these cells must certainly correspond to the heretofor unidentified population that delivers vasopressin to the hypophysial portal system. The co-occurrence of vasopressin and CRF in single neurons has important implications for understanding the interaction of these two peptides in the control of ACTH secretion and suggests the existence of a plasticity in the expression of biologically active peptides by neuroendocrine neurons as a function of physiological state.

It is important, however, to consider factors that bear on the validity of this observation. First, the result was obtained with an antiserum to vasopressin that had been adsorbed against oxytocin, as well as rat or ovine CRF, and that in normal histological material fails to stain cell bodies outside the regions in which vasopressinergic neurons have been reported to reside. Second, identical results were obtained with cross-absorbed CRF antisera that are directed against different parts of the ovine CRF molecule and with a serum against rat CRF itself, which differs by seven residues from the ovine peptide (21). Thus, it is quite unlikely that our results can be attributed to spurious crossreactivity of our CRF antibodies with vasopressin (or its prohormone), and vice versa, or with any of the other peptides recently shown (25) to coexist with CRF in the parvocellular division of the PVH.

The finding that a large population of parvocellular neurosecretory neurons expresses vasopressin immunoreactivity after adrenalectomy, but not after long-term treatment with colchicine (a procedure that is commonly required to visualize neuropeptide immunoreactivity in perikarya in the brain) indicates that vasopressin is normally expressed at very low levels, if at all, by most of these neurons and that adrenal steroids exert powerful inhibitory effects on vasopressin production in these cells. To our knowledge, comparable numbers of vasopressin-immunoreactive neurons have not previously been reported in the parvocellular division of the PVH under any experimental conditions. Because vasopressin immunoreactivity in untreated animals is readily demonstrable in magnocellular neurons, regulation of the synthesis of this peptide in the two major divisions of the PVH appears to be at least quantitatively different. On the other hand, CRF immunoreactivity in the same population of parvocellular neurons can be enhanced by colchicine treatment or by adrenalectomy (7–9), findings consistent with evidence that CRF is usually produced in at least moderate amounts at this locus (26, 27) and is subject to feedback inhibition by adrenal steroids (2). The observation that sites producing the CRF and vasopressin involved in the control of anterior pituitary corticotropes appear to be one and the same suggests that the integration of neural and humoral factors in the regulation of ACTH secretion takes place at the level of parvocellular cell bodies in the PVH. It will thus be important to identify factors that might differentially control the expression of the two peptides by this cell group.

The effects of adrenalectomy appear to be at least somewhat specific to both particular peptides and a particular cell type. No apparent alteration in the number or the distribution of oxytocin-stained neurons in the PVH was detected in tissue from the same experimental animals in which the unusual localization of vasopressin immunoreactivity was obvious. In addition, the adrenalectomy-induced enhancement of CRF immunostaining was quite strictly limited to cells in the parvocellular division of the PVH. Magnocellular neurosecretory neurons that have been shown in colchicine-treated rats to express both CRF and oxytocin immunoreactivity (14, 15) were not stained by any of our CRF antisera in the adrenalectomized rat. Therefore, it appears unlikely that the effects that we have described are a consequence of nonspecific influences of adrenal steroid withdrawal on cellular physiology or metabolism. It remains to be determined, however, whether qualitative changes in the expression of neuropeptides may be manifest over the course of normal fluctuations of adrenal steroid titers.

Of more general significance, the results raise the possibility that neurons in this system, at least, are capable of a "chemical switching" whereby a particular neuroactive substance is expressed only under a specified set of physiological conditions. In view of the well-established interaction of CRF and vasopressin in promoting ACTH secretion (2–5), there can be little doubt that in this instance the phenomenon is functionally meaningful. Magnocellular neurosecretory neurons appear to contain at least five peptides in addition to oxytocin and vasopressin (28). Similarly, the population of CRF- and vasopressin-stained cells under consideration here

may also contain enkephalin and PHI immunoreactivity (25). Together with other evidence that alterations in endocrine status can influence differentially the occurrence, or co-occurrence, of peptide expression in particular classes of PVH neurons (29), the present results support the view that the PVH is a dynamic mosaic of chemically specified cell types in which the level of expression of neuropeptides varies systematically as a function of physiological state.

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