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Characterization of a morphine-like molecule in secretory granules of chromaffin cells

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A Study Design
B Data Collection
C Statistical Analysis
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Summary

Background: Morphine and morphine-like molecules were identified in numerous mammalian tissues. In particularly, morphine was present in adrenal gland and in PC-12 cells, corresponding to the cell line derived from rat adrenal medullary chromaffin cells.

Material/Methods: Morphine-like compounds were isolated after a deproteinization followed by a reverse phase high pressure chromatography purification coupled to a UV detector. This alkaloid was identified using nano-eletrospray ionization quadripole time of flight tandem mass spectrometry.

Results: We demonstrate the presence of a morphine-like molecule inside the bovine chromaffin cell secretory granules. This alkaloid was also detected into the secretion medium of primary chromaffin cells in culture following nicotinic stimulation.

Conclusions: Our findings demonstrate that a morphine-like component is synthesized in chromaffin cells and secreted into the incubation medium upon stimulation. This finding strongly suggests that this material may be released from adrenal tissues in response to stressors.

key words: **morphine • chromaffin cells • secretion • adrenal medulla • opiates**

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BACKGROUND

For the last 30 years, several studies have been directed toward proving that endogenous opiate alkaloids exist in animals and represent new chemical messengers within neural and other tissues [1,2]. In the past we demonstrated the *in vivo* production of morphine in an animal parasite [3] and recently the *de novo* morphine biosynthesis was demonstrated in human cancer cells [4]. This last study, as well as others, demonstrate that the morphine biosynthesis pathway can occur *via* dopamine [1,2,4].

In this regard, chromaffin cells are catecholaminergic neuroendocrine cells that mainly constitute the adrenal medulla. Chromaffin secretory granules contain a complex mixture of proteins (chromogranins), peptides and catecholamines [5], including dopamine, all co-released into the circulation in response to splanchnic nerve stimulation [5].

Using both biochemistry and cell biology approaches, we investigated the subcellular localization of morphine components. We show here for the first time the presence of morphine-6-glucuronide (M6G) in chromaffin cell granules and its secretion, suggesting a stress related use because of their adrenal gland localization.

MATERIAL AND METHODS

Isolation of the subcellular fractions of chromaffin cells

Intragranular matrix of chromaffin cells was isolated from bovine adrenal medulla dissected from 20 glands, according to procedure first described by Smith and Winkler [6] and previously modified by our group [7,8]. Purity of the subcellular fractionation was assayed as previously described by using subcellular markers [8].

Isolation of proteins released from stimulated chromaffin cells

Chromaffin cells were isolated from fresh bovine adrenal glands, cultured as previously reported [9] and plated in plastic Petri dishes. After three days in culture, chromaffin cells (2.5×10^6 cells) were subsequently stimulated for 10 min with 10 µM nicotine or 59 mM KCl in Locke's solution as previously described [9]. The extracellular medium of 2.5×10^6 non-stimulated cells (control) was collected, centrifuged at 800 g for 10min at 4°C to remove cells that might be present in secretions and acidified up to 0.1% trifluoroacetic acid in order to prevent proteolytic degradation [7].

Isolation of alkaloids present in chromaffin granules of chromaffin cells

Experiments using opiate alkaloid standards were performed in a different room to avoid morphine contamination of the biological samples tested. Siliconized microtubes were used to minimize the loss of morphine and morphine-like compounds. Intragranular matrix fraction (33 mg) was successively extracted with 1N HCl (1 ml) and 5 ml chloroform/isopropanol (9:1) [10]. After a quick shake and a 5 min stand at 4°C, homogenates were centrifuged (2,500 g, 15 min). The aqueous supernatant, containing alkaloids was adjusted to pH 5 with 2 M KOH, and then passed through a Sep Pak prior to HPLC analysis.

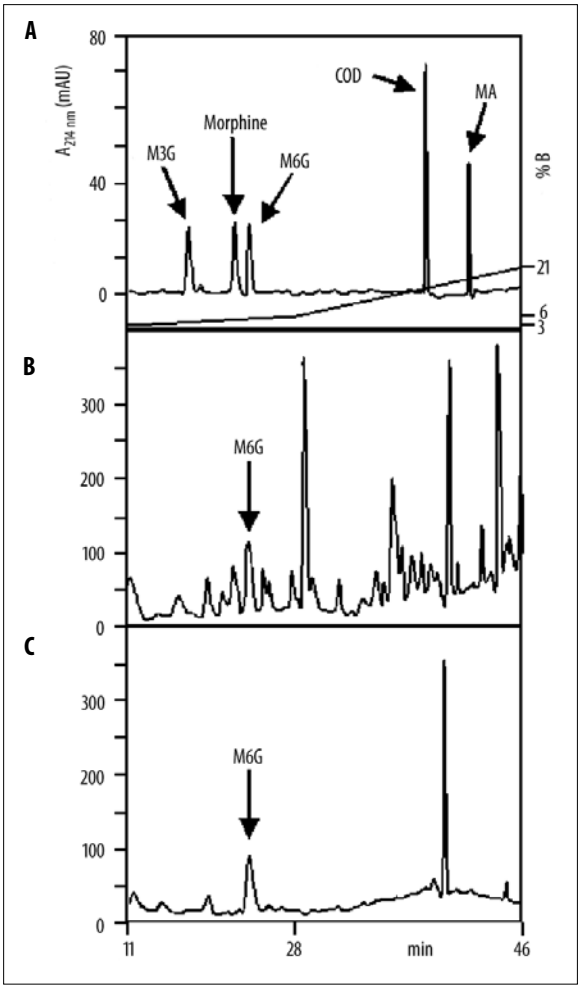

Figure 1. (**A**) HPLC of 500 pmoles of alkaloid standards (morphine, M3G, M6G, MA and COD). (**B**) HPLC purification of an extract from 33 mg of soluble bovine intragranular material. (**C**) HPLC purification of an extract of from primary chromaffin cell secretions after a stimulation with 10 µM of nicotine.

Aqueous supernatants containing morphine and its derivatives were loaded onto a Sep-Pak Plus C-18 cartridge (Waters, Milford, Massachusetts), previously activated with 100% acetonitrile (ACN) and washed with 0.1% trifluoroacetic acid (TFA) in water at a flow rate of 1ml per min. A first elution was performed using ACN/TFA/H_2O (5:0.1:94.9; v/v/v) in order to get rid of the highly hydrophilic components, whereas an elution at 50% ACN/TFA/H_2O (50:0.1:49.9; v/v/v) was performed in order to concentrate the morphine like-components. The collected fractions were concentrated with a Speed Vac (Savant Instruments, New York, USA) and stored at –20°C.

Purification of alkaloids by reverse phase HPLC

Alkaloid extractions and affinity elution were purified using an Äkta Purifier HPLC system (Amersham Pharmacia Biotech, Uppsala, Sweeden) and a Nucleosil reverse phase 300-5C18-column (4×250 mm; particle size 5 µm, porosity 300Å; Macherey Nagel, Hoerdt, France). Absorbance was monitored at 214 nm and the solvent system consisted of 0.1% (v/v) TFA in water (solvent A) and 0.09% (v/v) TFA

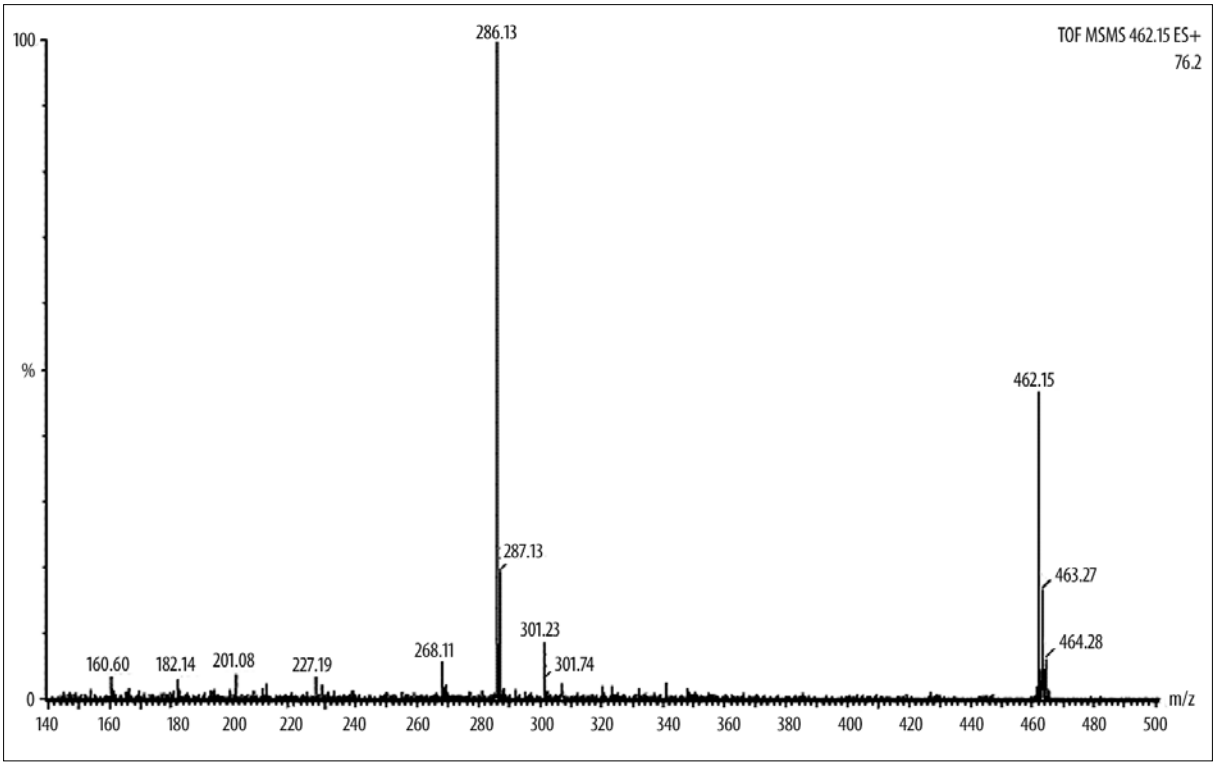

Figure 2. Q-TOF MS-MS analysis of a standard of the HPLC fraction corresponding to M6G (462.15 Da) present in the intragranular material (arrow Figure 1B).

in 70% ACN in water (solvent B). Elutions were performed at a flow rate of 700 µl/min using the gradient indicated on chromatograms. Internal standards corresponding to a fixed quantity of morphine, morphine-3-glucuronide (M3G), morphine-6-glucuronide (M6G), morphine acetate (MA) and codeine (COD), submitted alone or mixed with subcellular fractions, were loaded on HPLC in order to (i) determine of the retention times of these molecules and to (ii) to evaluate the recovery of morphine-like components after the same treatments as the subcellular fraction.

Mass spectra analysis

MS and MS-MS analyses were performed using electrospray mass spectrometry (ES-MS) on a Q-TOF II (Bio-Tech, Manchester, UK) in positive mode. Scanning was done from 50 to 700 m/z in 1s and calibration was performed by using phosphoric acid 0.1% in water/acetonitrinile 50/50. MS and MS-MS analysis were done by nanospray of line using "NanoES spray capillaries" from Protana (Odense, Denmark). For MS-MS experiments, argon 5.5 gas was used for the collision gas and the collision energy was set to 10–30 eV. MS-MS spectra were acquired using the selection of the precursor ion by the quadrupole and fragments were analyzed by the time of flight.

Results

After chromaffin cell subcellular fractionation, intragranular localization of morphine-like components was study using biochemical techniques. The morphine-like material was first extracted from the intragranular matrix. The alkaloid extract was loaded on a reverse phase HPLC column to purify and characterize the alkaloids present. The HPLC gradient was specifically designed to separate morphine, M3G, M6G, MA and COD (Figure 1A). Comparison of the chromatography profile of the intragranular material (Figure 1B) with the elution profile of standards (Figure 1A) shows the presence of a peak with the same retention time as M6G. In order to confirm the identity of this alkaloid, Q-TOF MS-MS analysis of the peak corresponding to M6G in this subcellular fraction was performed and positively identified as M6G (462.15 Da), according to its fragmentation profile generating morphine (*i.e.,* 286.13 Da; Figure 1B).

In order to characterize M6G in secretion from stimulated chromaffin cells, the extracellular medium was extracted and analyzed by reverse phase HPLC. After stimulation with 10 µM nicotine, the secreted material was submitted to HCl and chloroform/isopropanol extractions, followed a Sep Pak desalting. Alkaloids present in the aqueous phase were separated by HPLC on a reverse-phase C-18 column (Figure 1C), demonstrating a major peak corresponding to the M6G standard (Figure 1A). In experiments using the medium of resting chromaffin cells (same conditions without nicotine), no M6G release was detected.

Discussion

The present report demonstrates for the first time that M6G is present among the constituents of the secretory chromaffin granules of bovine adrenal medulla. Furthermore, M6G was identified in media incubating these cells after nicotinic stimulation, using biochemistry techniques (Figures 1,2). Interestingly, morphine is usually described to be catabolized in the liver by UDP-glucuronosyltransferase 2B enzyme family

(UGT2B) [11], leading to its transformation into M3G, which is totally inactive, and morphine M6G that displays stronger analgesic activity than morphine (50–600 times) [12].

Since M6G is secreted by the bovine chromaffin cells upon nicotinic stimulation (Figure 1C), we surmise that M6G is present in blood after stressful stimulation. In addition, our data suggests that the analgesia observed upon acute stress, usually related to enkephalins and corticoids release [13], might be due, in part, to M6G. Thus, after secretion, M6G may mediate several effects based on its affinity for μ opioid receptors (*i.e.,* analgesia), present on the numerous cell surfaces (neuroendocrine, endothelium and immune cells) [2,14–16]. Interestingly, there is literature supporting a separate and select μ opiate receptor for M6G. Thus, Rossi and colleagues, using an antisense probe targeting Gi alpha 1 in mammals, found that it blocked both heroin and M6G analgesia, but not morphine [15,16]. These results indicate that heroin, 6-acetylmorphine, fentanyl and etonitazine can all produce analgesia through a novel μ analgesic system, which is similar to that activated by M6G [17]. Antisense mapping studies on exons 1, 2 and 3 of MOR-1 (*i.e.,* μ1 opioid receptor) in mice suggest the presence of a novel mu receptor subtype responsible for M6G analgesia that may represent a splice variant of MOR-1 [18,19].

CONCLUSIONS

Taken together, the present study demonstrates the presence of specific biochemical and anatomical processes for mediating an opiate alkaloid signaling system, revealing a new chemical messenger system mediating neuroendocrine phenomena.

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