Reflex splanchnic nerve stimulation increases levels of proenkephalin A mRNA and proenkephalin A-related peptides in the rat adrenal medulla

(enkephalin biosynthesis/blot hybridization/insulin hypoglycemia/adrenal medulla)

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ABSTRACT   The effect of reflex splanchnic nerve stimulation on proenkephalin A biosynthesis was investigated in the rat adrenal medulla. Tissue levels of native [Met^5]enkephalin-like immunoreactivity (IR) (measured by direct RIA of tissue extracts), cryptic [Met^5]enkephalin-like IR (calculated as the increase in [Met^5]enkephalin-like IR detected in tissue extracts after sequential digestion with trypsin and carboxypeptidase B), and proenkephalin A mRNA were determined in adrenal medulla from rats sacrificed at various times after a period of insulin-induced hypoglycemia. Two hours of insulin hypoglycemia, which produced intense reflex stimulation of the splanchnic nerves as evidenced by a 55% decrease in the adrenal medulla catecholamine levels, resulted in a 3-fold increase in proenkephalin A mRNA levels in this tissue. The proenkephalin A mRNA levels reached a maximum 15-fold increase over control values 24 hr after this period of hypoglycemic stress and then gradually declined with an approximate half-life of 4 days. Native and cryptic [Met^5]enkephalin-like IR had increased 9-fold and 12-fold, respectively, 24 hr after this period of hypoglycemia, and both demonstrated maximum increases of 130-fold and 50-fold, respectively, after 96 hr. Combined pretreatment (i.p. administration) with the ganglionic and muscarinic blocking agents chlorisondamine (5 mg/kg of body weight) and atropine (1 mg/kg) blocked the increase in levels of proenkephalin A mRNA seen in the rat adrenal medulla following insulin hypoglycemia. These data indicate that reflex splanchnic nerve discharge stimulates proenkephalin biosynthesis, probably at the level of gene expression.

The role of the splanchnic innervation in the regulation of opioid peptide (OP) biosynthesis in the rat adrenal medulla has been a matter of controversy. Denervation of the rat adrenal gland has been reported to produce an increase in proenkephalin A-related peptides and proenkephalin A mRNA in the adrenal medulla (1–3). This evidence suggested that the splanchnic innervation may exert a tonic inhibitory influence on the expression of the proenkephalin A gene. In contrast, other reports indicated that reflex activation of the splanchnic nerve by insulin-induced hypoglycemia in the cat, guinea pig, or rat produced an initial secretion of adreno-medullary OP, which was followed by a rapid recovery in the OP content of this tissue to levels that exceeded prestimulation values (4, 5). Since the recovery of OP levels was blocked by cycloheximide, it was suggested that reflex splanchnic stimulation led to an increase in OP biosynthesis (4, 5). The aim of this study was to further examine the effect of reflex splanchnic nerve stimulation on the levels of

proenkephalin A mRNA, [Met^5]enkephalin, and [Met^5]enkephalin-related peptides in the rat adrenal medulla.

MATERIALS AND METHODS

Treatment of Animals. Male Sprague–Dawley rats weighing 200–300 g were fasted overnight. On the following day, experimental animals were injected s.c. with insulin (10 units per kg of body weight; Iletin, Eli Lilly) to induce hypoglycemia and subsequent reflex splanchnic nerve discharge. Unless otherwise stated, the insulin shock was terminated after 2 hr by oral administration of 1.5 ml of 40% sucrose. The effectiveness of this procedure was evident by the visible recovery of the animals within 20 min of sucrose administration. Control animals were untreated. The animals were sacrificed at various times after this 2-hr period of insulin hypoglycemia. The adrenal glands were rapidly removed, and the medullae were dissected free of cortical tissue on ice by using a stereoscopic microscope. Paired adrenal medullae were rapidly frozen in liquid nitrogen and stored at −70°C.

Tissue Preparation for Determinations of [Met^5]Enkephalin-like IR, Catecholamines, and Protein. One pair of adrenal medulla was homogenized in 200 μl of 0.5 M acetic acid by sonication. A 20-μl aliquot of the homogenate was diluted in four vol of 2 M perchloric acid and centrifuged. Proteins were determined by dissolving the perchloric acid precipitates in 0.1 M NaOH as described by Lowry *et al.* (6) with bovine serum albumin as standard. The perchloric acid supernatant was used for catecholamine determinations. The remainder of the acetic acid homogenate was centrifuged (25,000 × *g* for 10 min), and supernatant was used for determinations of [Met^5]enkephalin-like IR.

Catecholamine Assays. Levels of norepinephrine and epinephrine were determined by HPLC with dual-electrode coulometric detection (Environmental Sciences Associates, Bedford, MA) (F. S. Menniti and E.J.D., unpublished data). The perchloric acid extracts were chromatographed on a C$_{18}$ column (10 cm, 3-μm particle size; Rainin, Woburn, MA) eluted isocratically with 10 mM NaH$_2$PO$_4$/0.6 mM sodium octyl sulfate/16% methanol (vol/vol), pH 2.6, as the mobile phase. Catecholamines were oxidized at the first electrode at a potential of +0.45 V.

RIA of Native and Cryptic [Met^5]Enkephalin-like IR. The tissue levels of native [Met^5]enkephalin-like IR were determined by a RIA method described previously (7). A modified method of Liston *et al.* (8) and Cupo *et al.* (9) was used for the RIA of cryptic [Met^5]enkephalin-like IR. An aliquot of supernatant of acetic acid homogenate was lyophilized and reconstituted in twice the original volume of buffer (50 mM Tris·HCl/5 mM CaCl$_2$, pH 8.4). The mixture was incubated

with trypsin (final concentration, 10 μg/ml) for 4 hr at 37°C, boiled for 15 min, and cooled on ice. This mixture was incubated with carboxypeptidase B (final concentration, 0.1 μg/ml in a mixture 2.5 times the original volume of the aliquot) for 2 hr at 37°C, boiled for 15 min, and cooled on ice. The mixture was centrifuged at 25,000 × g for 30 min. The supernatant was used for RIA with [Met⁵]enkephalin assay kits (Immuno Nuclear, Stillwater, MN). The crossreactivities of the antiserum against [Met⁵]enkephalin toward other OP are as follows (on a molar basis): [Leu⁵]enkephalin, 0.45%; [Met⁵]enkephalin-Arg-Phe, [Met⁵]enkephalin-Arg-Gly-Leu, dynorphin A-(1–8), and β-endorphin, <0.1%.

Measurement of Proenkephalin A mRNA. *Preparation of RNA.* Total RNA was extracted from adrenal medulla as described (10) with slight modifications (11). Briefly, the frozen tissue was homogenized in 4 M guanidinium thiocyanate and centrifuged at 13,000 × g for 20 min. The supernatant was treated with acetic acid and ethanol and kept overnight at −20°C. The precipitate was recovered by centrifugation and treated with guanidine hydrochloride followed by precipitation with ethanol. The guanidine hydrochloride extraction step was repeated once. The final pellets were extracted with water, and the water extract containing RNA was precipitated with ethanol, recovered by centrifugation, dried *in vacuo*, and finally dissolved in water. Absorbance measurements were obtained at 260 and 280 nm with a spectrophotometer. The A_{260}/A_{280} ratio was between 1.70 and 2.0. The RNA samples were routinely stored at −70°C. For blot hybridization, a pool of 12 pairs of the rat adrenal medullae was used, which yielded 20–25 μg of total RNA from which an aliquot of 20 μg was used (see Fig. 1). For dot-blot hybridization, a pool of 8 pairs of the adrenal medullae was used. An aliquot of 5 μg of total RNA was used (see Figs. 4 and 5).

Preparation of cDNA probe for hybridization. The plasmids containing the rat brain proenkephalin A cDNA (pRPE2) were isolated from *Escherichia coli* lysates by CsCl/ethidium bromide equilibrium density gradient centrifugation. A 941-base-pair Bsp-1286 fragment was isolated from pRPE2 by endonuclease digestion followed by agarose gel electrophoresis. The fragment was nick-translated with [α-³²P]dCTP to a specific radioactivity of 1–3 × 10⁸ cpm/μg of DNA. The detailed procedures involved in the above steps have been described by Yoshikawa *et al.* (12). A nick-translation kit from New England Nuclear was used for ³²P-labeling.

Blot preparation. Total RNA (20 μg) was denatured in 50% (vol/vol) formamide and 6% (vol/vol) formaldehyde at 55°C for 15 min and electrophoresed in a 1.0% agarose gel containing 6% formaldehyde and 20 mM sodium phosphate buffer (pH 7.0) (13). After electrophoresis, the gel was soaked in 10 mM sodium phosphate buffer (pH 7.0), and the RNA was transferred to a GeneScreen membrane (New England Nuclear) by the capillary-blot procedure and left overnight. The membrane was washed twice with 10× NaCl/Cit (1× NaCl/Cit = 0.15 M NaCl/0.015 M sodium citrate, pH 7) for 15 min, air-dried at room temperature, and baked at 80°C for 2 hr.

Dot-blot preparation. Total RNA (5–10 μg) was denatured in 6% formaldehyde containing 6× NaCl/Cit for 15 min at 58°C and serially diluted with 3.7% formaldehyde in 8× NaCl/Cit. Denatured RNA was filtered under slight vacuum through a GeneScreen membrane held in a Hybri-Dot manifold (Bethesda Research Laboratories). The membrane was washed with 10× NaCl/Cit for 15 min twice, air-dried at room temperature, and baked at 80°C for 2 hr.

Blot hybridization. Each membrane was incubated for at least 2 hr at 42°C with 10 ml of prehybridizing solution [50% formamide/0.2% polyvinyl pyrrolidone/0.2% bovine serum albumin/0.2% Ficoll/0.05 M Tris·HCl buffer, pH 7.5/1.0 M

NaCl/0.1% sodium pyrophosphate/1% NaDodSO₄/10% (wt/vol) dextran sulfate/100 μg of denatured herring sperm DNA per ml]. A 941-base-pair nick-translated cDNA was added to the prehybridization solution (≈10⁵–10⁶ cpm/ml) and incubated for 18–24 hr at 42°C. The membrane was washed twice with 100 ml of 2× NaCl/Cit containing 0.1% NaDodSO₄ for 15–20 min at 42°C and then washed once with 100 ml of 0.1× NaCl/Cit containing 0.1% NaDodSO₄ for 20 min at 52°C. The GeneScreen membranes were exposed to Kodak XAR-5 film at −70°C in the presence of a DuPont Lightning Plus intensifying screen. The density of each dot was measured by scanning densitometry (Gilford multimedia densitometer with a Hewlett–Packard 3390 A integrator).

Statistics. One-way analysis of variance was used to test for overall statistical significance. If a significant overall effect of treatment was observed after one-way analysis of variance, *post hoc* comparisons between group means were made by using Fischer's least significant difference test (14). A significance of $P < 0.05$ was required for rejection of the null hypothesis.

RESULTS

Blot Hybridization of Rat Adrenal Medullary Total RNA with a ³²P-Labeled cDNA Probe Cloned from Rat Brain. The effect of insulin-induced hypoglycemia on the proenkephalin A mRNA abundance in the rat adrenal medulla, determined by the blot-hybridization method, is shown in Fig. 1. The ³²P-labeled cDNA probe coding for proenkephalin A hybridized with a single mRNA species of ≈1450 bases, in accordance with previous determinations for rat brain proenkephalin A mRNA (15). The intensity of the band from rats subjected to 2 hr of insulin hypoglycemia followed by 24 hr
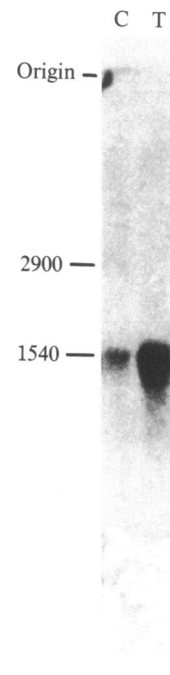

FIG. 1. Effect of 2 hr of insulin hypoglycemia on the level of proenkephalin A mRNA in the rat adrenal medulla. Rats were injected s.c. with insulin (10 units per kg of body weight). Two hours later, insulin shock was stopped by oral administration of 1.5 ml of 40% sucrose, and rats were killed 24 hr thereafter. Total RNA (20 μg) from control (lane C) and treated rats (lane T) was electrophoresed in a 1.3% agarose gel and transferred to a GeneScreen membrane. The membrane was then hybridized with ³²P-labeled cDNA probes coding for proenkephalin A derived from rat brain. Sizes are shown in base pairs.
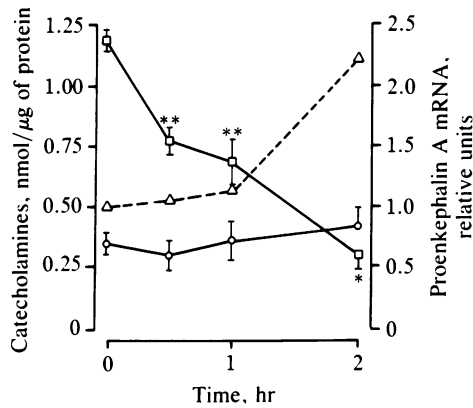
FIG. 2. Short-term effects of insulin hypoglycemia on the levels of proenkephalin A mRNA (△) and catecholamines [norepinephrine (○) and epinephrine (□)] in the rat adrenal medulla. Rats were injected s.c. with insulin (10 units per kg of body weight) and were sacrificed 30, 60, and 120 min later ($n = 8$; *, $P < 0.05$; **, $P < 0.005$). Data for mRNA were not subjected to statistical analysis. Each value represents the proenkephalin A mRNA from a pool of eight rats. The experiment was replicated once.

of recovery is much higher than that of control rats. Because the proenkephalin cDNA probes hybridize with a single species of mRNA, dot-blot hybridization was used for quantitating the relative abundance of proenkephalin A mRNA in further studies.

Acute Effects of Insulin Hypoglycemia on Proenkephalin A mRNA and Catecholamine Levels in the Rat Adrenal Medulla. Insulin hypoglycemia produced reflex splanchnic nerve stimulation and secretion from the epinephrine-containing cells of the rat adrenal medulla, which was apparent after 30 min and continued during 2 hr of hypoglycemia (see Fig. 2). The abundance of proenkephalin A mRNA remained unchanged

at the 30- and 60-min time points but was increased 2-fold 2 hr after insulin administration (Fig. 2).

Long-Term Effects of Insulin Hypoglycemia on Proenkephalin A mRNA, Native and Cryptic [Met⁵]Enkephalin-like IR, and Catecholamine Levels. To study the long-term changes in proenkephalin A mRNA, [Met⁵]enkephalin-like IR, and catecholamine metabolism after splanchnic nerve stimulation, rats were sacrificed at various times after the 2-hr period of insulin hypoglycemia. As previously described, there was a decrease in epinephrine levels in the adrenal medullae of insulin-treated rats (Fig. 3). The maximal decrease (75%) was seen 3 hr after the termination of insulin shock. Epinephrine levels had recovered to control levels after 4 days. Insulin treatment caused a rapid increase in the abundance of proenkephalin A mRNA, with a 3-fold increase apparent immediately after the 2 hr of hypoglycemia (Figs. 3 and 4). The proenkephalin A mRNA levels continued to increase, reaching a maximal level (15-fold increase) 24 hr after the period of insulin hypoglycemia. The level then gradually declined, with an approximate half-life of 4 days (Figs. 3 and 4). This increase in the abundance of proenkephalin A mRNA was followed by an increase in the levels of both cryptic and native [Met⁵]enkephalin-like IR, which was maximal 4 days after the insulin hypoglycemia (cryptic, 50-fold; native, 130-fold). The levels of both native and cryptic [Met⁵]enkephalin-like IR remained significantly higher than those of the control group 7 days after the period of insulin-induced hypoglycemia (Fig. 3).

Effects of Cholinergic Blocking Agents on Insulin-Induced Increase in the Levels of Proenkephalin A mRNA in the Rat Adrenal Medulla. To test further the hypothesis that the insulin-induced changes in levels of proenkephalin A mRNA are a consequence of splanchnic nerve activation, cholinergic blocking agents were used. Rats were administered i.p. chlorisondamine (5 mg/kg of body weight) and atropine (1 mg/kg) 15 min prior to insulin treatment (10 μ/kg). Rats received another injection of the same doses of chlorison-
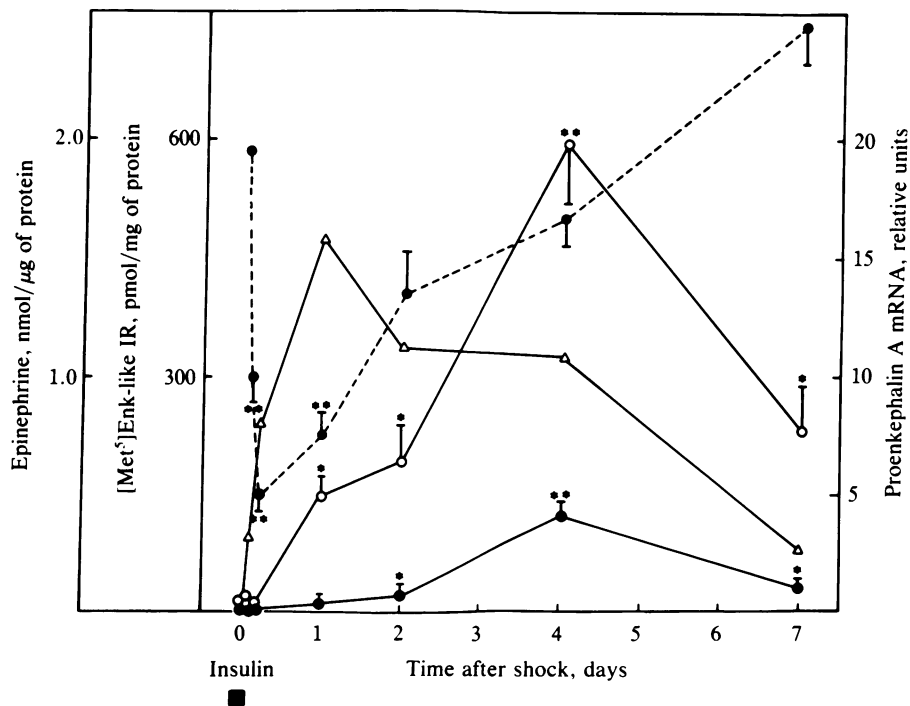

FIG. 3. Long-term effects of insulin hypoglycemia on the levels of [Met⁵]enkephalin ([Met⁵]Enk)-like IR [native (●—●) and cryptic (○—○)], proenkephalin A mRNA (△—△), and epinephrine (●---●) in the rat adrenal medulla. Rats were injected s.c. with insulin (10 units per kg of body weight). Two hours later, insulin shock was stopped by oral administration of 1.5 ml of 40% sucrose, and rats were killed at various times thereafter ($n = 8$; *, $P < 0.02$; **, $P < 0.005$). Data for mRNA were not subjected to statistical analysis. Each value represents the proenkephalin A mRNA from a pool of eight rats. The experiment was replicated once.
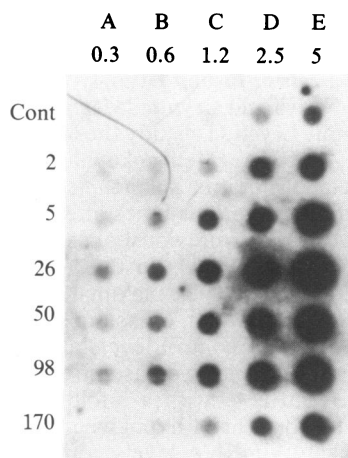
FIG. 4. Effect of insulin hypoglycemia on adrenal medullary proenkephalin A abundance as determined by dot-blot hybridization. Total RNA from control [Cont (uninjected)] and treated rats was hybridized with ^{32}P-labeled cDNA probes coding for proenkephalin A derived from rat brain. The amount of total RNA used for each dot was 0.3 μg in lane A, 0.6 μg in lane B, 1.2 μg in lane C, 2.5 μg in lane D, and 5 μg in lane E. The time period in hours after insulin treatment identifies each row.
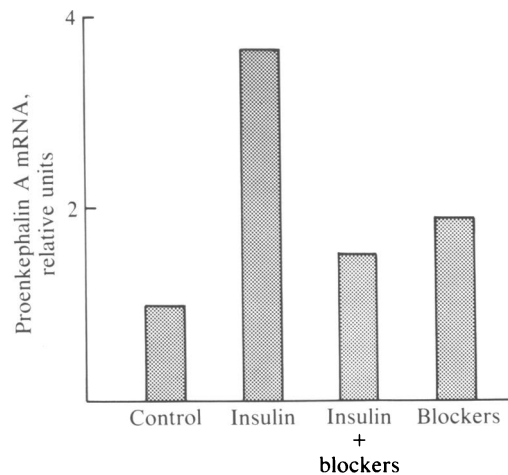

FIG. 5. Effects of cholinergic blocking agents on levels of proenkephalin A mRNA in the rat adrenal medulla following insulin hypoglycemia. Rats were administered i.p. chlorisondamine (5 mg/kg of body weight) and atropine (1 mg/kg) 15 min prior to insulin treatment. Rats received another injection of the same dose of chlorisondamine and atropine 45 min after insulin treatment, at which time the rats were recovered from hypoglycemic shock by administering 20% glucose (1.0 ml, i.p.) and 40% sucrose (1.5 ml, oral). Rats were sacrificed 2.25 hr after insulin injection. The measurement of proenkephalin A mRNA was replicated once.

damine and atropine 45 min after insulin treatment to ensure that the cholinergic blockade was maintained. Probably because of the inhibition of epinephrine secretion by the cholinergic antagonists, the animals receiving both insulin and the blockers were markedly more sensitive to the hypoglycemia, as evidenced by an earlier onset of convulsions compared to animals receiving only insulin. Consequently, for this part of the study, animals were subjected to hypoglycemia for 45 min, after which they were rapidly recovered by i.p. administration of 20% glucose (1 ml) and an oral dose of 40% sucrose (1.5 ml). Animals were then sacrificed 1.5 hr after termination of the hypoglycemia. Insulin hypoglycemia again produced a 3-fold increase in the abundance of proenkephalin A mRNA, which was blocked by the cholinergic antagonists (Fig. 5).

DISCUSSION

Combined determination of native and cryptic [Met^5]enkephalin-like IR and abundance of proenkephalin A mRNA levels has been used successfully as an index of enkephalin biosynthesis in the adrenal medulla (16) and brain tissue (17, 18). In this study, such measurements have been used to examine the effects of reflex activation of the splanchnic nerve, induced by insulin treatment, on the biosynthesis of proenkephalin A in the rat adrenal medulla. This treatment produced a rapid increase in levels of proenkephalin A mRNA, which was followed by a large increase in the levels of cryptic and native [Met^5]enkephalin-like IR. Moreover, the increase of cryptic [Met^5]enkephalin-like IR preceded the increase of the native IR. This temporal relationship suggests that reflex activation of the splanchnic nerve increases the biosynthesis of proenkephalin A. This conclusion is consistent with the previous findings that cycloheximide prevents the increase in OP levels following insulin hypoglycemia (4, 5) and that cycloheximide and actinomycin D markedly reduce the increase in OP seen in chromaffin cell cultures after catecholamine depletion (19). If the half-life of proenkephalin A mRNA in control rats is also 4 days, as observed after increased neurogenic stimulation (see *Results*), complete inhibition of proenkephalin A mRNA degradation after splanchnic activation would result in only a doubling of its levels in a 24 hr period. Though the stability of proenkephalin

A mRNA was not directly measured in these studies, the 15-fold increase in its levels in the 24 hr after the 2 hr of neurogenic stimulation strongly supports increase in gene transcription as the main mechanism for the increase in proenkephalin A mRNA levels. Consistent with this idea, we have observed that immobilization of rats for 2 hr, which also increases the rate of splanchnic nerve discharge (20), caused a 2-fold increase in proenkephalin A mRNA in the adrenal medulla (unpublished observations).

This evidence that increased splanchnic nerve stimulation produces a rapid and large increase in proenkephalin A biosynthesis, thus enhancing the rate of recovery of OP to levels higher than those prior to stimulation, is interesting in the light of previous reports that have concluded that the splanchnic innervation tonically represses proenkephalin A expression in the rat adrenal medulla (3, 21). Unilateral denervation of the rat adrenal gland has been reported to produce increases in the levels of enkephalin-related peptides and proenkephalin A mRNA (3, 22). Similarly, chronic treatment of rats with chlorisondamine to block the cholinergic splanchnic innervation of the adrenal medulla led to an increase in [Leu^5]enkephalin-like IR in this tissue (22). Furthermore, when rat adrenal medullae were explanted to tissue culture, there was an increase in levels of [Leu^5]enkephalin-like IR as well as proenkephalin A mRNA with time in culture (21). These increases were blocked by depolarizing conditions, and this effect could be antagonized by tetrodotoxin (22). Again this was taken as evidence that the splanchnic innervation tonically inhibits proenkephalin A expression and biosynthesis in the rat adrenal medulla. Such regulation would seem to be peculiar to the rat as primary cultures of bovine adrenal medulla chromaffin cells show stable proenkephalin A mRNA and OP levels with time (23, 24).

The present study demonstrates the marked sensitivity of the rat adrenal medulla proenkephalin A system to relatively short periods of splanchnic nerve stimulation and cholinergic receptor activation. Denervation studies must be interpreted cautiously because of the possibility of either surgical stress activating this system prior to denervation (5) or release of acetylcholine from degenerating nerve terminals after

transection of the splanchnic nerve. Such a release of acetylcholine from degenerating nerve terminals may also occur in explanted adrenal medullae. Rat adrenal medullae explanted to tissue culture show marked changes in protein and catecholamine as well as OP content. The observed increases in proenkephalin A mRNA and enkephalin-related peptides in rat adrenal medulla explants may be a consequence of depleted catecholamine stores, as reported in primary cultures of bovine adrenal medullae chromaffin cells (19, 24). A recent report by Siegel *et al.* (25), which indicated that increased potassium stimulates enkephalin biosynthesis in bovine chromaffin cells, is in accord with our findings.

In summary, the present study clearly demonstrates that physiological stimulation of the rat adrenal medulla by reflex splanchnic nerve activation not only produces secretion of chromaffin vesicle [Met^5]enkephalin-like IR but also stimulates proenkephalin A biosynthesis, thus facilitating a rapid and long-lasting recovery of [Met^5]enkephalin-like IR to levels in excess of prestimulation values. Studies on chromaffin cells in culture indicate that the long-lasting changes in the ratio of OP/catecholamine induced by catecholamine-depleting agents result in a proportional increase in the amount of OP secreted and a decrease in catecholamine secretion when these cultures are exposed to secretagogues (26). It has been suggested that the elevation of OP content and increase in the OP/catecholamine ratio found in the adrenal medulla of rats treated with reserpine also results in an increase in OP secretion *in vivo* and consequently in an increased opioid- and adrenal medulla-dependent analgesia induced by inescapable foot-shock (27, 28). The dynamic changes in the content and ratio of cotransmitters observed in the adrenal medulla after neurogenic stimulation or catecholamine-depleting agents may be the expression of a more general phenomena of chemical synaptic plasticity characteristic of neurons that store and secrete more than one neurotransmitter.

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