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Developmental Regulation of Leucine-Enkephalin Expression in Adrenal Chromaffin Cells by Glucocorticoids and Innervation

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Most catecholaminergic cells derived from the sympathoadrenal lineage of the neural crest contain one or more neuropeptides. Although a great deal is known about the development and regulation of catecholaminergic properties in these cells, relatively little is known about the developmental control of their neuropeptidergic properties. We have investigated the possible role of glucocorticoids and preganglionic innervation in the regulation of leucine-enkephalin (L-Enk) expression in cultures of embryonic and neonatal adrenal chromaffin cells and in mature chromaffin cells *in vivo*. Exposure of embryonic and neonatal chromaffin cells to the synthetic glucocorticoid dexamethasone increases L-Enk content. Neonatal chromaffin cells grown in medium containing elevated levels of potassium to mimic depolarization also exhibited increased L-Enk levels. The depolarization-induced increase in L-Enk was selectively inhibited by treatment with the enkephalin analog [D-Ala, d-Leu]-enkephalin to mimic the enkephalinergic component of the preganglionic innervation. Denervation of the adrenal gland *in vivo* resulted in a dramatic increase in L-enk expression that could be partially mimicked by selectively blocking enkephalinergic transmission with administration of the opiate receptor antagonist naloxone. Taken together with the developmental time course and pattern of L-Enk expression *in vivo*, our results suggest that glucocorticoids and the preganglionic innervation regulate the developmental expression of this peptide in adrenal chromaffin cells and therefore participate in the generation of the mature neurochemical phenotypes present in the adrenal medulla. Further, in adult chromaffin cells similar factors appear to regulate the expression of L-Enk, which could in turn participate in physiological responses to stress.

Catecholaminergic derivatives of the sympathoadrenal lineage of the neural crest, which include sympathetic neurons, small intensely fluorescent cells, and adrenal chromaffin cells, contain neuropeptides as well as small molecule neurotransmitters. For example, subpopulations of sympathetic neurons and adrenal chromaffin cells contain enkephalin (Schultzberg et al., 1978, 1979; Bohn et al., 1983; Henion and Landis, 1990) and neuropeptide Y (NPY) (Lundberg et al., 1982; deQuidt and Emson, 1986; Schalling et al., 1988a; Henion and Landis, 1990). In some cases, an important functional role has been established for the colocalized neuropeptide. For example, NPY released from sympathetic nerve terminals causes a long-lasting vasoconstriction in target vascular smooth muscle (Lundberg and Tatemoto, 1982; Lundberg et al., 1982, 1984; Franco-Cereceda et al., 1985). Thus, during development, the establishment of the mature neurochemical phenotypes of sympathoadrenal derivatives involves the acquisition and regulation of not only a classical transmitter but also one or more neuropeptides.

The appearance of catecholaminergic properties and the mechanisms that regulate their expression in sympathoadrenal derivatives have been studied extensively. The acquisition of noradrenergic traits by neural crest–derived sympathoadrenal precursors occurs early in development when neural crest cells begin to aggregate to form the primary sympathetic chain (DeChamplain et al., 1970; Cochard et al., 1979; Teitelman et al., 1979; Rothman et al., 1980). The migration route and/or the local ganglionic environment appear to be important for the induction of tyrosine hydroxylase (TH) and catecholamine synthesis (Cohen, 1972; Norr, 1973; LeDouarin and Teillet, 1974; Teillet et al., 1978; Howard and Bronner-Fraser, 1985, 1986). Subsequent to the initial expression of TH, NGF (Thoenen et al., 1971; Otten et al., 1977; Max et al., 1978), cell contact (Acheson and Thoenen, 1983), and impulse activity (Meuller et al., 1969a,b; Joh et al., 1973; Zigmond, 1985) influence levels of the enzyme. In the adrenal medullary system, a subpopulation of TH-immunoreactive (IR) cells undergoes a secondary migration from the sympathetic chain to the adrenal anlage. After their arrival in the adrenal gland, a subset of TH-IR precursor cells express phenylethanolamine *N*-methyltransferase (PNMT; Bohn et al., 1981; Henion and Landis, 1990), the enzyme that catalyzes the conversion of norepinephrine to epinephrine. In late embryonic, neonatal, and adult chromaffin cells, PNMT expression is regulated by glucocorticoids (Wurtman and Axelrod, 1966; Bohn et al., 1981; Teitelman et al., 1982; Grothe et al., 1985; Ehrlich et al., 1989), and glucocorticoids appear to be necessary for the induction of PNMT expression by chromaffin cells (Seidl and Unsicker, 1989). Thus, the induction and subsequent regulation of catecholaminergic properties in sympathoadrenal derivatives are mediated by environmental influences.

While a great deal is known about the regulation of catecholaminergic phenotypes during the development of sympathoadrenal derivatives, comparatively little is known about the factors that influence neuropeptide expression. The control of neuropeptide expression, however, has been studied in mature sympathoadrenal derivatives, particularly in adrenal chromaffin cells. Subpopulations of rat and bovine adrenal chromaffin cells express leucine-enkephalin (L-Enk) and NPY (Schultzberg et

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al., 1978; Bohn et al., 1983; deQuidt and Emson, 1986; Bastiaensen et al., 1988; Schalling et al., 1988a,b; Henion and Landis, 1990). Attention has focused on the effects of glucocorticoids and impulse activity on L-Enk levels in postnatal bovine and rat adrenal chromaffin cells both *in vitro* and *in vivo*. For example, the synthetic glucocorticoid dexamethasone increases L-Enk levels in adult chromaffin cells (Naranjo et al., 1986; LaGamma and Adler, 1987; Stachowiak et al., 1988). Surgical denervation of the adrenal gland results in a dramatic increase in the proportion of immunocytochemically detectable L-Enk-IR cells (Schultzberg et al., 1978; Lewis et al., 1981; Bohn et al., 1983) and the total amount of L-Enk per gland (LaGamma et al., 1984), suggesting that depolarization of chromaffin cells inhibits L-Enk expression. Paradoxically, reflex splanchnic nerve stimulation caused by insulin-induced hypoglycemia, administration of the amine-depleting drug reserpine, and cold stress causes an increase in L-Enk peptides and proenkephalin A mRNA *in vivo* (Bohn et al., 1983; Kanamatsu et al., 1986; Sietzen et al., 1987; DeCristofaro and La Gamma, 1991). Further, reserpine and depolarization increase L-Enk expression in cultured chromaffin cells (Wilson et al., 1981; Siegel et al., 1985; see, however, LaGamma et al., 1984). Taken together, these results suggest that L-Enk levels in adult adrenal chromaffin cells are regulated, at least to some extent, by the local hormonal environment and preganglionic innervation. The developmental mechanisms, however, that determine which neuropeptides are expressed and the proportion of cells within a population that express a particular neuropeptide are not well understood.

In previous studies of the development of L-Enk expression in rat adrenal chromaffin cells (Henion and Landis, 1990), we found that the proportion of cells that expressed immunocytochemically detectable peptide varied dramatically during development. L-Enk-IR first appeared in TH-IR cells at embryonic day (E) 16, and the proportion that expressed L-Enk increased in a striking fashion between E16 and E20. Subsequently, the proportions of L-Enk–containing cells decreased dramatically within the first postnatal week. The appearance and increase in L-Enk-IR cells are correlated temporally with the onset and subsequent increase in glucocorticoid synthesis by adrenal cortical cells (Seidl and Unsicker, 1989; Michelson and Anderson, 1992), while their decrease is correlated with the initial appearance of choline acetyltransferase (ChAT) and L-Enk-IR in the preganglionic innervation (Henion and Landis, 1990) and the onset of responsiveness of chromaffin cells to preganglionic stimulation (Lau et al., 1987).

The correspondence between the time and pattern of L-Enk expression during development and the factors that affect L-Enk expression in mature chromaffin cells raised the possibility that glucocorticoids and impulse activity regulate the expression of L-Enk in rat adrenal chromaffin cells during development. To address this question, we established cultures highly enriched for embryonic and postnatal chromaffin cells and assayed the effects of glucocorticoids and factors that mimic influences of the preganglionic innervation of chromaffin cells *in situ* on neuropeptide expression. Exposure to glucocorticoids increased the expression of L-Enk in cultures of E15 and neonatal chromaffin cells. We have also found that neonatal chromaffin cells express significantly more L-Enk when grown under depolarizing conditions. This depolarization-induced increase in L-Enk levels can be completely blocked by addition of the enkephalin agonist [D-Ala, D-Leu]-enkephalin. Paradoxically, in adult rats, denervation of the adrenal gland resulted in a marked increase in adrenal L-Enk levels. Administration of the opiate receptor antagonist naloxone, however, partially mimicked the effects of denervation both qualitatively and quantitatively. Our results suggest that glucocorticoids and two distinct aspects of the preganglionic innervation regulate the determination of the neuropeptidergic phenotypes of rat adrenal chromaffin cells and that levels of L-Enk expression in adult chromaffin cells are dynamically regulated by the preganglionic innervation.

Materials and Methods

Animals. Timed pregnant, newborn, and operated and control adult rats were obtained from Zivic-Miller (Zelienople, PA). Embryos were considered to be embryonic day (E) 0.5 the morning after a midnight breeding. Additionally, at the time of death, some embryos were staged by examination of external features according to Christie (1962). Newborn rat pups were used within 12 hr after birth. Pregnant mothers were killed by ether inhalation, and the entire uterus was removed and placed in a Petri dish containing phosphate-buffered saline (PBS). Embryos were removed and adrenals were dissected quickly and placed in ice-cold Hank's balanced salt solution (HBSS; GIBCO). Newborn rat pups were killed by decapitation, and the adrenal glands were dissected and placed in ice-cold HBSS. For perfusion fixation, adult rats were killed by ether inhalation and perfused through the heart with 4% paraformaldehyde in 0.1 M phosphate buffer for 10 min. Adrenal glands were dissected and postfixed for 50 min, infiltrated with 30% sucrose, and frozen onto cryostat chucks with OCT compound (Miles Inc., Elkhart, IN). Seven micrometer cryostat sections were cut and mounted on gelatin-coated slides and processed for immunocytochemistry.

In vivo *drug experiments.* The effects of the opiate receptor antagonist naloxone (Sigma) on L-Enk expression in chromaffin cells *in vivo* was examined using an osmotic pump delivery system. Rats weighing 150 gm were used in all experiments. Osmotic pumps (1 μl/hr, 7 d; ALZA Corp., Palo Alto, CA) were loaded with 410 mM (150 mg/ml) naloxone in 0.9% NaCl (200 μl/pump). Rats were anesthetized with chloral hydrate, and two pumps were implanted subcutaneously in the region above the forelimbs. The dose of naloxone delivered by the two pumps was in the range shown to elicit classical opiate-abstinence behavioral effects in rats (Malin et al., 1986). Animals were killed 4 d later by ether inhalation; either they were perfused with 4% paraformaldehyde for immunocytochemical analysis or adrenal glands were quickly dissected and processed for radioimmunoassay (RIA) determination of L-Enk content.

Cell culture. Adrenal glands from two litters of E15 embryos or one litter of postnatal day (P) 0 rat pups were used for each experiment. PO adrenals were decapsulated with watchmaker's forceps before dissociation. Adrenal glands were enzymatically dissociated in Dispase (5 mg/ml) and collagenase (1 mg/ml) followed by gentle trituration with a fire-polished glass pipette. Dissociated cells were washed in serum-containing medium, centrifuged, and resuspended in 1 ml of medium. The cell suspension was carefully layered on top of a self-generated, discontinuous Percoll (Pharmacia LKB, Uppsala, Sweden) density gradient and centrifuged at 400 × *g* for 15 min at room temperature. Fractions corresponding to a buoyant density range of 1.075–1.06 gm/ml were collected and washed with a sixfold excess of HBSS. After centrifugation, the cells were resuspended in an appropriate volume of medium and plated in 96-well tissue culture plates that had been coated sequentially with poly-L-lysine and laminin at a density of 3000–4000 cells per well. Cell counts were made at the beginning and end of each experiment using a grid and at least three randomly selected fields. In addition, a fraction of the cells was plated onto laminin-coated glass coverslips in 24-well plates to determine the purity of each set of cultures at various times after plating.

Chromaffin cell cultures were grown in several different experimental media. The basal growth medium for all experiments consisted of Liebovitz's L15-CO_2 medium supplemented with 5% serum from rats that had been adrenalectomized 5 d previously. Other additives used in different experiments included 20 mM KCl, 20 mM NaCl, 5 μM dexamethasone (Sigma), 10 nM [D-Ala, D-Leu]-enkephalin (Peninsula) and 1 μM naloxone (Sigma).

The purity of chromaffin cell cultures was determined by staining coverslips with a monoclonal antibody that recognized tyrosine hydroxylase (TH; kind gift of A. Acheson, University of Alberta, Edmon-

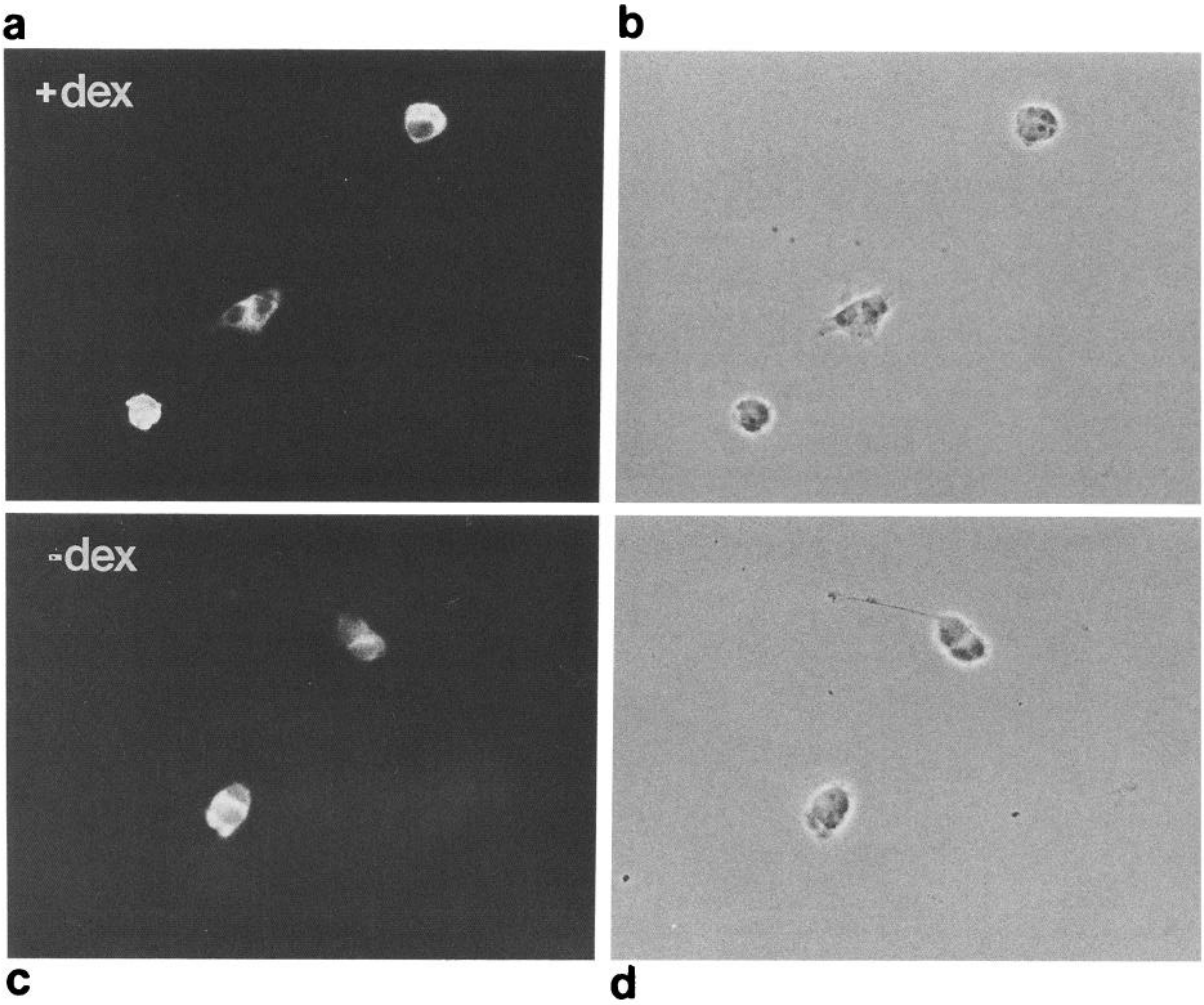

Figure 1. Embryonic adrenal cultures contain primary TH-IR cells that exhibit different morphologies depending upon culture conditions. Cultures of E15 adrenals were grown in the presence (*a, b*) or absence (*c, d*) of dexamethasone for 48 hr and are shown by TH immunofluorescence (*a, c*) and phase (*b, d*). Approximately 90% of cells plated were TH-IR after 12 hr *in vitro*. After an additional 48 hr, cultures containing dexamethasone comprised 85% TH-IR cells while those without hormone contained 75% TH-IR cells. TH-IR cells grown in the presence of dexamethasone were either round or flat and generally lacked processes (*b*). Many TH-IR cells grown in the absence of dexamethasone had short, unbranched processes (*d*). Scale bar, 50 μm.

ton, Alberta, Canada). Briefly, coverslips were removed from 24 well plates and rinsed three times in HBSS that had been warmed to 37°C. Cells were fixed with 85 μl of a paraformaldehyde solution (4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4) added to the surface of each coverslip for 15–30 min at room temperature (RT) in a humid chamber. The coverslips were then rinsed in PBS and incubated for 1–2 hr at RT with antisera diluted in a buffer containing 2% BSA, 0.5 M NaCl, 0.01 M phosphate buffer, pH 7.3, 0.1% sodium azide, and 0.3% Triton X-100. The coverslips were then rinsed in PBS and incubated for 1–2 hr at RT with antisera diluted in a buffer containing 2% BSA, 0.5 M NaCl, 0.01 M phosphate buffer, pH 7.3, 0.1% sodium azide, and 0.3% Triton X-100. The coverslips were then rinsed in PBS and incubated with species-appropriate secondary antisera diluted in the same buffer used for dilution of primary antisera for 30 min to 1 hr at RT. Coverslips were then rinsed in PBS and mounted on slides with glycerol: PBS (1:1). Mounted coverslips were examined with a Zeiss microscope equipped with epifluorescence and rhodamine and fluorescein filter sets. After 12–24 hr *in vitro*, 90% of the cells in E15 cultures and 95% in P0 cultures were TH-IR. Two days later, 75–85% of the cells in E15 cultures and 95% in P0 cultures were TH-IR. The majority of contaminating cells in E15 and P0 cultures were fibroblasts as determined by morphology and immunoreactivity for an antiserum that recognized rat fibronectin (Telios Pharmaceuticals, San Diego, CA). Polyclonal antisera that recognized phenylethanolamine *N*-methyltransferase (Eugene Tech, Allendale, NJ), neuropeptide Y (Henion and Landis, 1990), L-Enk (Incstar, Stillwater, MN), and SCG10 (kind gift of D. J. Anderson, California Institute of Technology, Pasadena, CA) were also used in some experiments to characterize the cultured cells. Species specific secondary antisera (see Henion and Landis, 1990) used included tetramethylrhodamine isothiocyanate–conjugated goat anti-rabbit (Tago, Burlingame, CA), 7-amino-4-methylcoumarin-3-acetic acid–conjugated goat anti-rabbit (Jackson ImmunoResearch, West Grove, PA), Texas red–conjugated goat anti-guinea pig (Jackson ImmunoResearch), and fluorescein isothiocyanate–conjugated goat anti-mouse (Antibodies Inc., Davis, CA).

The immunocytochemistry protocol used for tissue sections has been described in detail previously (Henion and Landis, 1990). Briefly, sections were rinsed in PBS and incubated overnight with primary antisera at RT in a humid chamber. Sections were then rinsed in PBS and incubated with appropriate secondary antisera for 2 hr at RT in a humid chamber. The sections were rinsed in PBS, coverslipped with glycerol: PBS (1:1), and examined.

Radioimmunoassay. Culture wells were rinsed once with PBS and homogenized in 100 μl of 2 M acetic acid. Adult adrenal glands were dissected free of the surrounding cortical layers, minced with a double-edge razor blade, and homogenized in 200 μl of 2 M acetic acid. Samples were then boiled for 5 min in a water bath and centrifuged for 5 min in an Eppendorf microfuge. The supernatants were dried under vacuum and stored at −80°C for subsequent assays. L-Enk was assayed using kits obtained from Peninsula and Incstar, both of which contained a primary antiserum previously demonstrated to show minimal cross-reactivity with other opioid peptides. The primary antiserum of the

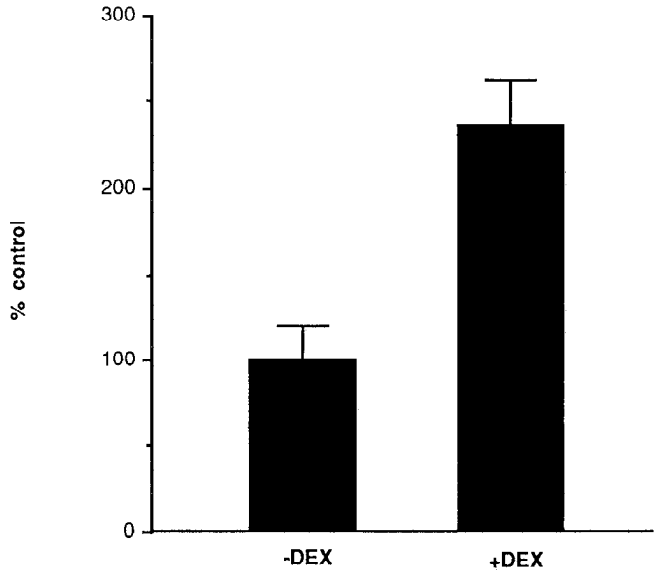

Figure 2. Exposure of enriched embryonic chromaffin cell cultures to dexamethasone stimulates L-Enk expression. Cultures of E15 chromaffin cells were grown in the presence (*+DEX*) or absence (*−DEX*) of 5 μM dexamethasone for 2 d, and L-Enk levels were measured by RIA. Exposure to dexamethasone resulted in a 2.5-fold increase in L-Enk levels compared to cultures grown in the absence of dexamethasone. Error bars are ±SEM.

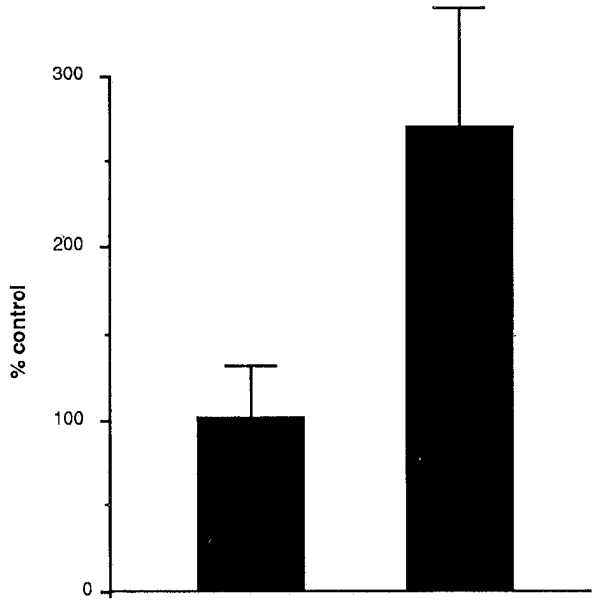

Figure 3. Elevated L-Enk levels are present in enriched cultures of neonatal chromaffin cells grown in the presence of dexamethasone. Cultures of P0 chromaffin cells were grown in medium with (*left column*) and without (*right column*) 5 μM dexamethasone for 2 d. At the end of the culture period, the cells were harvested and L-Enk levels measured by RIA. L-Enk levels were 2.5-fold higher in cultures grown in medium containing dexamethasone compared with cultures not exposed to dexamethasone. Error bars are ±SEM.

Incstar kit, however, that was used in most of the experiments with E15 chromaffin cells was found to cross-react with a high molecular weight protein(s) present in NIH 3T3 cells, a fibroblast cell line.

Results

Glucocorticoids elevate L-Enk levels in developing chromaffin cells

The effect of glucocorticoids on the expression of L-Enk by embryonic adrenal chromaffin cells was tested by exposing enriched cultures of E15 chromaffin cell precursors to dexamethasone. Cultures highly enriched for chromaffin cell precursors and devoid, to the greatest extent possible, of adrenal cortical cells were generated using Percoll density gradients. This procedure consistently yielded cultures consisting of 90% TH-IR cells (Fig. 1). After the 48 hr experimental period, the total number of chromaffin precursors at the end of the experiments was the same in experimental and control cultures. Since the number of contaminating cells, which were predominantly fibroblasts, was greater in control cultures, cultures grown in the presence or absence of dexamethasone contained 85% and 75% TH-IR cells, respectively. As previously described by Anderson and colleagues (Anderson and Axel, 1986; Michelson and Anderson, 1992), many TH-IR cells in cultures grown in the absence of dexamethasone had a single short process while virtually all of the cells grown in the presence of dexamethasone lacked processes (Fig. 1).

The L-Enk levels in cultures of E15 chromaffin precursors exposed to 5 μM dexamethasone for 2 d were compared to those of control cultures grown in the absence of glucocorticoid. Dissociated cells were plated on a laminin substrate in 96-well plates and grown for 12–18 hr in medium that did not contain dexamethasone. During this 12–18 hr period, the cells attached and the medium was then changed to the experimental media. The media were changed 24 hr later and cells were harvested after an additional 24 hr so that cells were exposed to experimental media for a total of 48 hr. Chromaffin precursors exposed to dexamethasone for 2 d were found to contain greater than 2.5-fold more L-Enk than chromaffin precursors grown in its absence (Fig. 2). The Enk-IR present in the control cultures most likely represents cross-reactive material present in the contaminating fibroblasts (see Materials and Methods). Consistent with this possibility, when L-Enk content was assayed in cultures of E15 chromaffin precursors with a primary antiserum that did not recognize this component, L-Enk was undetectable in control cultures (data not shown). These observations suggest that the apparent L-Enk content of control cultures in Figure 2 represents cross-reactive material and that the difference between control and dexamethasone-treated cultures is an underestimate of L-Enk induction.

The effect of glucocorticoids on L-Enk expression was also examined in cultures of P0 chromaffin cells. Highly enriched cultures of neonatal chromaffin cells were generated to test the effects of glucocorticoids on L-Enk expression. Control and experimental cultures contained approximately 95% TH-IR cells at the beginning and end of the experiments. The total number and morphology of TH-IR cells were the same for both conditions at all times during the experiments. In addition, the proportion of cells expressing immunocytochemically detectable levels of PNMT was the same in both media (not shown). After longer periods in the absence of dexamethasone, however, the number of TH-IR cells progressively declined. Cultures of P0 chromaffin cells were grown in the presence or absence of 5 μM dexamethasone for 2 d and L-Enk levels were determined by RIA. Neonatal chromaffin cells grown in the presence of dexamethasone contained approximately 2.5-fold more L-Enk than control cultures, as was the case for embryonic chromaffin cells (Fig. 3). Thus, exposure to glucocorticoids stimulates the L-Enk expression in chromaffin precursors and chromaffin cells developing in culture.

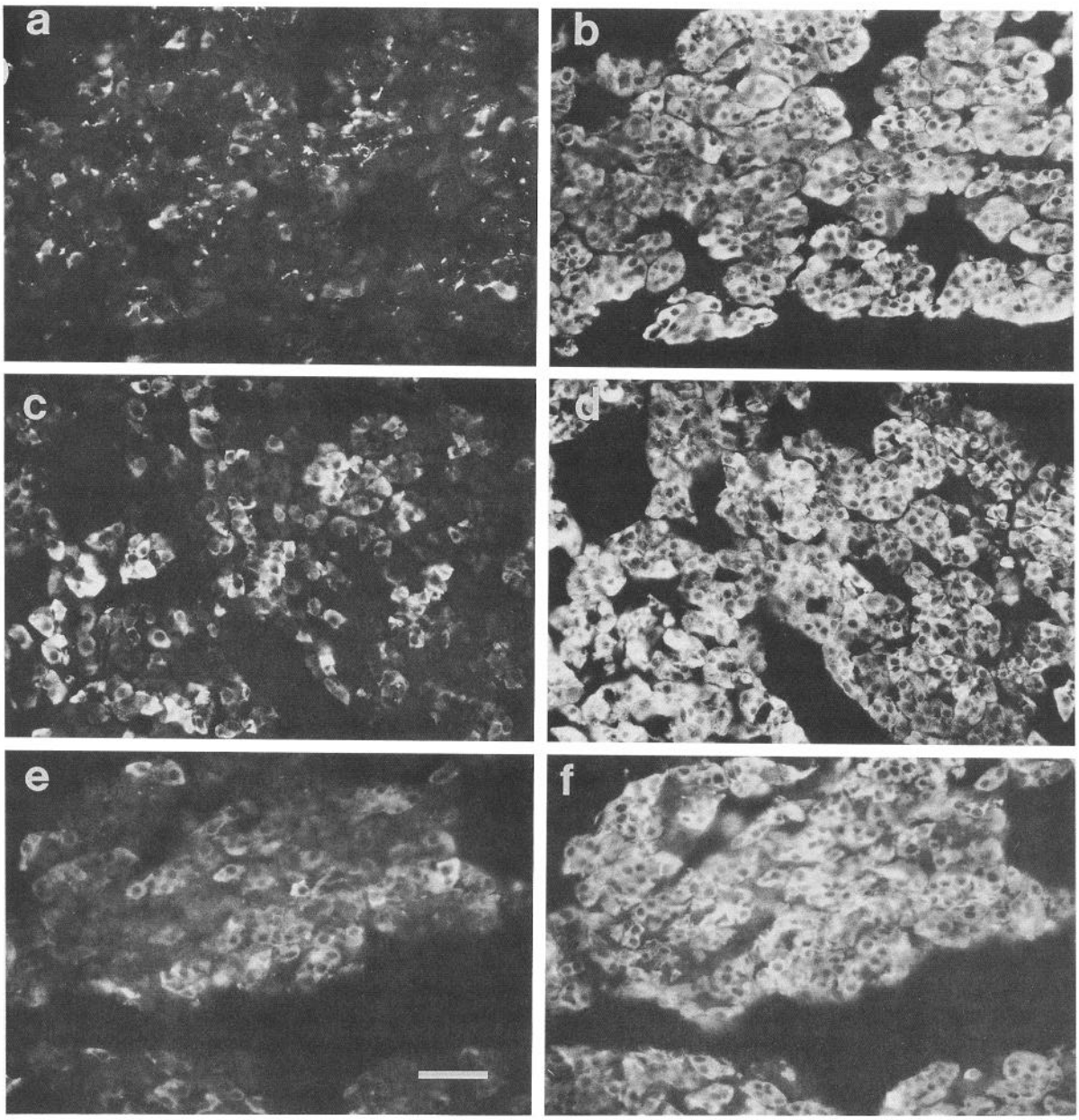

Figure 4. Surgical denervation of the adrenal gland and exposure to naloxone *in vivo* result in a dramatic increase in the number of L-Enk-IR chromaffin cells. Unilateral denervation of adrenal glands in adult rats via splanchnic nerve transection and implantation of naloxone-loaded osmotic pumps were performed 3–5 d before death. Sections of control, naloxone-treated, and denervated adrenal glands were double labeled with antisera directed against L-Enk (*a, c, e*) and TH (*b, d, f*). The number and intensity of L-Enk-IR cells were much greater in denervated adrenals (*c*) compared to contralateral controls (*a*). A significantly greater number of L-Enk-IR cells were present in adrenals from naloxone-treated animals (*e*) compared to controls (*a*). Scale bar, 40 μm.

Differential effects of preganglionic factors on L-Enk expression

The possible influence of the preganglionic innervation on L-Enk expression in adrenal chromaffin cells was initially tested *in vivo*. Since it was not possible to denervate the adrenal glands of early postnatal rats, the adrenal medulla of adult rats was surgically denervated by cutting the splanchnic nerve, which provides preganglionic input to the adrenal. Three and five days after denervation, sections of adrenal glands from perfusion-fixed rats were examined with an antiserum directed against L-Enk. L-Enk-IR fibers that are normally associated with L-Enk–negative chromaffin cells in unoperated, innervated adrenal glands were absent from glands following denervation. The denervated adrenal medullae exhibited a dramatic increase in the number and intensity of L-Enk-IR chromaffin cells when compared with the contralateral unoperated adrenal gland and with the adrenal glands of sham-operated control rats (Fig. 4). Further, denervated adrenal glands contained over fivefold more L-Enk than control adrenal glands as determined by RIA (Fig. 5). Sections of unoperated and denervated adrenals were stained with antisera to TH, PNMT, and NPY. In contrast to the striking changes observed in L-Enk-IR, no differences were evident in the number of immunoreactive chromaffin cells or the intensity of immunoreactivity for these three markers (data not shown).

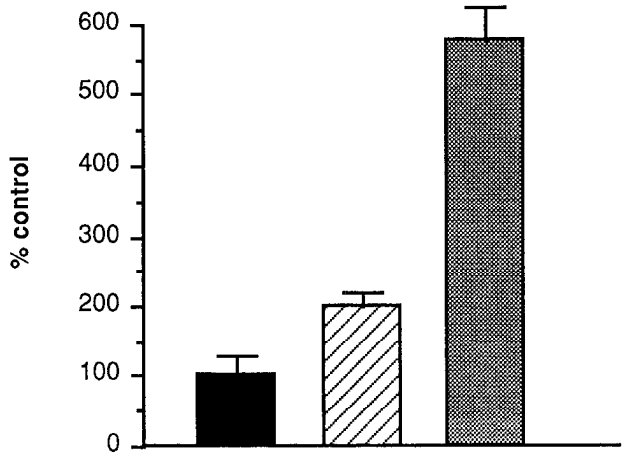

Figure 5. Denervation and naloxone treatment result in elevated adrenal L-Enk content. Surgical denervation of the adrenal gland results in increased adrenal L-Enk levels that can be partially mimicked by exposure to naloxone. L-Enk content of adrenal glands from control (*solid bar*), naloxone treated (*hatched bar*), and operated (*shaded bar*) animals (see Fig. 4 caption) was assayed by RIA. Denervation resulted in a greater than fivefold increase in L-Enk levels, while naloxone treatment resulted in a twofold elevation in adrenal L-Enk content compared to controls. Error bars are ±SEM.

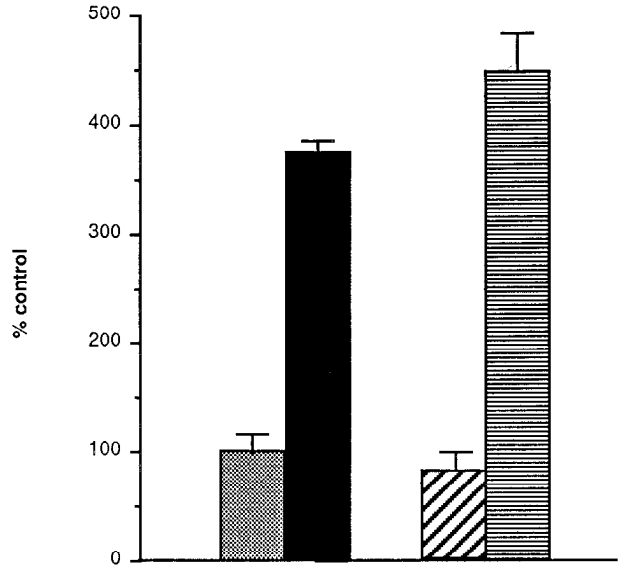

Figure 6. Factors that mimic the preganglionic innervation of chromaffin cells differentially affect L-Enk expression *in vitro*. Depolarization stimulates L-Enk expression in cultures of neonatal chromaffin cells that is completely inhibited by addition of the enkephalin analog DADLE. Enriched cultures of P0 chromaffin cells were exposed to 20 mM potassium for 2 d, and L-Enk levels, determined by RIA, were compared to cultures grown under nondepolarizing conditions. Cultures grown in high potassium medium (*solid bar*) contained fourfold higher L-Enk levels than control cultures (*shaded bar*). L-Enk levels in cultures exposed to high potassium and DADLE (*diagonally hatched bar*) were equivalent to cultures grown under nondepolarizing conditions. The inhibitory effect of DADLE was blocked by addition of the opiate receptor antagonist naloxone (*horizontally hatched bar*). Error bars are ±SEM.

To examine more directly different aspects of the preganglionic innervation on L-Enk expression, we first tested the effects of depolarizing conditions on L-Enk expression in cultures of neonatal chromaffin cells. Neonatal chromaffin cell cultures were grown in the presence of dexamethasone either with or without added potassium (20 mM). As a control for the possible osmotic effects of the added potassium, some cells were grown in medium containing dexamethasone and 20 mM NaCl. Cells were plated in control (−dex/−K) medium, and experimental media were added after 12 hr. The medium was changed twice during the 48 hr duration of the experiments. At the end of each experiment, samples were collected and L-Enk levels were determined by RIA. No differences in cell survival or morphology were observed between cells grown in the different media. In cultures exposed to depolarizing conditions for 2 d, L-Enk levels were fourfold higher than in cultures grown without elevated K and cultures with added NaCl (Fig. 6).

The increase in L-Enk levels following depolarization appeared inconsistent with the increased L-Enk expression observed after denervation since denervation is usually associated with a decrease in activity. A majority of the fibers that constitute the preganglionic innervation of the adrenal medulla not only are cholinergic but also contain L-Enk-IR. Thus, denervation would remove this peptidergic component of the innervation as well. Therefore, we tested the effects of the enkephalin agonist DADLE on the expression of L-Enk by cultured neonatal chromaffin cells. In addition, the opiate receptor antagonist naloxone was added to some cultures. Cells were plated in control medium (−dex/−K) for 12 hr, at which time the following media were added to sister cultures: (1) +dex, (2) +dex/+K, (3) +dex/+DADLE, (4) +dex/+K/+DADLE, (5) +dex/+K/+DADLE/+naloxone. The medium was changed twice during the 48 hr duration of the experiments, and L-Enk levels were quantified by RIA. Cultures grown under depolarizing conditions exhibited a fourfold increase in L-Enk levels, which was completely inhibited by addition of DADLE. The inhibitory effect of DADLE on the depolarization-induced increase in L-Enk was abolished by naloxone (Fig. 6). Exposure of control cultures to DADLE alone or addition of naloxone alone to control or depolarized cultures did not affect L-Enk levels (not shown).

The inhibitory effect of DADLE on the depolarization-induced elevation of L-Enk content in cultured chromaffin cells suggested that the increase in the number and intensity of L-Enk-IR cells observed after denervation results from the elimination of an inhibitory influence of the L-Enk normally released from preganglionic fibers. To test this possibility, we exposed adult rats to the opiate receptor antagonist naloxone released continuously from surgically implanted osmotic pumps for 4 d. We then examined L-Enk expression in adrenal glands from control and naloxone-treated animals using immunocytochemistry and RIA. Adrenal glands from naloxone-treated animals contained a significantly greater number of L-Enk-IR chromaffin cells compared to control (Fig. 4). In addition, the total L-Enk content of adrenal glands from naloxone-treated rats was over twofold greater than adrenal glands from control rats (Fig. 5). Since no change was evident in the density or intensity of the L-Enk-IR preganglionic fibers, this change appears to reflect increased L-Enk content in chromaffin cells.

Discussion

Since previous studies revealed striking changes in L-Enk levels during the development of chromaffin cells *in vivo*, we used cultures of embryonic and neonatal chromaffin cells to examine the possible role of local environmental signals on L-Enk expression. L-Enk is initially expressed on E16, and the proportion of L-Enk-IR cells increases dramatically shortly thereafter (Hen-

ion and Landis, 1990). This pattern of L-Enk expression is correlated temporally with the initial production and subsequent increase in adrenal cortical-derived glucocorticoids (Seidl and Unsicker, 1989; Michelson and Anderson, 1992). Therefore, we tested the effects of glucocorticoids on L-Enk expression in highly enriched chromaffin cell cultures. E15 precursors and P0 chromaffin cells grown for 2 d in glucocorticoid-containing medium exhibit a greater than 2.5-fold increase in L-Enk levels compared with cells grown without steroid. It is important to note that the absence of glucocorticoids did not affect the survival of TH-IR chromaffin cells during the 2 d duration of the experiments. Some TH-IR cells in E15 cultures grown without dexamethasone, however, elaborated short processes while those grown with dexamethasone did not, consistent with previous findings (Anderson and Axel, 1986; Michelson and Anderson, 1992). While the lack of dexamethasone did not affect the survival or morphological appearance of TH-IR cells in neonatal cultures during the 48 hr period, cultures maintained for longer periods (more than 4 d) in the absence of dexamethasone did show a decrease in TH-IR cells as previously described (Doupe et al., 1985; Seidl and Unsicker, 1989). These results demonstrate that glucocorticoids stimulate L-Enk expression in embryonic and neonatal chromaffin cells *in vitro* and are consistent with the hypothesis that glucocorticoid production stimulates expression of L-Enk by chromaffin cells during development *in vivo*.

While our results suggest an important role for glucocorticoids in the regulation of L-Enk in chromaffin cells during development, it is not clear whether glucocorticoids per se are required and/or sufficient for the *de novo* induction of L-Enk expression. Glucocorticoid receptor mRNA has been detected in chromaffin precursors as early as E15 (Anderson and Michelsohn, 1989), before the initial expression of L-Enk, consistent with the possibility that glucocorticoids trigger the production of L-Enk in chromaffin cells. Further, the morphological difference between control and dexamethasone-treated cultures suggests that the E15 precursor cells respond to glucocorticoids (Anderson and Axel, 1986; Michelson and Anderson, 1992; present results). Our results indicate that glucocorticoids increase L-Enk levels in cultures of embryonic chromaffin cells. Due to the cross-reactivity observed with the primary antiserum used in most RIA determinations of L-Enk levels of E15 cultures, we were not able to determine whether L-Enk was detectable in cultures grown without dexamethasone. In fact, since more non-chromaffin cells were present in the cultures grown without dexamethasone than in those grown with dexamethasone, our results underestimate the glucocorticoid-stimulated increase in L-Enk. Further, it is possible that the L-Enk measured in control cultures actually represented only cross-reactive material. Consistent with this possibility, control cultures assayed with a recently available RIA using an antiserum that is more specific but less sensitive contained undetectable levels of L-Enk, while cultures exposed to dexamethasone contained L-Enk levels approximating those obtained previously.

Several observations raise the possibility that L-Enk and PNMT are coordinately expressed in a subpopulation of chromaffin precursors during development. Both L-Enk and PNMT are initially expressed at the same time (E16), and L-Enk expression is restricted to the PNMT-IR population of chromaffin cells during development and in the adult (Henion and Landis, 1990). It has been suggested that the initial expression of PNMT is dependent upon glucocorticoids (Seidl and Unsicker, 1989) and glucocorticoids regulate PNMT levels subsequently (Wurtman and Axelrod, 1966; Bohn et al., 1981, 1982; Teitelman et al., 1982). Two observations, however, are inconsistent with the notion of coordinate regulation. First, L-Enk is detectable in only a subset of PNMT-IR cells. Further, shortly after birth there is a large decrease in the proportion of L-Enk-IR cells while the proportion of PNMT-IR cells increases slightly. Therefore, while L-Enk and PNMT levels are each influenced by glucocorticoids, it appears that these neurochemical properties can be controlled independently.

It should be noted that the methods employed to assess the effects of glucocorticoids and, as will be discussed below, factors that mimic influences of the preganglionic innervation on L-Enk expression measure steady state levels of L-Enk-IR material. Therefore, it is possible that changes in the levels of L-Enk could be the result of differential processing of precursor peptides (see, e.g., Stachowiak et al., 1988). It has been shown, however, that dexamethasone and depolarization elevate both proenkephalin mRNA and enkephalin peptide levels in cultured bovine chromaffin cells (Naranjo et al., 1986), strongly suggesting that changes in the steady state L-Enk levels that we have observed are a reflection of altered enkephalin synthesis.

The possible effects of the preganglionic innervation of the adrenal medulla on L-Enk expression were examined in adrenal glands that had been denervated via splanchnic nerve transection. Denervations were performed on adult rats because splanchnic nerve transections were not technically feasible in neonatal pups. Examination of tissue sections from denervated and control adrenals labeled with antiserum to L-Enk revealed that denervated adrenals contained a significantly greater number of L-Enk-IR cells and many chromaffin cells exhibited much more intense immunoreactivity. Further, adrenal L-Enk content quantified by RIA was also dramatically increased. These results confirm previous reports (Schultzberg et al., 1978; Bohn et al., 1983) and suggest that the L-Enk expression in mature chromaffin cells is regulated, at least in part, by influences of the preganglionic innervation. During development, ChAT-IR is initially detectable in preganglionic fibers shortly after birth, when the proportion of L-Enk-IR cells decreases dramatically (Henion and Landis, 1990). Taken together, the results of denervation and the correlation between the appearance of ChAT-IR in preganglionic fibers and the decrease in the proportion of L-Enk-IR cells suggested that the depolarizing influence of ACh released during the first postnatal week inhibits L-Enk expression in chromaffin cells and thus plays a role in establishing the adult pattern of L-Enk expression.

To assess the role of the depolarizing aspects of the innervation in the developmental regulation of L-Enk, we examined the effects of depolarization on L-Enk levels in cultures of neonatal chromaffin cells. Exposure of chromaffin cell cultures to elevated potassium for 48 hr caused a fourfold increase in L-Enk levels. Although this result appears to be inconsistent with the effects of denervation *in vivo*, which also results in a dramatic increase in the proportion of L-Enk-IR cells, several studies on the effects of impulse activity and reserpine administration *in vivo* and depolarization and cholinergic stimulation *in vitro* on L-Enk levels of older bovine and rat chromaffin cells have yielded similar results (Siegel et al., 1985; Kanamatsu et al., 1986; Sietzen et al., 1987; DeCristofaro and La Gamma, 1991; see, however, LaGamma et al., 1984; LaGamma and Adler, 1988). In the context of development, the onset of functional innervation shortly after birth (Lau et al., 1987) correlates temporally with the dramatic decrease in the proportion of L-Enk-IR cells.

Our results suggest that this decrease in L-Enk expression by neonatal chromaffin cells is not due to depolarizing influences of the preganglionic innervation since depolarization of cells *in vitro* does not mimic the decrease in L-Enk expression observed *in vivo*. The possibility exists, however, that chromaffin cells of different ages respond differently, in terms of L-Enk expression, to depolarizing stimuli as has been suggested by LaGamma and Adler (1988).

In addition to the initial appearance of ChAT-IR in the preganglionic innervation of the adrenal medulla, L-Enk-IR is also initially detectable in preganglionic fibers shortly after birth. We tested the effects of the enkephalin analog DADLE on L-Enk expression in cultures of neonatal chromaffin cells. We have found that exposure to DADLE completely inhibits the depolarization-induced increase in L-Enk levels. DADLE, however, had no effect on L-Enk expression in cultures grown under nondepolarizing conditions. Further, the inhibition of the depolarization-induced increase in L-Enk levels by DADLE was abolished by opiate receptor blockade via addition of naloxone. That naloxone addition to control cultures or cultures grown under depolarizing conditions had no effect on L-Enk levels suggests that endogenously released L-Enk does not significantly affect L-Enk expression in chromaffin cells. These results raise the possibility that, subsequent to the initial expression of ChAT and L-Enk in preganglionic fibers and onset of function, chromaffin cells exposed to the depolarizing influence of ACh express increased levels of L-Enk while cells exposed to ACh and L-Enk express significantly lower levels. Consistent with this possibility, most mature chromaffin cells appear to be innervated by fibers that exhibit ChAT and L-Enk-IR (P. D. Henion and S. C. Landis, unpublished observation) and most chromaffin cells do not normally contain immunocytochemically detectable levels of L-Enk (Henion and Landis, 1990).

How might ACh and L-Enk interact to influence the expression of enkephalin by chromaffin cells? Enkephalin and enkephalin analogs act postsynaptically to reduce calcium-dependent action potentials and transmitter release by opiate receptor–mediated mechanisms in several different classes of neurons (Mudge et al., 1979; Werz and MacDonald, 1982; Bixby and Spitzer, 1983; Cherubini and North, 1985). In addition, enkephalins can inhibit transmitter release by increasing potassium conductance (Williams et al., 1982; Cherubini and North, 1985). Of interest, enkephalin analogs act presynaptically to inhibit ACh release from preganglionic fibers in sympathetic ganglia (Konishi et al., 1980; Araujo and Collier, 1987). By analogy, in the adrenal, L-Enk released by preganglionic fibers may inhibit the corelease of ACh, reducing the depolarizing effects of the innervation. Conversely, released enkephalin could act postsynaptically to decrease L-Enk synthesis in chromaffin cells, possibly by means of a calcium-dependent mechanism (e.g., Bunn and Dunkley, 1991). Based on our cell culture results, inhibition of L-Enk expression in chromaffin cells by L-Enk released from preganglionic fibers would be reflected by lower L-Enk levels in cells innervated by fibers that release both ACh and L-Enk than in cells innervated by fibers that release only ACh. Therefore, denervation of the adrenal would not only eliminate the stimulatory effects of depolarization but would also remove the inhibitory effects of L-Enk. The net result of denervation may be higher L-Enk levels as a consequence of the removal of an inhibitory influence in combination with the stimulatory effects of glucocorticoids. Thus, during development, the expression in and release of L-Enk from preganglionic fibers could be responsible for the reduction in the number of L-Enk-IR chromaffin cells observed shortly after birth.

Based on the effects of DADLE on L-Enk expression by chromaffin cells *in vitro*, the neurotransmitter properties of the preganglionic innervation, and the effect of denervation *in vivo*, inhibition of the effects of preganglionically released enkephalin would be predicted to mimic the effects of denervation. We continuously exposed adult rats to the opiate receptor antagonist naloxone and found that adrenal L-Enk content and the number of L-Enk-IR chromaffin cells were significantly increased in treated animals compared to controls. Naloxone treatment, however, did not completely mimic the effect of denervation on L-Enk expression. There are several possible explanations for this observation. First, the concentration of naloxone may not have been sufficient to inhibit completely the receptor-mediated effects of released L-Enk. The rats in these experiments, however, were infused with 2 mg/kg/hr naloxone, which is in excess of concentrations capable of eliciting the expected behavioral responses to naloxone in other studies (e.g., see Malin et al., 1986). Another possible explanation for the difference between naloxone treatment and denervation may be chronic opiate receptor blockade affects other systems, such as the pituitary and ACTH release (Lotti et al., 1969; Buckingham and Cooper, 1984) and therefore affects adrenal L-Enk levels independent of preganglionic influences.

Taken together with the pattern of L-Enk expression during development *in vivo*, our present results indicate that local environmental cues regulate the expression of L-Enk during adrenal development. Glucocorticoids and depolarization stimulate L-Enk expression, while the enkephalin analog DADLE inhibits the depolarization-induced increase in L-Enk levels. Our results are consistent with a role for glucocorticoids in increasing L-Enk levels and/or the number of chromaffin cells expressing immunocytochemically detectable levels of L-Enk during embryonic development. In addition, the possibility exists that glucocorticoids may be responsible for the induction of L-Enk expression in chromaffin cells. The distinct effects of the different neurotransmitters released by the preganglionic innervation could result in the establishment of the mature proportion of L-Enk-IR cells among the chromaffin cell population. Our results indicate that the preganglionic innervation regulates L-Enk levels in adult chromaffin cells and suggest that it could modulate adrenal L-Enk levels in response to environmental stimuli such as stress.

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