Endothelin 1-Like Immunoreactivity in the Hypothalamo-Hypophysial System of the Bullfrog, *Rana catesbeiana*

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ABSTRACT

We investigated the distribution of endothelin 1 (ET1)-like immuno-reactivity and its colocalization with oxytocin-like immunoreactivity in the hypothalamo-hypophysial system of the bullfrog, *Rana catesbeiana*. ET1-immunoreactive (ET1-IR) somata were scattered in the preoptic nucleus and exhibited granular appearance. ET1-IR somata were classified into large (average 24 μm in diameter) and small (average 13 μm in diameter) cell types, and these somata extended ET1-IR varicose fibers in the ventro-lateral direction. In the neurohypophysis, dense accumulation of ET1-IR fibers was seen near blood vessels. ET1-IR neurons corresponded to a subpopulation of oxytocin-immunoreactive neurons. These observations suggest that ET1-like peptide is colocalized with a subpopulation of classical neurohypophysial hormones and the peptide may regulate neurohypophysial hormone secretion in the frog, as suggested in the mammalian hypothalamo-hypophysial system.

INTRODUCTION

Endothelins (ETs) are a family of acidic, 21-amino acid peptides with potent vasoconstrictive properties, including three isoforms, ET1, ET2 and ET3. ET1 was originally purified from cultured vascular epithelial cells (Yanagisawa et al., 1988). In the mammalian brain, including the hypothalamo-hypophysial system, immunoreactivity for ETs and expression of ET mRNAs have been detected (Cintra et al., 1989; MacCumber et al., 1989; Lee et al., 1990; Yoshizawa et al., 1990; Fuxe et al., 1991; Giaid et al., 1991; Takahashi et al., 1991; Nakamura et al., 1993). Furthermore, specific [^{125}I]ET1 binding sites were detected in the neurohypophysis (Jones et al., 1989; Ritz et al., 1992). In addition to diverse biological functions of ETs in peripheral organs (Simonson and Dunn, 1990; Masaki et al., 1991), it has been suggested that ETs modulate the secretion of arginine vasopressin (Yamamoto et al., 1991; Samson et al., 1991; Ritz et al., 1992). Furthermore, intracranial administration of ETs

altered drinking behavior in the rat (Samson et al., 1991) and the Japanese quail (Tezuka et al., 1992), and hemorrhage increased ET1 contents in the rat neurohypophysis (Uemura et al., 1994). Thus, such accumulating evidence suggests that ETs contribute to the regulation of body fluid homeostasis. However, most of these studies have been conducted in mammalian species. To study ETs in relation to water balance, amphibians should serve as interesting models, in view of their characteristic life cycle.

In this study, we investigated the distribution of ET1-like immunoreactivity in the bullfrog hypothalamo-hypophysial system, and demonstrated the coexistence of ET1 with oxytocin-like immunoreactivity in the classical neurosecretory neurons of the frog.

MATERIALS AND METHODS

Five bullfrogs (*Rana catesbeiana*) were deeply anesthetized with ethyl *m*-aminobenzoate (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and the brain with the pituitary was carefully dissected out. Specimens were then fixed for 16 hr in Bouin-Hollande solution at room temperature. After washing in distilled water, the tissues were dehydrated and wax-embedded according to the conventional method. Sagittal and transverse sections 6 μm thick were cut at intervals of 100 μm and collected onto slides for immunohistochemical analyses. Consecutive 3-μm sections of the area of the preoptic nucleus and the hypophysis were also mounted on different slides for comparison of ET1- and oxytocin-like immunoreactivity.

Sections on slides were dewaxed in xylene and gradually hydrated with a graded series of alcohol solutions. The sections were then washed overnight in 0.1 M sodium phosphate buffer containing 0.9 % saline (PBS) at pH 7.4 and incubated for 48 hr at 4 ^{o}C with anti-ET1 antiserum (Peptide Institute Inc., Osaka, Japan) diluted 1:1,000 in PBS containing 0.3 % Triton X-100 (PBST). This antiserum cross-reacts 60% with ET2 and 40% with ET1. After washing in PBST, the sections were incubated for 1 hr at room temperature with biotinylated anti-rabbit IgG (Vector Laboratories, Inc., Burlingame, CA, USA) diluted 1:100 in PBST. The sections were then washed again in PBST and incubated for 1 hr at room temperature with avidin-biotin-horseradish peroxidase complex (Vector) diluted 1:100 in PBST. After a final wash in PBST the sections were reacted with a 0.05 M Tris-HCl buffer solution (pH 7.4) containing 0.02 % 3,3'-diamonobenzidine hydrochloride and 0.005 % hydrogen

peroxide. Then, the sections were dehydrated, cleared in xylene, and coverslipped with Malinol (Muto Pure Chemicals Ltd., Tokyo, Japan). For comparison of ET1- and oxytocin-like immunoreactivity, sections 3 μm thick were stained with anti-ET1 antiserum and alternate sections were stained with an anti-oxytocin antibody (UCB Bioproducts S.A., Belgium) at a dilution of 1:5,000 to label the frog neurosecretory cells. This antiserum has cross-reactivity with the amphibian classical neurohypophysial hormones, vasotocin and mesotocin, and there is a very weak cross-reaction with vasopressin.

Immunohistochemical controls were carried out with normal rabbit serum, or with antiserum preabsorbed with synthetic ET1 or ET3 (10 μg/ml; Novabiochem, Läufelfingen, Switzerland) as the primary antibody, or by omission of antiserum in the first incubation.

RESULTS

ET1-like immunoreactivity was seen in the preoptic nucleus, hypothalamo-hypophysial tracts, and neurohypophysis, but not in other areas of the brain, nor in the intermediate or distal lobes. ET1-immunoreactive (ET1-IR) somata and fibers were scattered in the preoptic nucleus (Fig. 1A-C). ET1-IR somata showed punctate immunoreactivity and were classified into large (about 24 μm in diameter) and small (about 13 μm in diameter) neurons (Fig. 1D, E). Within these immunoreactive somata and proximal processes, ET1-IR puncta were unevenly distributed (Fig. 1D, E). These ET1-IR neurons extended immunoreactive varicose fibers in the ventro-lateral direction (Fig. 1B) and these fibers seemed to travel to the infundibulum. In the infundibulum and median eminence, most of ET1-IR fibers were very fine but a few were large (Fig. 1F, G).

In the neurohypophysis, ET1-IR fibers were scattered and dense accumulation of ET1-IR fibers was seen near blood vessels (Fig. 2A, B). The size of ET1-IR terminals abutting on sinusoidal spaces varied from 3 to 10 μm in diameter. Small immunoreactive puncta were scattered among these large terminals (Fig. 2B). ET1-IR fibers were very sparse in the lateral portion of the neurohypophysis (Fig. 4C). Figure 3 schematically shows the distribution of ET1-like immunoreactivity in transverse sections from anterior (Fig. 3A) to posterior (Fig. 3F). These immunoreactivity profiles

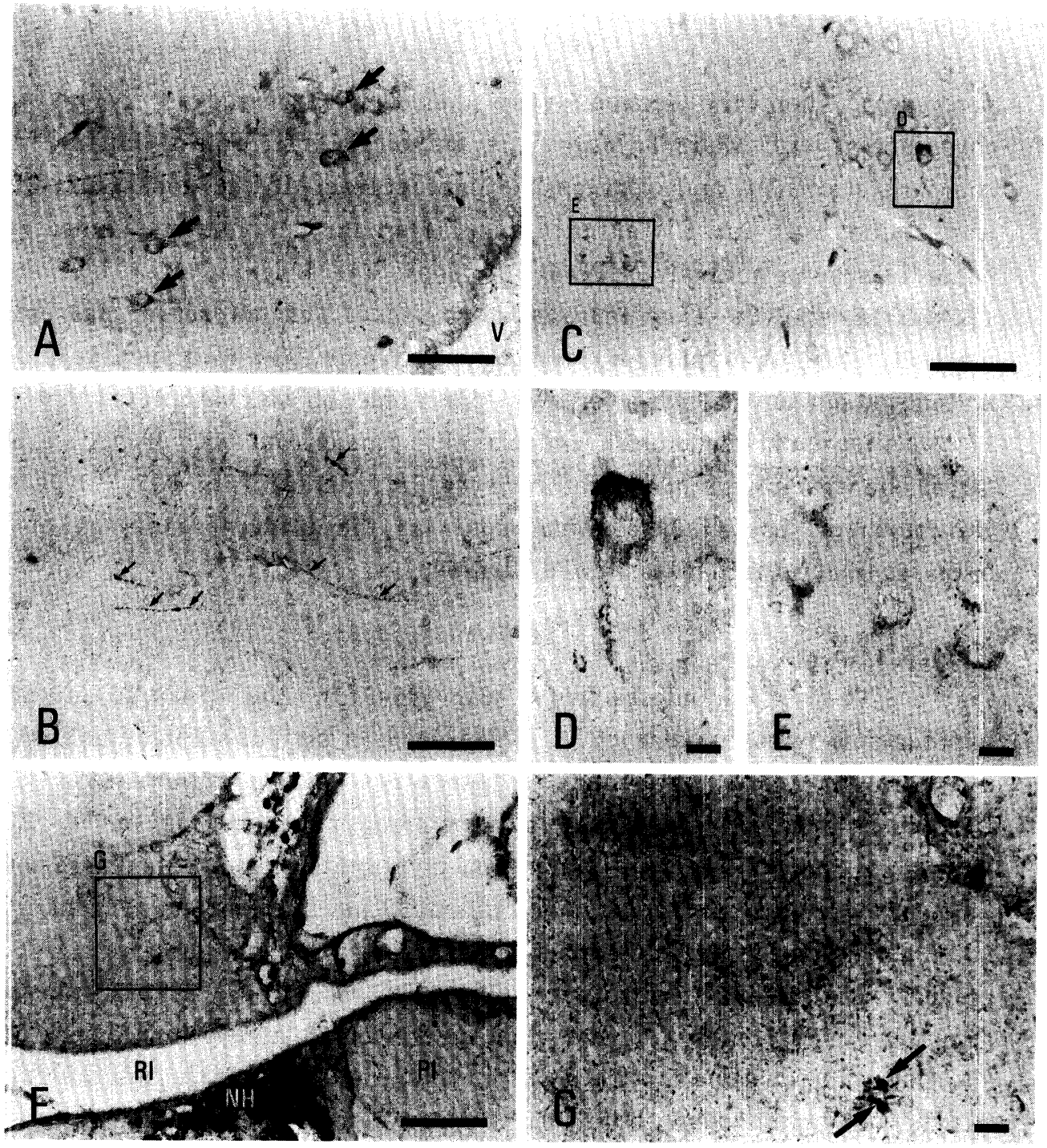

Fig. 1. Photomicrographs of transverse (A-E) and sagittal (F, G) sections showing ET1-like immunoreactivity in the hypothalamus. **A**: Immunoreactive somata (arrows) in the pars magnocellularis of the preoptic nucleus. V indicates the third ventricle. **B**: ET1-IR fibers (arrows) located in the ventro-lateral area of the preoptic nucleus at the level of optic chiasma. Left, lateral; right, median. **C**: Photomicrograph showing ET1-IR somata in the pars magnocellularis (shown by box D) and pars parvocellularis (shown by box E) of the preoptic nucleus. Left, lateral; right, median. **D**: High magnification of a large ET1-IR soma shown in box D in panel C. Note granular appearance in the ET1-IR soma. **E**: High magnification of small ET1-IR somata shown by box E in panel C. Note a few immunopositive puncta in the small somata. **F**: Low magnification view of a sagittal section showing ET1-like immunoreactivity in the hypothalamus. Left, anterior; right, posterior. Abbreviations: NH, neurohypophysis; PI, pars intermedia; RI, recessus infundibulum. **G**: High magnification view of the ventral infundibular nucleus indicated by box G in panel F. Note fine (small arrows) and large (large arrows) punctate immunoreactivity in this area. Bars in A-C, F, 100 μm; bars in D, E, G, 10 μm.

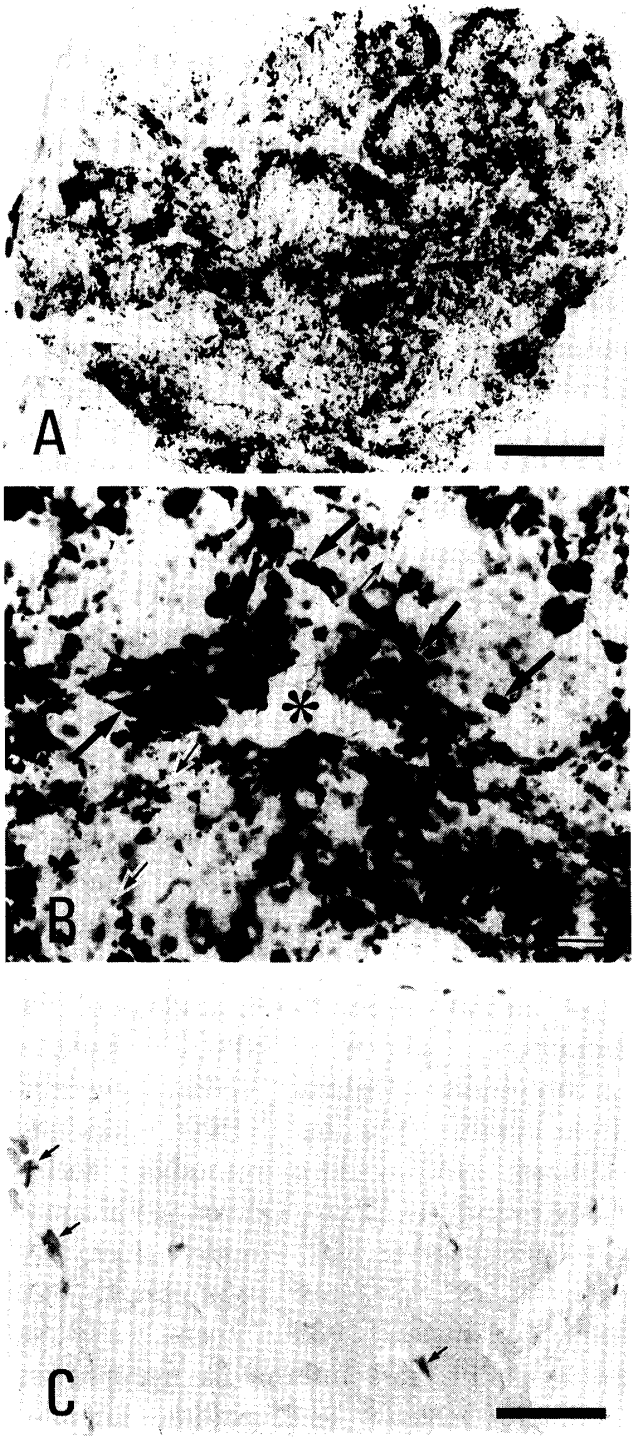

Fig. 2. Photomicrographs of sagittal sections near the median showing ET1-like immunoreactivity in the neuro-hypophysis. **A**: Low magnification view of the neurohypophysis. **B**: High magnification view of the neuro-hypophysis. Note dense accumulation of immunoreative terminals near a blood vessel (asterisk). Large arrows indicate large ET1-IR terminals and small arrows indicate fine ET1-IR puncta. **C**: A section processed with preabsorbed antiserum. No immunoreactive deposition was seen but erythrocytes (arrows) showed deposition caused by endogeneous peroxidase activity. Bars in A, C, 100 μm; bar in B, 10 μm.

were abolished by preabsorption with synthetic ET1 (Fig. 2C), but preabsorption with ET3 had no effect on ET1-like immunoreactivity. Immunostaining with normal rabbit serum (1:50) or omission of the primary antibody yielded no immunoreative deposition although endogeneous peroxidase activity was seen in erythrocytes as seen in the absorption test (Fig. 2C).

Only some of the oxytocin-immunoreactive somata showed ET1-like immunoreactivity and all ET1-IR somata were intensely immunopositive for

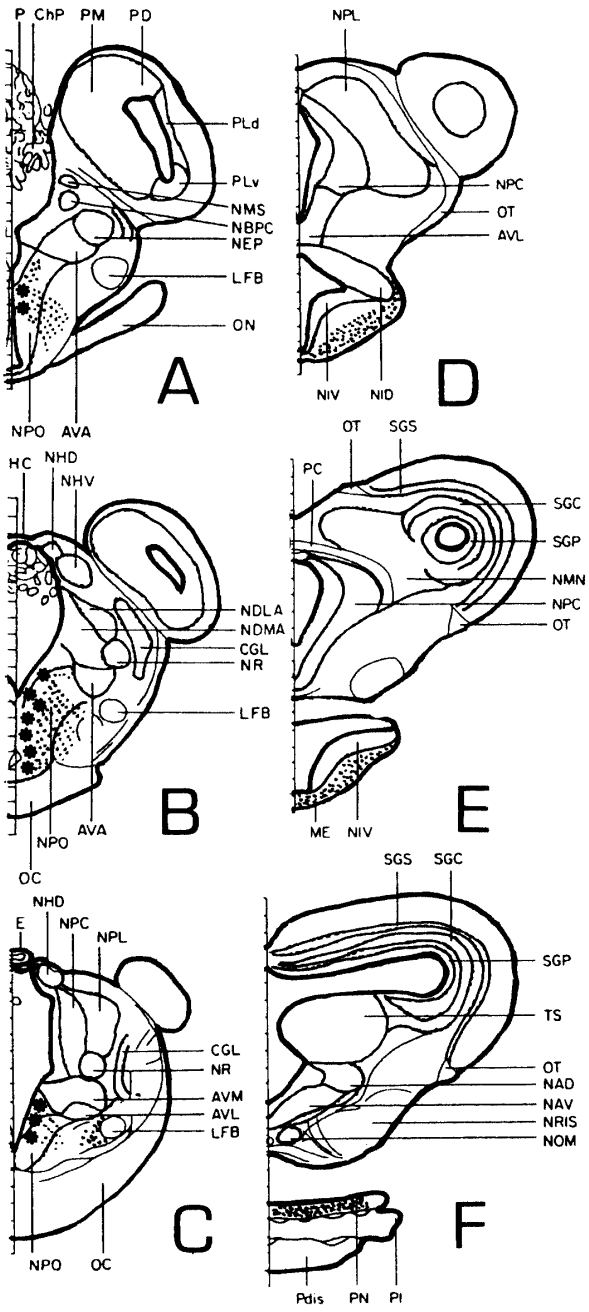

Fig. 3. Schematic representation of the distribution of ET1-IR somata (asterisks) and fibers (dotted lines) in transverse sections from anterior (A) to posterior (F). The frog brain atlas is drawn based on the description by Wada et al. (1980). Abbreviations: AVA, area ventralis anterior thalami; AVL, area ventrolateralis thalami; AVM, area ventromedialis thalami; CGL, corpus geniculatus laterale; ChP choroid plexus; E, epiphysis; HC, habenular commissure; LFB, lateral forebrain bundle; ME, median eminence; NAD, nucleus anterodorsalis tegmenti mesencephali; NAV, nucleus anteroventralis tegmenti mesencephali; NBPC, bed nucleus of the pallial commissure; NDLA, nucleus dorsolateralis anterior thalami; NDMA, nucleus dorsomedialis anterior thalami; NEP, nucleus entopeduncularis; NHD, nucleus habenularis dorsalis; NHV, nucleus habenularis ventralis; NID, nucleus infundibularis dorsalis; NIV, nucleus infundibularis ventralis; NMN, nucleus mesencephalicus nervi trigemini; NMS, nucleus medialis septi; NOM, nucleus of the oculomotor nerve; NPC, nucleus posterocentralis thalami; NPL, nucleus posterolateralis thalami; NPO, nucleus preopticus; NR, nucleus rotundus; NRIS, nucleus reticularis isthmi; OC, optic chiasma; ON, optic nerve; OT, optic tract; P, paraphysis; PC, posterior commissure; PD, pallium dorsale; Pdis, pars distalis hypophysis; PI, pars intermedia hypophysis; PL, pallium laterale; PLd, pallium laterale, pars dorsalis; PLv, pallium laterale, pars ventralis; PM, pallium mediale; PN, pars nervosa hypophysis; SGC, stratum griseum centrale tecti; SGP, stratum griseum periventricularis tecti; SGS, stratum griseum superficiale tecti; TS, torus semicircularis.

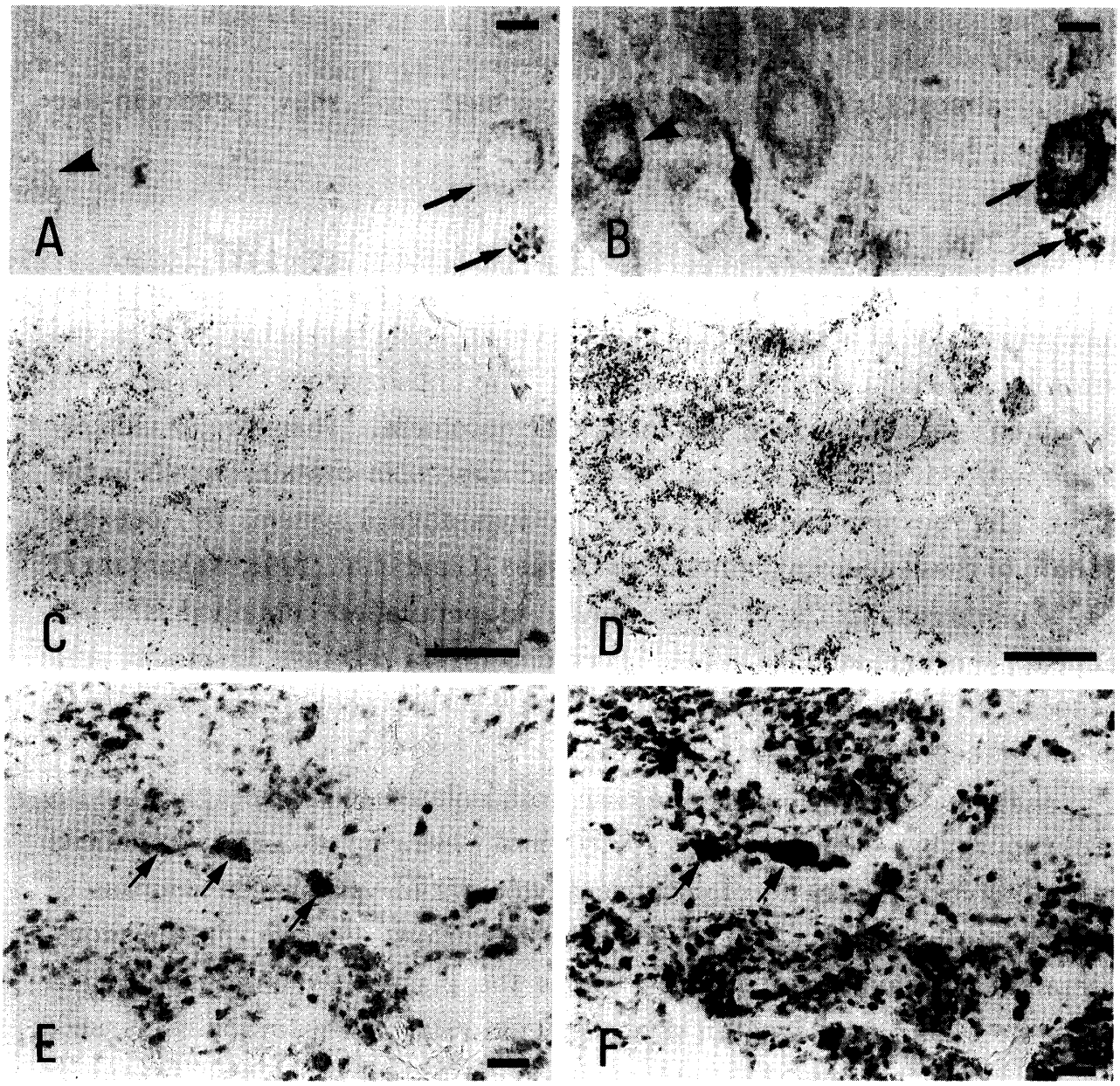

Fig. 4. Photomicrographs of pairs of adjacent transverse sections of the preoptic nucleus (A, B) and adjacent horizontal sections of the neurohypophysis (C-F). **A, B**: ET1-IR (A) and oxytocin-IR (B) somata in the preoptic nucleus. Arrows indicate identical somata immunopositive for both ET1 and oxytocin, and arrowheads indicate an identical soma immunopositive for oxytocin but not for ET1. **C, D**: Low magnification of ET1-IR (C) and oxytocin-IR (D) terminals in the neurohypophysis. Note similar distribution patterns of immunoreactive terminals except in the lateral portion (right side of the panel) of the neurohypophysis. **E, F**: High magnification view of ET1-IR (E) and oxytocin-IR (F) terminals. Note some varicose fibers were labeled with both antibodies (arrows). Bars in A, B, E, F, 10 μm; bars in C, D, 100 μm.

oxytocin in the preoptic nucleus (Fig. 4A, B). In the neurohypophysis, the overall distribution of ET1-IR fibers was similar to that of oxytocin-immunoreactive fibers (Fig. 4C, D). However, oxytocin-immunoreactive fibers dominated over ET1-IR fibers, especially in the lateral portion of the neurohypophysis. Although it was difficult to confirm using consecutive 3-μm

sections, almost all ET1-IR fibers seemed to show oxytocin-like immunoreactivity (Fig. 4E, F).

DISCUSSION

In this study, we demonstrated the presence of ET1-like immunoreactivity in a subpopulation of oxytocin-immunoreactive cells in the bullfrog hypothalamo-hypophysial system. The anti-oxytocin antiserum used here had cross-reactivity with vasotocin and mesotocin. Therefore, it is likely that this antibody labeled both vasotocin- and mesotocin-containing cells in the bullfrog. In the mammalian hypothalamo-hypophysial system, ET1 coexists with both of these neurohypophysial hormones (Giaid et al., 1991; Nakamura et al., 1993), whereas in the bullfrog, only a subpopulation contained ET1-like peptide. Further studies using specific antisera against vasotocin and mesotocin are required to determine whether ET1-like peptide was colocalized with one of these hormones or with both.

ETs modulate vasopressin release from isolated nerve endings of the rat neurohypophysis (Ritz et al., 1992) and intravenous ET1 enhanced electrophysiological activity of putative oxytocin and vasopressin neurons of rats (Yamashita et al., 1991; Wall and Ferguson, 1992). Furthermore, intracranial administration of ETs elevates the plasma levels of vasopressin (Yamamoto et al., 1991; Samson et al., 1991) in rats. Dehydration decreased, and hemorrhage increased ET1 contents in the rat neurohypophysis (Uemura et al., 1994). Together with these results, the peptide seems to be involved in regulation of neurohypophysial hormone secretion at loci in either the hypothalamus or the neurohypophysis in relation to water balance in both amphibians and mammals.

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