Localization of immunoreactive dynorphin in neurons cultured from spinal cord and dorsal root ganglia

(endorphin/peptidergic neurons/spinal sensory neurons/immunohistochemistry/radioimmunoassay)

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ABSTRACT Antisera specific for dynorphin were used to study the cellular distribution of opioid peptides in spinal cord and dorsal root ganglion neurons in dissociated cell culture. Radioimmunoassay of 4-wk-old cultures yielded levels of dynorphin immunoreactivity similar to those in adult rodent spinal cord. Immunohistochemistry showed staining confined to the perinuclear region of neuronal cell bodies. In contrast, enkephalin immunoreactivity was found in extensive neurite fields as well as in neuronal perikarya. Opioid peptide immunoreactivity was observed in $\approx 5\%$ of the spinal cord neurons either with dynorphin or enkephalin antiserum. No substantial increase in the number of reactive cells was observed when the two sera were applied simultaneously. These results suggest that the perinuclear region of opioid spinal cord neurons in culture contains peptide with an amino acid sequence similar to that of the midportion of dynorphin, whereas the neurites appear to contain smaller peptides related to NH_2 -terminal fragments of dynorphin. By using simple morphological criteria, spinal sensory neurons can be identified in these cell cultures and in cultures prepared from dorsal root ganglia without spinal cord. Approximately 1–2% of these ganglion cells showed intense immunostaining with an affinity-purified dynorphin antiserum. An additional few percent of the sensory neurons showed less intense opioid immunoreactivity. This result extends the observations of opioid peptides one step further along the pathway that processes sensory information.

Considerable evidence has been obtained to support the involvement of opioid peptides in spinal cord function (1). Spinal cord tissue contains a relatively high concentration of opiate receptors (2) and enkephalin-like molecules, as defined by immunohistochemistry (IHC; refs. 3 and 4). Using spinal cord cell cultures as a model for the study of these peptides, we have reported multiple, distinctly different actions of opioid peptides on the excitability of spinal cord neurons (5, 6) and have demonstrated the presence of immunoreactive (ir)-opioid peptide in cell bodies and neurites, using enkephalin antisera (7). Further, we have demonstrated (8) that cultured spinal neurons synthesize [Met]enkephalin in a ribosome-dependent manner and that the newly synthesized peptide can be released by elevating the extracellular potassium concentration. However, beyond this latter study, very little is known about the precise structure of the opioid peptides within spinal cord neurons.

Highly specific antiserum, "Lucia" (9), prepared against the first 13 amino acids of the 17-residue dynorphin, has revealed that spinal cord contains a rather high concentration of this opioid peptide (10, 11). This antiserum has been characterized by radioimmunoassay (RIA) as exhibiting maximal affinity for the sequence 3–12 of dynorphin, with $<10^{-6}\%$ crossreactivity

with [Leu]enkephalin (9). Low levels of ir-dynorphin also have been observed in adult rabbit and rat dorsal root ganglia (11) with the "Lucia" antiserum, whereas ir-enkephalin has been reported in spinal ganglia that were maintained in explant culture with spinal cord tissue (12).

In the present study, we used the "Lucia" antiserum and affinity-purified dynorphin antibodies from a very similar antiserum ("Paul") to study the cellular distribution of ir-dynorphin within spinal cord and sensory neurons in dissociated cell cultures, and we have compared the results with those obtained using enkephalin antisera.

MATERIALS AND METHODS

Preparation of Cell Cultures. Spinal cord cell cultures were prepared and maintained as described (13). Spinal cords with associated sensory ganglia were removed from 13.5- to 14.5-day (gestational age) fetal mice (C57BL/6), mechanically dissociated, and sterilely inoculated in Eagle's minimal essential medium with 10% horse serum and 10% fetal calf serum. For fluorescence IHC, one-third of a spinal cord was inoculated onto collagen-coated 22×22 mm glass coverslips (Clay Adams). For peroxidase-antiperoxidase assays, cells from one-third of a spinal cord were inoculated onto collagen-coated 35-mm plastic culture dishes, and for RIA, the cells were inoculated onto 60-mm plastic culture dishes at a density of three spinal cords per dish. Background cell growth was inhibited after one treatment with uridine at $50 \mu\text{g/ml}$ and fluorouridine deoxyribose at $20 \mu\text{g/ml}$ (courtesy of W. E. Scott, Hoffmann-LaRoche) applied between day 4 and day 7 in culture.

Cells were maintained between day 4 and day 28 in Eagle's minimal essential medium with 10% horse serum. The cultures that were prepared for RIA determination of dynorphin were maintained for 4 wk in medium with sodium bicarbonate at 3.5 g/liter and an atmosphere of 7% CO_2 in air. Cells prepared for IHC were grown either in the above medium or were maintained in an air atmosphere with similar medium containing sodium bicarbonate at 0.35 g/liter and 30 mM Hepes buffer (pH 7.35). We have found the Hepes-buffered medium to provide a suitable environment for the differentiation of murine spinal cord and brain cells in dissociated culture. There was no apparent difference in the expression of peptide development between cultures maintained in either buffer system.

IHC. Cultures were rinsed with medium and fixed for 40 min in freshly depolymerized, cold, 4% paraformaldehyde in 0.1 M phosphate buffer. Rabbit antiserum to dynorphin ("Lucia" or "Paul"), [Met]enkephalin (lot no. 49279, ImmunoNuclear), or

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Abbreviations: RIA, radioimmunoassay; IHC, immunohistochemistry; ir, immunoreactive.

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[Leu]enkephalin (generously provided by June L. Dahl, Department of Pharmacology, University of Wisconsin Medical School) was adsorbed with mouse liver powder at 20 $\mu\text{g}/\text{ml}$ (Cappel Laboratories, Cochranville, PA) to decrease nonspecific reactivity in the IHC.

For fluorescence studies, the fixed cultures were incubated with 0.5 ml of a 1:100 dilution of rabbit antiserum with phosphate-buffered saline (pH 7.4) for up to 24 hr in a humid environment at 4°C. By using the indirect immunofluorescence method of Coons (14), the cultures were incubated for 30 min at 4°C with a 1:40 dilution with phosphate-buffered saline (pH 7.4) of fluorescein-conjugated goat antiserum against rabbit IgG (Cappel Laboratories). Cultures were inverted on a glass slide with a drop of glycerol and phosphate-buffered saline, 3:1 (vol/vol), and visualized in a Zeiss Photomicroscope. For the enzyme-mediated histochemistry (15), fixed cells were incubated as follows: 1 hr at room temperature with 3% normal goat serum in 50 mM Tris-HCl-buffered (pH 7.4) saline; 24 hr at 4°C or 3 hr at room temperature in a 1:600 dilution of primary dynorphin or enkephalin antiserum with 0.25% Triton X-100/1% normal goat serum/50 mM Tris-HCl-buffered saline; 30 min at room temperature in a 1:40 dilution of goat anti-rabbit IgG (Sternberger-Meyer Immunochemicals); 30 min at room temperature with a 1:80 dilution of the peroxidase-antiperoxidase complex (Sternberger-Meyer Immunochemicals); and finally, 6–20 min at 22°C with 0.06% 3,3'-diaminobenzidine tetrahydrochloride/0.01% hydrogen peroxide in Tris buffer.

RIA. Spinal cord cells in 60-mm culture dishes were rinsed and incubated in 2.5 ml of Earle's balanced salt solution containing 0.5% bovine serum albumin, bacitracin at 150 mg/liter, and glucose at 4.5 g/liter for 30 min at 37°C. Replicate cultures were exposed for 30 min to similar medium in which the concentration of potassium was raised to 40 mM and the osmolarity of the solution was balanced by an equivalent reduction in the concentration of the other ionic constituents. The medium from 13–16 dishes was pooled and diluted with methanol/HCl to give final concentrations of 50% methanol/0.05 M HCl. Medium from similar cultures was acidified to a final concentration of 0.1 M acetic acid and heated to 90°C for 30 min. The samples were centrifuged at $54,000 \times g$ for 30 min and the supernatants were dried under nitrogen gas. The cells were frozen and stored at -80°C for up to several weeks before they were scraped from the culture dishes and the peptide extracted in a medium of either methanol/0.1 M HCl, 1:1 (vol/vol), or 0.1 M acetic acid at 90°C for 30 min.

After centrifugation, the extraction supernatant was frozen and dried under vacuum. The dried samples were resuspended in 0.1 M HCl with 0.1% Triton X-100 and were centrifuged to remove undissolved material. Supernatant solutions were applied to Sep-Pak C_{18} cartridges and washed with 5 mM trifluoroacetic acid. Inclusion of ^{125}I -labeled dynorphin as an internal control under these conditions confirmed that 91–99% of the dynorphin was retained by the Sep-Pak and was eluted by 50% acetonitrile in 5% trifluoroacetic acid. The trifluoroacetic acid rinses and acetonitrile elutions were lyophilized and subsequently solubilized in 0.5 ml of 0.1 M HCl with 0.1% Triton X-100. Dilutions were assayed in the standard RIA (9) with "Lucia" antiserum and dynorphin-(1–13) as standard.

Preparation and Specificity of Dynorphin Antisera. Antiserum "Lucia" has been fully described elsewhere (9). In RIA, natural dynorphin-17, dynorphin-(2–13), and dynorphin-(1–12) are fully crossreactive with dynorphin-(1–13) standard, whereas [Leu]enkephalin [dynorphin-(1–5)] crossreacts $<10^{-6}\%$. Thus, Lucia antibodies recognize a central portion of the dynorphin sequence. Antiserum "Paul" was raised in the same way as "Lucia"; it behaves identically in all respects. Specific "Paul"

antibodies were obtained by immunoaffinity purification on Sepharose 4B coupled to dynorphin-(1–13). In RIA, crossreactivities of this antibody preparation against dynorphin-(1–13) standard were: dynorphin-17, 100%; α -neo-enkephalin, $8 \times 10^{-5}\%$; dynorphin B (16), $7 \times 10^{-5}\%$; [Leu]enkephalin, $3 \times 10^{-6}\%$. Specificity of "Lucia" in IHC, determined by blocking experiments, is reported in *Results and Discussion*.

RESULTS AND DISCUSSION

Cells dissociated from embryonic mouse spinal cord and sensory ganglia contained ir-dynorphin after 4 wk of differentiation in culture (Table 1). This level of peptide is equivalent to that reported in the dorsal spinal cord of adult rodents (11). The data are consistent with the results obtained after spinal cord transection, which indicated that most of this peptide was present in the spinal neurons rather than in descending spinal afferents (11). The RIA employed in this study was quite specific for the midportion amino acid sequence in dynorphin. The lack of significant crossreactivity with the enkephalins in this assay together with our previous demonstration—by using HPLC—of [Met]enkephalin synthesis and release by similar spinal cord cell cultures clearly indicate the presence of two different opioid peptides in spinal cord neurons.

Using RIA we were unable to demonstrate release of a significant amount of dynorphin from these cultures. However, this negative result may not be conclusive for several reasons. We might anticipate release of $<5\%$ of total neuronal dynorphin under the conditions of elevated extracellular potassium. This would represent a level of peptide close to the assay background produced by culture medium alone and this quantity may be difficult to detect consistently. Beyond this, the propensity of dynorphin to adhere to physical substrates may have interfered with our ability to collect the released peptide. It is also possible that some peptidase activity remained in the incubation medium or on culture surfaces. Alternatively, the peptide that the spinal cord cells release may be a small dynorphin-related peptide that is not detected by the "Lucia" antiserum.

Table 1. ir-Dynorphin in spinal cord/dorsal root ganglion cell cultures

Treatment	Cultures, no.	Extraction	ir-Dynorphin, fmol per culture	
			Cells	Medium*
Control	16	Acetic acid	125	4.0
High K	13	Acetic acid	171	6.7
Control	16	Methanol/HCl	118	3.0
High K	13	Methanol/HCl	125	4.0

The dynorphin content of 60-mm spinal cord cell cultures was determined by RIA by using "Lucia" antiserum (9). To assay for peptide release, spinal cord cells that had differentiated for 4 wk in culture were incubated for 30 min in a modified balanced salt solution (see text) containing 6 mM potassium (control) or 40 mM potassium (high K). The dynorphin in the release media, as well as the peptide remaining in the spinal cord cells, was extracted either with acetic acid or methanol/HCl (see text) and the ir-dynorphin content was determined for each sample. Determinations were carried out in triplicate across three dilution conditions for each pooled sample from 13–16 cultures. These 60-mm spinal cord cultures generally contained 650–700 μg of total protein. If we estimate protein content as 10% of the wet tissue weight, we may calculate the dynorphin concentration to be ≈ 20 pmol/g of tissue, to compare these results to those published (11) for adult rodent spinal cord tissue.

* Balanced salt solution similar to that used in this experiment, but not incubated with cells, produced background values of between 3 and 4 fmol per culture in this assay. These background values have not been subtracted from the data presented in this table.

FIG. 1. Spinal cord neurons containing ir-dynorphin are presented in A (peroxidase-antiperoxidase method) and B (fluorescein second antibody method). Positive reaction product is confined to the perinuclear region (arrows) and cannot be found within the neurites of these cells. In C, ir-enkephalin is localized in neurites that envelop several unreactive neuronal somata. "Lucia" was the primary antiserum for A and B, whereas antiserum prepared against [Leu]enkephalin (from June Dahl) was used in C.

Data consistent with this latter possibility were obtained from IHC analysis of spinal cord neurons. We observed intense immunoreactivity in the perinuclear region of the cell body of spinal cord neurons (Fig. 1 A and B) using "Lucia." In the fluorescence studies, ir-dynorphin often appeared as groups of lines or waves of reaction product possibly reflecting stacks of cytoplasmic membranes, such as Golgi complex or endoplasmic reticulum. Typically, the ir-dynorphin terminated abruptly at the base of the neurites. In general, neither fluorescence nor peroxidase reaction product could be detected in neuronal processes. However, when affinity-purified "Paul" antibodies were used with peroxidase IHC, a few cases of apparently reactive, isolated neurites were found. This is in contrast to cultures reacted with enkephalin antisera, in which ir-peptide was distributed within cell bodies as well as in extensive fields of neurites (Fig. 1C and refs. 7 and 17).

These results suggest that the enkephalin antisera may be reacting in the neurites with constituents that the dynorphin antisera do not recognize. Blocking experiments performed under IHC conditions (Table 2) provide an index of the specificity of two antisera used in this study. The enkephalin antiserum used for peroxidase IHC of the spinal cord neurons (Table 3) was broadly reactive with both enkephalin peptides and dynorphin-(1-13), whereas the "Lucia" antiserum was selective for peptides larger than [Leu]enkephalin, an observation which is consistent with its specificity under RIA conditions (9). Because of the reactivity of the enkephalin antiserum with the common NH₂ terminus of the enkephalins and dynorphin, we may conclude only that the cell bodies of opioid spinal cord neurons contain a peptide sequence similar to the midportion sequence of dynorphin, whereas the neurites probably contain a smaller opioid peptide sequence common to dynorphin and the enkephalins.

In an attempt to gain insight into the relationship between the cell body reactivity to enkephalin and dynorphin antisera, we determined the percentage of spinal cord neurons that stained after incubation with antisera applied individually or simultaneously (Table 3). We found that $\approx 5\%$ of the neurons contained ir-enkephalin and a similar percentage contained ir-dynorphin. There was little, if any, consistent increase in the percentage of the ir-cells when both of the antisera were applied to the same culture. These results, together with data from the blocking studies, suggest that the dynorphin sequence is

present in the majority of the opioid-containing spinal cord neurons.

The affinity-purified "Paul" antibodies produced an extremely low background stain with the peroxidase-antiperoxidase method, allowing us to demonstrate clearly the presence of ir-dynorphin in 1-2% of the morphologically identified dorsal root ganglion neurons (Fig. 2) also normally present in our spinal cord cell cultures. In other experiments, we have observed ir-dynorphin in these sensory neurons cultured without spinal

Table 2. Specificity of antisera

Condition	Antiserum against	
	Dynorphin-(1-13)	[Met]-Enkephalin
Blocking agent, μ M		
[Leu]enkephalin, 2-20	-	+
[Met]enkephalin, 2	-	+
Dynorphin-(1-13), 2	+	+
Dynorphin-(1-9), 20	+/-	*
Dynorphin-(1-8), 20	+/-	*
Specificity of antiserum	Midportion	NH ₂ terminus
IHC localization in spinal cord cells		
Neuronal perikarya	+	+
Neurites	-	+

The specificities of two antisera used to obtain the data in Table 3 are presented. The dynorphin-(1-13) antiserum was "Lucia" and the enkephalin antiserum was that from ImmunoNuclear. Immunoreactivity that was lost in the presence of the blocking agent is indicated by +, whereas blocking agents that had no significant effect on the immunoreactivity are indicated by -. Dynorphin-(1-9) and dynorphin-(1-8) substantially decreased the intensity of the histochemical reaction, as indicated by +/-, but they did not completely block the reaction as did dynorphin-(1-13). The differing specificities of the antisera are further indicated by their reactivity (+) with spinal cord neuronal cell bodies or neurites. Although the enkephalin antiserum from ImmunoNuclear was prepared against [Met]enkephalin, our blocking studies indicated that under IHC conditions, it readily recognized both [Leu]enkephalin and [Met]enkephalin.

* Our supply of this antiserum was exhausted prior to the execution of the blocking studies with dynorphin-(1-8) and dynorphin-(1-9). However, the efficacy of both [Leu]enkephalin and dynorphin-(1-13) in eliminating the reactivity strongly suggests that it would also be blocked by the intermediate dynorphin molecules.

Table 3. Neuronal cell bodies in spinal cord cell cultures demonstrating ir-enkephalin and ir-dynorphin

Culture conditions	% positive neuronal cell bodies		Enkephalin and dynorphin
	Enkephalin	Dynorphin	
CO ₂ /NaHCO ₃	4.7	5.3	4.8
Air/Hepes	4.3	5.1	7.1

Spinal cord neurons (20,600) were sampled in 250 microscopic fields under $\times 200$ magnification in 35-mm cell cultures. Data were obtained from two different inoculations of embryonic spinal cord cells that were maintained in different buffering conditions for 4 wk. Cells were evaluated by using the peroxidase-antiperoxidase IHC.

cord cells but in the presence of nerve growth factor and spinal cord-conditioned medium. These dorsal root ganglion cells have been characterized electrophysiologically (18) and can be identified in phase microscopy by their spherical shape, typically one to three fine neurites, large nucleus, and prominent nucleolus. In these cultures they develop individually (Fig. 2 B-E), in small groups, or in ganglion-like clusters (Fig. 2A).

Our observation of intensely staining ir-dynorphin in a small number of sensory neurons (Fig. 2) is consistent with the reported opioid peptide in explant cultured spinal ganglia (12) and the observation of much less ir-dynorphin in dorsal root ganglia than in the dorsal spinal cord of rabbits and rats (11).

In addition to the heavily stained sensory neurons described above, we also observed a rather broad spectrum of moderately stained cells. These neurons were generally larger than the intensely reactive cells and they comprised an additional few percent of the total dorsal root ganglion cells in the cultures. The overwhelming majority of the sensory neurons did not stain at all with this antiserum. Absorption of the affinity-purified antiserum with 2 μ M dynorphin-(1-13) was sufficient to eliminate the intense reactivity in sensory neurons, yet there remained

a decreased number of moderate to lightly stained sensory neurons. In light of the failure of the dynorphin absorption to completely eliminate the moderate-to-light reaction product and the resulting difficulty in establishing an unequivocal background staining density for the large round sensory cells, we have not attempted to define the number of positive dorsal root ganglion cells beyond those most intensely stained.

Our observation of ir-dynorphin in dorsal root ganglion neurons raises the possibility of the coexistence of other opioid peptides in these same cells. The presence within the recently cloned prodynorphin gene (19) of both α -neo-endorphin (20) and an opioid peptide that was recently subjected to sequence analysis, termed dynorphin B (16), supports the proposal that these opioid peptides also may be synthesized by this small population of sensory neurons.

Evidence to support the coexistence of dynorphin and α -neo-endorphin has been obtained with both RIA (21) and IHC (22). In preliminary studies, we have observed ir-dynorphin B in both spinal cord and sensory neurons in a distribution similar to that reported here for dynorphin. Beyond this, the possibility exists for the coexistence of these opioid peptides with other peptides previously observed in dorsal root ganglion cells in a manner analogous to the presence of both dynorphin and vasopressin in magnocellular neurons (23). In view of the effect of opiates in inhibiting the release of substance P from sensory neurons (24, 25) and in the suppression of quantal excitatory synaptic transmission between sensory and spinal neurons (26), it would be of interest to learn if these dynorphin sensory cells also contain somatostatin or substance P.

Dynorphin antisera have been used to study the IHC distribution of the peptide in the intact nervous system. In initial studies that used "Lucia" antiserum, the ir-peptide was readily observed in neuronal cell bodies in the hypothalamus and in neurites in the guinea pig ileum (27). Few reactive fibers were detected in the brain. In more recent studies with other antisera, ir-dynorphin and ir- α -neo-endorphin have been reported

FIG. 2. Cells identified morphologically as dorsal root ganglion neurons from the spinal-sensory cultures are presented. A cluster of undissociated sensory neurons (A) contains a single small intensely staining cell (arrow) and a larger lightly stained cell within a group of unreactive neurons. Cells in B-E present a morphology that is typical of neurons observed in pure dorsal root ganglion cell cultures and they each contain rather intense ir-dynorphin in the cell body. The typically fine neurites of these cells do not contain reaction product. The cells in this figure were reacted with dynorphin antibodies ("Paul") and the immunoreactivity was determined by using the peroxidase-antiperoxidase method.

in closely associated or identical cell bodies and neurites in a broad spectrum of brain regions (22). In research done in collaboration with H. Khachaturian, S. J. Watson, M. E. Lewis, D. Coy, and H. Akil, the "Paul" antiserum has been applied to the study of the ir-dynorphin distribution in the adult rat central nervous system. This has revealed both perikaryal and diffuse immunoreactivity in the marginal zone of the spinal cord as well as patches of ir-varicosities in deeper laminae. The significance of the colchicine treatment applied in the study of the intact spinal cord and the possible contribution of descending opioid pathways remain to be determined.

The pattern of perikaryal ir-dynorphin staining reported here for spinal cord and sensory neurons indicates the presence of the midportion dynorphin peptide sequence in these cell bodies. The absence of neurite staining, in the same preparation in which ir-enkephalin staining can consistently be observed in processes, suggests that either the two dynorphin antisera were unable to detect dynorphin within neurites or the neurites of these cells contained smaller opioid peptides. Though the blocking experiments confirmed that the "Lucia" antiserum does recognize the dynorphin-related peptides but not [Leu]enkephalin, the specificity of these sera, under conditions of aldehyde fixation and IHC, cannot fully be ascertained from these results. RIAs of the neurointermediate pituitary (28) and of other regions of the central nervous system (29) indicate the presence of dynorphin-(1-8). A fuller appreciation of the significance of these data and of the IHC presented here, with respect to the physiologic action of dynorphin-related peptides in spinal and sensory neurons, awaits rigorous analysis of the release and synaptic action of these molecules.

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