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SHORT COMMUNICATION

Opiate-Like Materials in the Adrenal Medulla: Evidence for Storage and Secretion with Catecholamines

O. HUMBERTO VIVEROS, EMANUEL J. DILIBERTO, JR., ELI HAZUM AND KWEN-JEN CHANG

Department of Medicinal Biochemistry and Department of Molecular Biology, Wellcome Research Laboratories, Research Triangle Park, N. C. 27709

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SUMMARY

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Opiate-like materials, as measured by the radioreceptor assay, were found in the adrenal gland of 7 mammalian species including man. The opiate-like materials are confined to the adrenal medulla, and followed a pattern identical to catecholamines on differential and isopycnic centrifugation. In the isolated perfused dog adrenal gland, acetylcholine stimulates a Ca^{2+}-dependent secretion of opiate-like materials and catecholamines in the same molar ratio as they are stored within the chromaffin cell. Ba^{2+} also stimulates the secretion of both products from the adrenal gland. Separation of the opiate-like materials by Sephadex G-50 and reverse phase high pressure liquid chromatography indicates the presence of four peaks of opiate-like materials of molecular weights below 2,000. Two of these peaks comigrate in these systems with authentic met- and leu-enkephalins and react with the corresponding antibodies. These results indicate that enkephalins and other low molecular weight opiate-like materials are stored in and secreted from the chromaffin vesicles with catecholamines in the adrenal glands. These findings support a role for enkephalins as neurotransmitters and/or neurohormones. Also, they suggest that enkephalins and other opiate-like materials may exert important neuroendocrine functions outside the central nervous system.

INTRODUCTION

Secretion from the adrenal medulla occurs as a quantal, all-or-none, release of all the soluble components within the chromaffin vesicles directly into the extracellular space (1, 2). During this process of exocytosis, soluble dopamine-β-hydroxylase (DBH[1]) (3) and chromogranins, a poorly characterized family of soluble intravesicular peptides, are secreted (4). This is a first attempt to characterize the function of the secreted proteins and peptides. Different forms of stressful stimuli elicit marked neurogenic stimulation of the adrenal medulla (5). Similar stimuli induce significant analgesia which can be, at least in part, blocked by opiate antagonists (6, 7). Thus, we first examined the possible storage and secretion of opiate-like materials (OLM) from chromaffin cells of several species. (Short accounts of these findings have been published elsewhere [8, 9].)

Adrenals from different animals were obtained immediately after death and the ad-

[1] Abbreviations used are: DBH, dopamine-β-hydroxylase; OLM, opiate-like materials; CA, catecholamines; HPLC, high pressure liquid chromatography.

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renal medullae were rapidly dissected from the cortices under a stereoscopic microscope in the cold. One human adrenal gland was obtained at autopsy 8 hours after death. Analysis for OLM in total organ was performed by direct homogenization of the medulla in cold 1 M acetic acid. For differential centrifugation and sucrose density gradient fractionation the medullae were homogenized in cold 0.3 M sucrose and a nuclear, mitochondrial, microsomal and postmicrosomal supernatant obtained as previously described (10). Immediately after preparation of each fraction, cold acetic acid was added to obtain a final concentration of 1 M. The acid fractions were centrifuged at 160,000 × *g* for 5 min (Beckman airfuge) and the clear supernatant saved for catecholamine (CA) (11) and OLM assays. OLM were measured by the radioreceptor assay using rat brain membrane preparations and [^{125}I] [D-ala^2, D-leu^5]-enkephalin (0.25 nM; specific activity 2 Ci/μmol) as ligand in the presence of 1 mM $MnCl_2$ (12, 13). OLM were expressed as the molar equivalent of a met-enkephalin standard. Partial purification and characterization of OLM was obtained through Sephadex G-50 chromatography, silica gel thin layer chromatography, and reverse phase high pressure liquid chromatography (HPLC) with a Partisil PXS-10, 10/25 ODS column (Whatman). The HPLC effluent was also characterized by radioimmunoassay against met- and leu-enkephalin specific antibodies (12). Dog adrenals were perfused retrogradely with Locke solution at 2 ml/min; collection and stimulation periods lasted 2 min (3). Aliquots of the adrenal effluent for OLM assay were introduced into a boiling water bath for 10 min immediately after collection of the sample and either assayed with no delay or stored at −70°.

Table 1 shows the OLM and CA content in the adrenal medulla of 7 species including man. While OLM content seems to be similar in the striatum of different species (see also ref. 14), there is a 100-fold span in the content of OLM in the adrenal medulla between the rat and the dog or cow. Currently we do not understand the reason for such widespread interspecies variation.

TABLE 1

Opiate-like materials in the adrenal medulla of several species

Brains and adrenal glands were removed immediately after the death of the animal and striatum and adrenal medullae and cortex dissected in the cold and rapidly homogenized in 1 M acetic acid. Opiate-like materials were measured by the radioreceptor assay (12, 13) and are expressed as the molar equivalent of a met-enkephalin standard. Values represent the mean ± S.E.M. of duplicate determinations of individual pair of organs from 6 Long Evans black hooded rats, 6 New Zealand white rabbits, 5 mongrel cats, 6 mongrel dogs, and 2 cows. The medullae of 5 hamsters were pooled for assay. The human adrenal gland was obtained at autopsy 8 hours after death.

Species	Adrenal Medulla		Striatum
	Catecholamines	Opiate-like material	Opiate-like material
	μmol/g wet wt.	*nmol/g wet wt.*	*nmol/g wet wt.*
Rat	——	0.29 ± 0.03	1.93 ± 0.29
Hamster	——	0.50	——
Rabbit	——	0.85 ± 0.15	——
Cat	31.73 ± 2.85	3.20 ± 0.66	2.10 ± 0.13
Dog	23.72 ± 2.70	21.20 ± 1.24	
(% in Medulla)	(93.8%)	(97.3%)	——
Cow			
Human	8.64	20.00 ± 5.00	——
	8.59	6.50	——

Certainly, it is not due to the presence of OLM stored preferentially or exclusively in epinephrine- or norepinephrine-containing cells (while rat and human adrenals contain 10–20% norepinephrine, dog, cow and cat contain approximately 30% and rabbit has exclusively epinephrine containing cells in its adrenal gland [15, 16]). As shown in the table less than 3% of the OLM content was found in the adrenal cortex and all of it can easily be accounted for by medullary contamination (6% of the CA were found in the cortex). Similar observations have been made using preparations of cat and cow adrenal glands.

The subcellular distribution of OLM and CA in the dog adrenal medulla is shown in Figure 1. Eighty percent of the OLM sediments at 26,000 × *g* for 20 min (P_2 fraction). When the P_2 fraction is further resolved by isopycnic centrifugation through a linear

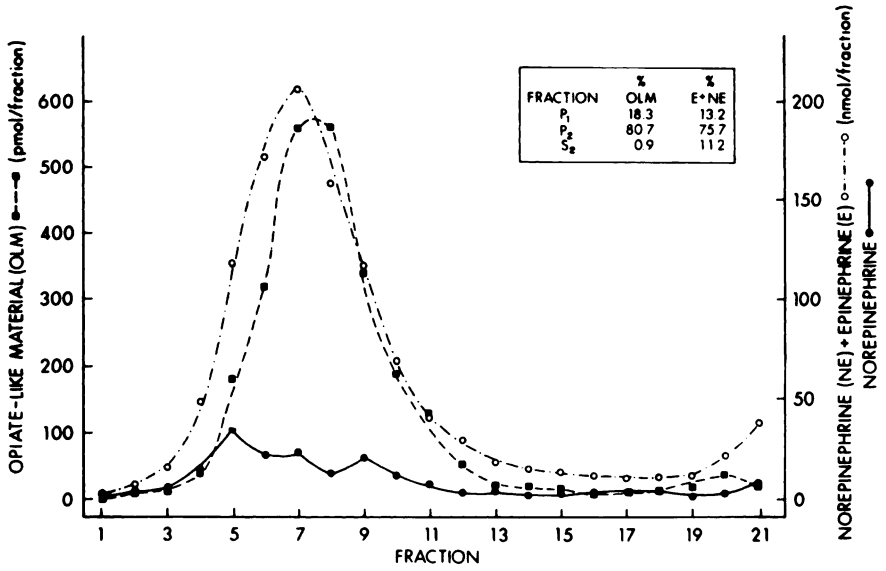

FIG. 1. *Distribution of opiate-like material and catecholamines on sucrose density gradient centrifugation of dog adrenal medulla large granular fraction*

One pair of adrenal medullae from one dog was homogenized 1:10 (w/v) in 0.3 M sucrose and the homogenate centrifuged in the cold to obtain a nuclear (P_1), large granular (P_2) and corresponding supernatant (S_2) fractions as previously described (10). The P_2 fraction was resuspended 1:20 in 0.3 M sucrose. A 200 μl aliquot was layered over a 4.3 ml linear gradient between 61.6% to 19.2% (w/w) sucrose in water and centrifuged to equilibrium at 60,000 rpm for 3 hr in a Beckman SW 60 Ti rotor. Nine drop fractions were collected (approximately 195 μl) and 9 μl of 4 N HCl was immediately added to 170 μl of each fraction and frozen at −70° for later analysis of opiate-like materials. The insert shows the distribution of opiate-like materials and catecholamines after differential centrifugation. The gradient distribution was repeated on another pair of adrenal medullae with qualitatively the same results.

sucrose gradient, the OLM follows very closely the CA profile. Similarly, the OLM from cow adrenal medulla sediments with the CA through 1.7 M sucrose in a discontinuous density gradient. The small difference in the position of the OLM and CA peaks in the continuous gradient (Fig. 1) is similar to that found when DBH is used as a marker for chromaffin vesicles (2, 10). This suggests that OLM may be incorporated into the vesicles together with the other soluble proteins, preceding the CA which are added later through uptake and synthesis (10). Those vesicles with higher CA content attain the highest buoyant density (10). The differential analysis of epinephrine and norepinephrine throughout the gradient does not support the contention that OLM may be preferentially associated with norepinephrine containing cells (17). The lower percentage of OLM in the high-speed supernatant (S_2) as compared to the CA may reflect a higher rate of degradation of the former after being released from vesicles broken during homogenization. In different experiments we have found that the molar ratio of CA/OLM in the large granular fraction from cow and dog adrenals is 347 to 817 and between 283 to 658 in the individual fractions comprising 2 tubes above and below the CA peak in the gradient of Figure 1. Since the concentration of CA within the chromaffin vesicle is considered to be close to 0.5 M (18) the concentration of OLM (if all were equipotent with met-enkephalin) would be between 0.6 and 1.8 mM.

Perfused dog adrenals spontaneously release low amounts of CA and OLM. In most experiments the released OLM was at or below the level of sensitivity of the receptor binding assay (Fig. 2). Introduction of acetylcholine into the Locke solution perfusing the gland results in an increased efflux of

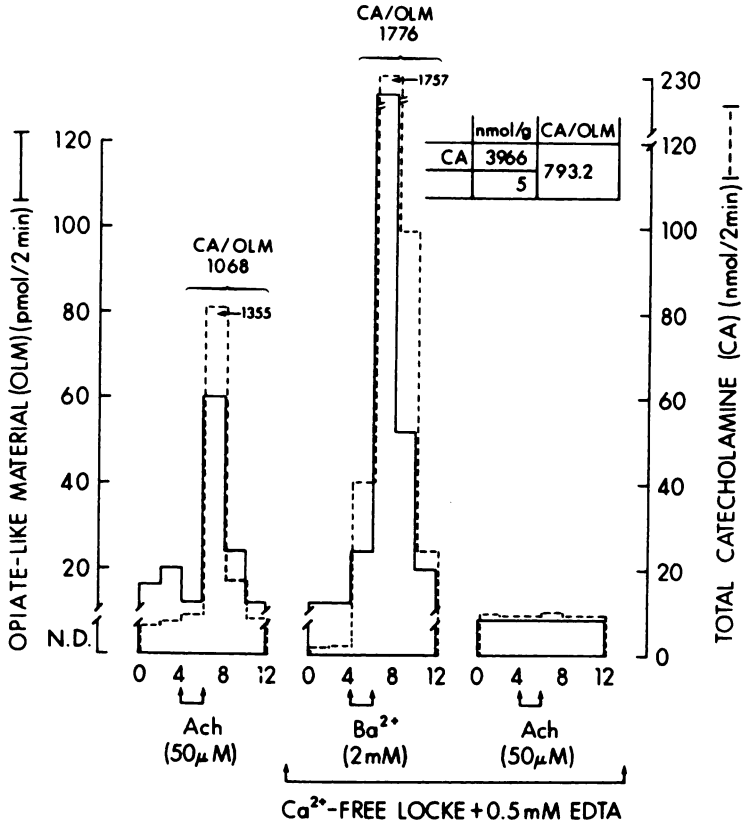

FIG. 2. *Secretion of opiate-like material, together with catecholamines, from the isolated, perfused adrenal gland of the dog*

Dog adrenals were perfused retrogradely through the adrenolumbar vein with modified Locke solution at 2 ml/min. Acetylcholine (5 mM) and $BaCl_2$ (200 mM) were dissolved in Locke solution and infused into the mainstream of the Locke entering the gland at 0.02 ml/min with a Harvard constant infusion pump. Collection and stimulation periods lasted 2 min. There is a 30 min washout period with Locke solution between two consecutive stimulations. Immediately after the first period of stimulation the gland was perfused with Ca^{2+}-free Locke containing 0.5 mM EDTA for the next 84 min. The insert shows the content of catecholamines and opiate-like materials in the medulla at the end of the experiment. The number above the brackets in each stimulation period corresponds to the molar ratio of CA/OLM for the accumulated output during the 2 min of stimulation and the 6 min following it. The number with the arrow is the molar ratio of CA/OLM for the highest secretion period. Note the similarity of the ratios in the secreted material and in the adrenal medulla.

both CA and OLM. The stimulation of secretion of both substances by acetylcholine requires Ca^{2+} in the medium, while Ba^{2+} is a potent secretagogue for both, even in the absence of Ca^{2+}. The insert to Figure 2 shows the CA and OLM values in the gland at the end of the perfusion. The ratio of CA/OLM in the medulla compared to the ratio of secreted material is amazingly close, considering the very short half-life of leu- and met-enkephalin in tissues or in blood (19). In other experiments to be reported later,[2] there is a close parallel in the secretion of OLM and CA induced by other Ca^{2+}-dependent secretagogues, as well as on the blockade of secretion by anticholinergic drugs.

The separation by HPLC of the OLM extracted from purified chromaffin vesicles from the dog and cow adrenal medulla, and from the dog adrenal perfusates, indicated the presence of four well defined peaks of

[2] O. H. Viveros, E. J. Diliberto, Jr., E. Hazum, and K.-J. Chang. Unpublished observations.

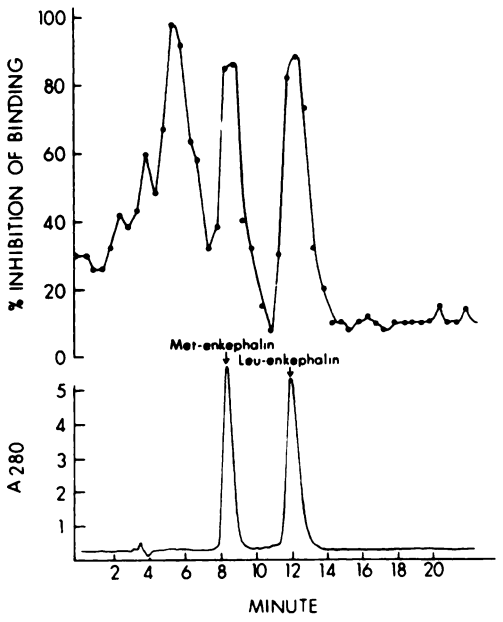

FIG. 3. *Separation of HPLC of opiate-like materials from chromaffin vesicles of cow adrenal medulla*

Chromaffin vesicles purified by centrifugation through 1.7 M sucrose were lysed in 5 mM Tris-HCl buffer, pH 7.4. The lysates were ultrafiltered through a UM-10 Amicon filter (cut-off point:10,000 M.W.). The filtrates were lyophilized and dissolved in a small volume of 0.1 M acetic acid. Enkephalin-like materials were initially separated on Sephadex G-50. All opiate-like materials eluted in a broad peak corresponding to fractions with molecular weight below 2,000. No opiate-like materials were detected at fractions corresponding to β-endorphin. The opiate-like materials were further separated on HPLC with a Partisil PXS-10 10/25 ODS column (Whatman) and eluted with 20% acetonitrile in 10 mM ammonium acetate pH 4.2. Flow rate was 1 ml/min. Aliquots were assayed for opiate-like activity and expressed as percent inhibition of the radioreceptor assay (12, 13). The upper part of the figure shows the profile of opiate-like materials, the lower part the absorbance profile of met- and leu-enkephalin standards. Four peaks of activity, with retention time 4, 6, 9, and 13 minutes were observed. The slight difference in retention time between the activity as measured by the radioreceptor assay and the absorbance profile of the met- and leu-enkephalin standards is due to the 0.3 ml dead space between the flow cell and fraction collection. The relative broadness of the OLM as compared with the standards is the result of collecting the HPLC effluent in 0.5 ml fractions, while absorbance is measured in a 10 μl flow cell. Similar results were found for the dog adrenal medulla and for the adrenal gland effluents.

activity (Fig. 3). The opiate receptor binding activity of all four peaks is lost by previous preincubation with pronase indicating that the OLM are probably peptides. The peaks of retention times 9 and 13 min co-elute with met- and leu-enkephalin, respectively. These peaks also give a single spot and co-migrate with the corresponding pentapeptides when analyzed by TLC in a silica plate using butanol/acetic acid/H_2O (4/1/4) as solvent. The HPLC effluent was further characterized by radioimmunoassay. Peaks 2 and 3 specifically cross-reacted with anti- met-enkephalin antibodies while peak 4 interacted with anti- leu-enkephalin antibodies. The nature of the first two peaks is currently under investigation. When the adrenal medulla extracts were submitted to molecular seive chromatography there was no OLM associated with a molecular weight corresponding to that of β-endorphin.

While this research was in progress two other groups provided evidence for the presence of OLM in gland cells and nerve terminals of the adrenal medulla: Schultzberg *et al.* (17) have recently identified enkephalin-like immunoreactivity in adrenomedullary cells of rat, cat and guinea pig and Costa *et al.* (20, 21) have isolated three different molecular forms of opiate-like peptides from the bovine adrenal medulla, one of which has been identified as met-enkephalin. The other two are of higher molecular weight and can generate smaller peptides with high affinity for opiate receptors on tryptic cleavage. They do not correspond to β-lipotropin or α, β or γ-endorphin.

Met- and leu-enkephalin immunoreactivity has previously been found in nerve terminals and perikarya of the ontogenically homologous sympathetic ganglion cells (14), as well as in the serotonin-containing enterochromaffin cells of the antrum and duodenum of the pig (22). In this latter report the enkephalin immunoperoxidase staining showed accumulation of reaction product over secretory granules. Enkephalin-like immunorectivity has also been found associated with large and small dense core vesicles in nerve terminals of locus

coeruleus and A_2 regions of rat brain (23).

Several recent reports have demonstrated *in vitro* release of OLM from brain synaptosomes (24), brain slices (24, 25), and from guinea pig ileum (26, 27) after electric field or high potassium stimulation. There is also evidence for enkephalin release *in vivo*, into the cerebrospinal fluid of man after stimulation of the periventricular grey region of the brain (28). These reports are suggestive of a physiological secretion of OLM, and the morphological data may indicate the coexistence of enkephalins with other transmitters in the same neuron. Nevertheless, these studies do not distinguish between the possibility that the two transmitters may be in separate monoaminergic and enkephalinergic cells or that they are in the same or different storage organelles. To our knowledge only Alumets *et al.* (22) and Pickel *et al.* (23) have tried to identify the subcellular storage components for these peptides. The morphological techniques used have the disadvantage, compared to cellular fractionation, that the peptides will be identified only where there are diffusion barriers that impede the washout of OLM during tissue preparation. These studies in the reports mentioned above do not determine if a neuron containing more than one transmitter will store them within the same compartment and secrete both simultaneously, or if preferential release of one or the other occurs.

The present experiments indicate conclusively that the large majority of the adrenomedullary leu- and met-enkephalin and the other OLM are contained within subcellular particles that sediment on centrifugation. Furthermore, the enkephalin storage particle is the same as the catecholamine storage vesicle, based on the following observations: a) identical distribution pattern of both group of substances in continuous and discontinuous sucrose gradients, b) concomitant, Ca^{2+}-dependent secretion of OLM and CA in the same ratios as are contained within the gland with diverse stimuli, c) parallel depletion of CA and OLM in glands where neurogenic secretion of the adrenal medulla has been induced by insulin hypoglycemia.[2] The fact that both neurohormones (or neurotransmitters) are stored in and secreted from the same storage vesicles obviously means that both pertain to the same individual cell. Since the adrenomedullary chromaffin cell is derived from the same sympathogonia as the noradrenergic sympathetic neuron, it is highly probable that a similar system may be present in sympathetic nerve terminals. Preliminary observations indicate that substantial amounts of OLM exist in the splenic nerves and spleen of the dog.[2] These results are probably to this date the most substantial challenge to the one neuron–one transmitter hypothesis. As far as we are aware, this is also the first report of a cholinergic interaction with an enkephalinergic cell. It will need to be proven if such a relationship is also present at enkephalinergic neurons in the peripheral and central nervous system.

The secretion of substantial amounts of OLM from the stimulated adrenomedullary chromaffin cell strongly supports the role of enkephalins and other OLM as neurotransmitters or neuromodulators. Furthermore, it suggests that OLM may have not only a localized action at the synapse, where they are released, but also that they may have more general effects in peripheral tissues and central nervous system, constituting a new class of neurohormones. These putative actions of the adrenomedullary peptides will be uncovered only by studies taking into account the masking pharmacological effects of the secreted catecholamines.

The stimuli for the secretion of OLM *in vivo* will be the same as those that induce secretion of catecholamines. We have already found that reflex discharge of the splanchnic nerve by hypoglycemia following insulin administration produces large depletion of the OLM stored in the gland.[2] This strongly suggests a role for the adrenomedullary enkephalins in stress-induced analgesia, particularly in those species with a large adrenal content of OLM. However, in one experiment, adrenalectomy failed to reduce the mild analgesia produced in the rat (a species with low content of OLM in its adrenal gland) by 2-deoxy-glucose, 700 mg/kg, i.p.[3]

[3] R. Vinegar, and O. H. Viveros. Unpublished observations.

An organ as easily accessible as the adrenal gland, with such large complement of stored OLM, should be useful to identify OLM other than met- and leu-enkephalin, and their possible precursors. The adrenal medulla may also be an excellent model to study the natural history of enkephalin-like-peptide-containing cells.

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