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CORTICOTROPIN-RELEASING FACTOR: IMMUNOHISTOCHEMICAL CO-LOCALIZATION WITH ADRENOCORTICOTROPIN AND ß-ENDORPHIN, BUT NOT WITH MET-ENKEPHALIN, IN SUBPOPULATIONS OF DUODENAL PERIKARYA OF RAT

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SUMMARY: By the use of two different double-staining techniques (simultaneous staining of adjacent serial sections and the double-staining elution method) it was possible to demonstrate that a corticotropin-releasing factor (CRF) immunofluorescence co-existed with an adrenocorticotropin (ACTH) and ß-endorphin (ß-END) immunoreactivity, but not with a Met-enkephalin (Met-ENK) immunostaining, within perikarya subpopulations of both the myenteric and submucousal plexus of the rat duodenum. Not a single Met-ENK-positive neuronal cell body was stained also for CRF, ACTH or ß-END. Even nerve fibres, localized in both the myenteric plexus and closely to submucousal blood vessels (probably arterioles), revealed a CRF immunofluorescence, which is also colocalized with an ß-END staining. These results are quite different to the recent observations in the mammalian hypothalamus, suggesting that some myenteric and submucousal plexus neurons may synthesize CRF as well as ß-END and ACTH, but not Met-ENK. The colocalized peptides might be concomitantly released into the synaptic cleft after terminal stimulation.

ACTH and the opioid peptides ß-END and Met-ENK as well as the 41-residue CRF (1,2) have been detected by biochemical and immunohistochemical methods in the brain, pituitary, and peripheral nervous system of various mammalian species. By using recombinant DNA methodology it could be demonstrated that the amino acid sequences of these peptides are contained in three different precursor peptides: in the bovine (3,4) and human (9) adrenal proenkephalin (preproenkephalin A) (Met-ENK) precursor peptide, in the human (5), rat (55), and bovine (6) pro-opiomelanocortin precursor molecule (ACTH and ß-END), and in the human (7) and ovine (8) hypothalamic prepro-CRF precursor peptide. They are cleaved enzymatically from their corresponding precursor peptides to free biologically active peptides at the peptide bonds -Arg-Arg-, -Lys-Arg-, and -Lys-Lys- (10).

In the mammalian brain, a CRF immunostaining has been described mainly in the parvocellular and magnocellular region of the hypothalamic paraventricular nucleus (PVN) and in cell populations of

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the supraoptic nucleus (SON) as well as within their axons, which project to the median eminence and neurohypophysis to form close contacts with blood capillaries (11-15). Met-ENK immunostaining was detected within the bovine magnocellular PVN and SON perikarya and their axons, which also trace to the neurohypophysis to form close associations with blood capillaries (16). Met-ENK does not co-exists with ß-END in the rat brain (17), but with CRF in subpopulations of parvocellular neurons of the rat PVN (18). ACTH and ß-END immunoreactivities are contained mainly within neuronal cell bodies of the hypothalamic arcuate nucleus (AR) (19) and the human infundibular nucleus (20) as well as within their axons, projecting to the median eminence. The mammalian neurohypophysis contains no ß-END or ACTH immunoreactivity, but has Met-ENK-positive nerve fibres (23,35-37). Both ß-END and ACTH were shown by immunohistochemical and biochemical methods in the rat pituitary within endocrine corticotroph anterior lobe and melanotroph pars intermedia cells (21,22,24,25) as well as within subpopulations of pars intermedia cells of the dog pituitary (53).

The release of ß-END and ACTH from corticotrophs and melanotrophs is mediated by CRF that originates from PVN and SON perikarya and that is transported within their axons to nerve terminals, which are close to blood vessels of the median eminence and the neurohypophysis. CRF is, then, released into the capillary lumen for transport to specific plasma membrane-bound CRF receptors of these corticotroph and melanotroph pituitary cells (54). Interestingly, ACTH- and ß-END stained nerve fibres, which may originate from AR perikarya, are also to detect in the rat PVN closely surrounding CRF-positive neuronal cell bodies (26). Both ACTH, ß-END and CRF immunoreactivities can be demonstrated by biochemical and immunohistochemical methods in the mammalian enteric nervous system. Neuronal cell bodies of the myenteric plexus of the rat duodenum contain ACTH (27), ß-END (28) as well as CRF (29) immunofluorescences. Met-ENK stained perikarya are to reveal in the guinea-pig duodenum (30). ß-END has been visualized also within submucous plexus perikarya of the rat duodenum (28). Nerve fibre-bundles, lying in the myenteric and submucousal plexus, were also stained by antibodies directed against Met-ENK, ACTH and ß-END. ACTH and ß-END are colocalized within myenteric plexus perikarya of the rat duodenum (27) as has been described for human infundibular nucleus perikarya (20), rat AN perikarya (31) and corticotrophs of the anterior lobe (24) and melanotrophs of the pars intermedia lobe (25) of the rat pituitary. Met-ENK (32), ACTH and ß-END (33) have been found by chromatographic methods in tissue extracts of the mammalian gastrointestinal tract. Met-ENK tissue concentrations were highest in the duodenum (rat and guinea-pig), but also the ß-END

immunoreactivity was very high in duodenal tissue extracts (rat) (32,33). Here I report that CRF co-exists with ACTH and ß-END, but not with Met-ENK, in subpopulations of myenteric and submucous plexus perikarya of the rat duodenum within one and the same neuronal cell body. However, no Met-ENK-positive perikaryon were stained for CRF, ACTH or ß-END. These results suggest that, first, these peptides, which originate from three different precursor peptides, have important physiological roles in the mammalian gastrointestinal tract, and, second, that within CRF stained myenteric and submucous plexus perikarya, ACTH and ß-END , but not Met-ENK, are synthetized and transported to nerve endings to be released into the synaptic cleft after stimulation.

MATERIALS and METHODS

Materials. The following synthetic peptides were used: α-melanotropin (α-MSH), ACTH, ACTH(1-14), α- and ß-END (Sigma); ovine CRF, sauvagine, renin, dynorphin (1-13) (Peninsula) as well as Met-ENK and Leu-ENK (Bachem). Antisera against ß-END, ACTH, ovine CRF and Met-ENK were purchased from Immuno Nucl. Corp. and fluorescein-isothiocyanate (FITC)-conjungated swine anti-rabbit IgG as well as swine anti-rabbit IgG were products of Dakopatts.

Tissue preparation. Male, colchicine-untreated Wistar rats (200-300 g) were anesthetized with Nembutal (60 mg/kg), the duodenum dissected and cut into 2,5 µm serial sections on a cryostat microtome (-25°C). Then, the sections were immersed in freshly depolymerized 4 % paraformaldehyde (in 100mM phosphate-buffered salin, PBS; pH 7.4) for 90 min at 4°C, rinsed several times in the same buffer and stored overnight in PBS containing 5 % sucrose and 4 % normal swine serum. The processings for immunofluorescence microscopy were the same as previously reported (27,28).

Tissue staining. Briefly, ACTH, ß-END, Met-ENK and CRF antisera (primary antibodies) were raised in rabbits and used at a dilution of 1:100 (in PBS, pH 7.4, containing 0.3 % Triton X-100 and 4 % normal swine serum). The sections were incubated with the primary antibodies for 16 hr at 4°C followed by incubation with FITC-conjungated swine anti-rabbit IgG (1:40 in PBS containing 0.3 % Triton X-100 and 4 % normal swine serum) for 30 min at 37°C. Then, the sections were extensively washed in PBS, examined and photographed with a Leitz Orthoplan microscope fitted with an epifluorescence attachement. For comparing sections stained with two different antisera, either adjacent serial sections through both the myenteric and submucousal plexus of the rat duodenum were stained simultaneously (CRF and ß-END, the latter antibodies could not be eluted from the sections) or according the double-staining elution method previously described by Tramu et al. (34). The latter method was performed by elution of the primary antibodies (CRF, ACTH or Met-ENK) with a solution consisting of 2,5 % potassium permanganate (dissolved in distilled water), which was further diluted with an equal volume 5 % H_2SO_4. This stock solution was further diluted with distilled water (1:60). The sections were incubated with this final solution for 1 min at room temperature and, then, rinsed several times in PBS. To block entirely any residual bindings of the primary antibodies, the sections were incubated with an excess of swine anti-rabbit IgG (1:5 in PBS) for 30 min at 37°C. To control the successful elution and blocking of the primary antiserum, the sections were

re-incubated with FITC-conjungated swine anti-rabbit IgG (1:40 in PBS) for 30 min at 37°C and, then, re-examined with an epifluorescence microscope. Sections, revealing no fluorescence, were choosen for further staining experiments. These sections were re-incubated with another antiserum under the same incubation conditions described above, and re-examined and re-photographed for comparison studies.

Specificity controls. All four antisera were pre-incubated with related or unrelated synthetic peptides (1-10 μM): the CRF antiserum with ovine CRF, sauvagine, renin, and the unrelated peptides ß-END, Met-ENK and ACTH: the ACTH antiserum with ACTH, ACTH (1-24), α-MSH and the unrelated peptides CRF, ß-END and Met-ENK; the ß-END antiserum with ß-END, Met-ENK, α-END and the unrelated peptides CRF, α-MSH and ACTH. Pre-incubation of the Met-ENK antiserum with ACTH, ß-END and CRF did not affect the Met-ENK staining. However, pre-absorption of the Met-ENK antiserum with dynorphin (1-13), an opioid peptide with an amino acid sequence at its N-terminal portion similar to that of Met-ENK, and Leu-ENK reduced the Met-ENK immunofluorescence slightly (dynorphin (1-13) or considerably (Leu-ENK).

RESULTS and DISCUSSION

Immunohistochemical staining experiments combined with two different double-staining techniques (the staining of adjacent serial sections and the double-staining elution method) revealed that a CRF immunostaining is co-existent with an ACTH and ß-END immunofluorescence,but not with a Met-ENK staining, within subpopulations of myenteric and submucous plexus perikarya of the rat duodenum. Neuronal cell bodies, stained for Met-ENK, showed, on the other hand, no immunoreactivity for CRF, ACTH or ß-END (unpublished observations). Nerve fibres, lying in the myenteric plexus or forming close associations with submucousal blood vessels, expressed the same immunostaining patterns for CRF and ß-END as described in neuronal cell bodies of the myenteric and submucousal plexus. A comparison of colocalization of CRF, ACTH, ß-END and Met-ENK in the rat duodenal nervous system with that of the rat or bovine hypothalamo-hypophysial axis should be accepted with caution because the occurrence of bioactive ß-END and Met-ENK material in the mammalian hypothalamus is controversially (Figures 1 and 2).

ß-END is present in the rat pituitary and brain in its authentic bioactive (des α-N-acetylated) and its inactive (α-N-acetylated) forms (35,36). Biochemical experiments, effected by Weber at al.(37), revealed, however, that bioactive ß-END was found only in tissue extracts of the rat pituitary, but not in the rat brain. These chromatographic findings are in contradiction of those of Zakarain and Smyth, in which the rat brain contained region-specifically bioactive ß-END and inactive N-terminal ß-END amino acid sequences (35,36). Still unclear is whether the duodenal ß-END is α-N-acetylated or desα -N-acetylated and which form represents the bioactive form there. It is also contro-

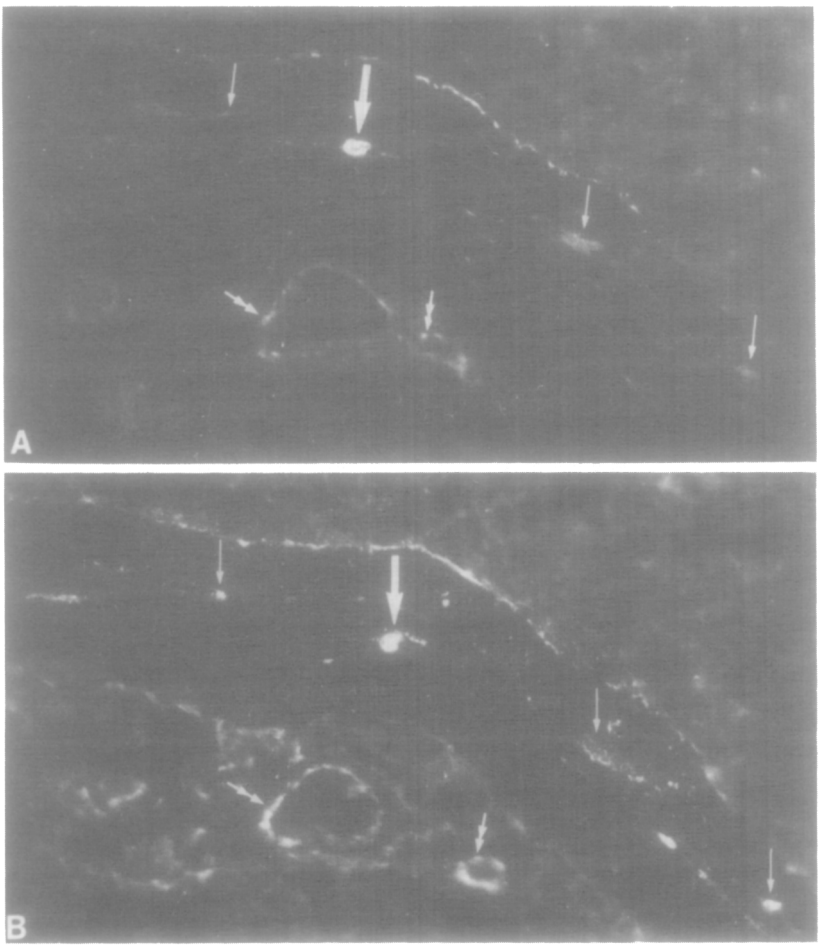

Fig. 1. Immunofluorescence photomicrographs of adjacent serial sections through the rat duodenum stained for CRF (A) or ß-END (B). One and the same neuronal cell body (solid arrows), which is located in the myenteric plexus, expresses a heavily ß-END or CRF immunofluorescence, indicating the co-localization of both neuropeptides. Longitudinally cut or tangentially cut nerve fibres (small arrows), lying also in the myenteric plexus, reveal nearly superimposable staining patterns for CRF and ß-END too. Some nerve terminals (double-headed arrows), which are in close contacts with sub-mucousal arterioles, contain both CRF and ß-END material.

versially if bioactive Met-ENK is present within perikarya of the mammalian hypothalamic PVN and SON perikarya because Watson et al. (38) and Weber et al. (39) found independently no authentic bioactive hypothalamic Met-ENK in biochemical and immunohistochemical studies.

Physiological experiments, performed by Orwoll and Kendall (33), revealed that the fasting rat has significant higher amounts of ACTH and ß-END in tissue extracts of the gastrointestinal tract than the *normal fed rat*. Intraventricular injection of synthetic ovine CRF into the rat brain led to a gastrin-independent inhibition of the gastric acid secretion via central, vagal and adrenergic mechanisms (40).

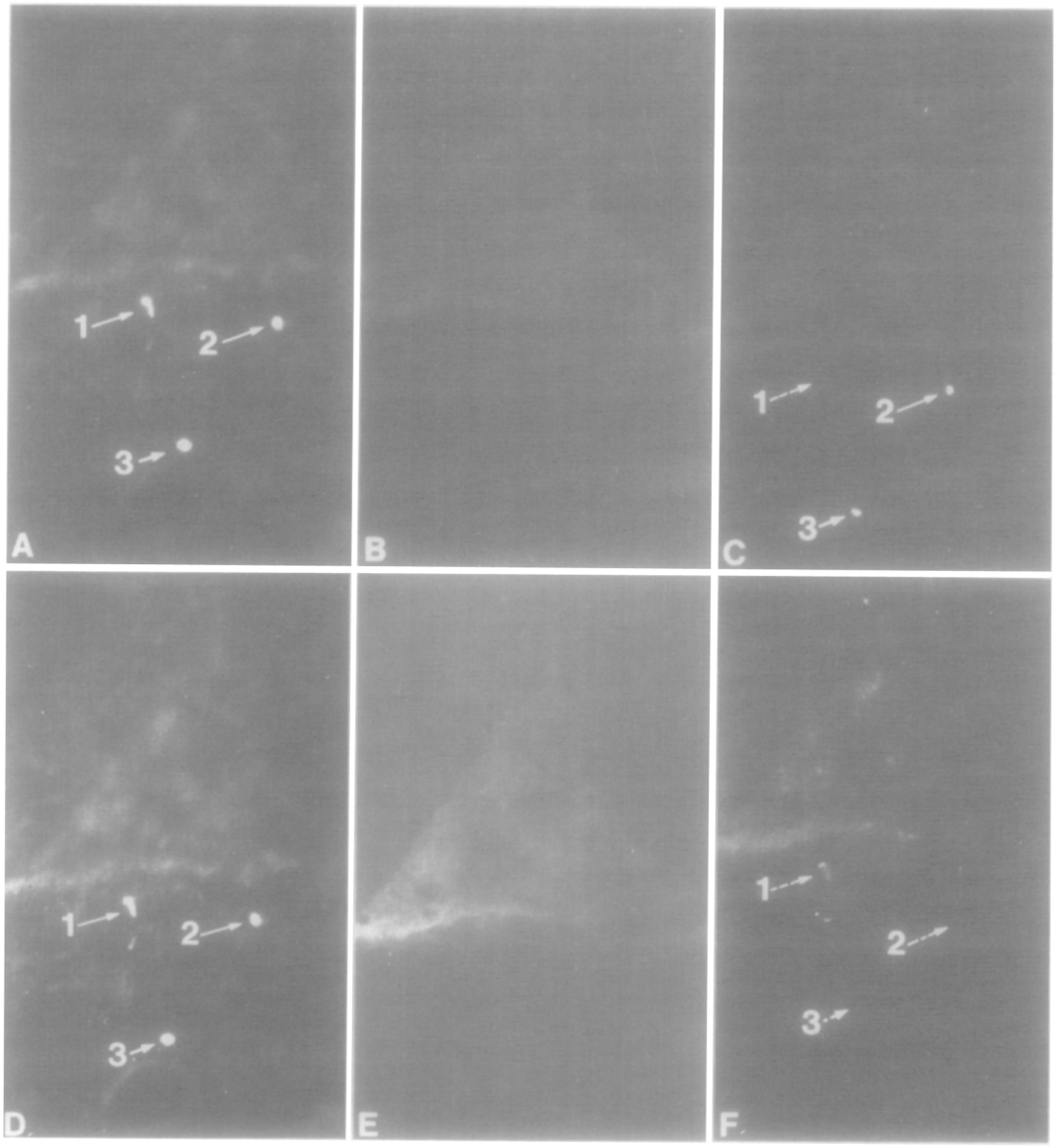

Fig. 2. Immunofluorescence photomicrographs ofcryostat sections through the rat duodenum. The sections were incubated with CRF-antiserum (A and D), photographed, the antibodies were eluted as described in Methods, re-photographed (B and E), re-incubated with ACTH (C) or Met-ENK (F) antiserum and photographed again. Immunoreactive perikarya are seen in both the myenteric (numer 1 and 2) and submucousal plexus (number 3). Met-ENK or ACTH-unstained neuronal cell bodies (dashed arrows) are localized in both nerve plexi. Comparison of the sections demonstrates that a CRF staining is linked with an ACTH immunofluorescence within the myenteric and submucousal plexus. However, not all CRF stained myenteric plexus perikarya are also immunofluorescent for ACTH. CRF-positive perikarya do not stain for Met-ENK either in the myenteric plexusor in the submucousal plexus of the rat duodenum.

Stress elevated both the ACTH and ß-END secretion from corticotroph and melanotroph rat pituitary cells *in vitro* and *in vivo*. These responses might be mediated by CRF, which is produced in PVN and SON perikarya

(41,56). Stress results, on the other hand, in ulcer of the human and mammalian stomach (gastric ulcer) and duodenum (duodenal ulcer). Smooth muscle cell preparations with adherent nerve tissue from the ileum (42-45) as well as isolated gastric smooth muscle cells devoid of neural elements (46), both from guinea-pig, shared specific receptor populations for the endogenous opioid ligands ß-END (μ-receptors) and Met-ENK (δ-receptors). Therefore, structures of the enteric nervous system as well as enteric smooth muscle cells have specific Met-ENK and ß-END receptor subpopulations. Application of ß-END and Met-ENK to in vitro preparations of the myenteric plexus of the guinea-pig ileum led to a dose-related, naloxone-reversible, rapid inhibition of the firing rate of these neurons (43,44). Exposure of isolated circular smooth muscle cells, devoid of neural elements, to Met-ENK resulted in a direct contractile action, which was also reversible by naloxone (46). These neuropharmacological and physiological findings may indicate that ß-END and Met-ENK are produced in myenteric plexus perikarya and that they have specific and potent receptor-dependent activities on both enteric neural structures and smooth muscle cells. In this manner they can influence the motility, peristalsis, and contractility of the gastrointestinal tract (47). As Met-ENK and ß-END are not colocalized within perikarya of the duodenal nervous system, different releasing mechanisms may occure in the mammalian gastrointestinal tract.

Comparison of these CRF, Met-ENK, ACTH and ß-END-positive duodenal neuronal cell bodies with those enteric perikarya, which were classified by Ramon y Cajal (48), Dogiel (49), and Stach (50) at light microscopic level and by Burnstock and Cook (51) and Wilson et al. (52) at ultrastructural level, may facilitate the understanding of possible different physiological roles of these peptides.

Further important conclusions from the colocalization of CRF material with ACTH and ß-END immunoreactivities within subpopulations of duodenal perikarya are, first, that the three peptides might be synthesized simultaneously in the same perikarya and, second, that they might be released concomitantly into the synaptic cleft after stimulation.

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