Identification of morphine-6-glucuronide in chromaffin cell secretory granules

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We report for the first time that morphine-6-glucuronide, a highly analgesic morphine-derived molecule, is present in adrenal chromaffin granules and secreted from chromaffin cells upon stimulation. We also demonstrate that PhosphatidylEthanolamine-Binding Protein (alternatively named Raf-1 Kinase Inhibitor Protein) acts as an endogenous morphine-6-glucuronide-binding protein. An UDP-glucuronosyltransferase 2B-like enzyme, described to transform morphine into morphine-6-glucuronide, has been immunodetected in chromaffin granule matrix and morphine-6-glucuronide de novo synthesis has been characterized demonstrating the possible involvement of intragranular UDP-glucuronosyltransferase 2B-like enzyme in morphine-6-glucuronide metabolism. Once secreted into circulation, morphine-6-glucuronide may mediate several systemic actions (e.g., on immune cells) based on its affinity for mu opioid receptors. These activities could be facilitated by PhosphatidylEthanolamine-Binding Protein, acting as a molecular shield and preventing morphine-6-glucuronide from rapid clearance. Taken together, our data represent an important observation on the role of morphine-6-glucuronide as a new endocrine factor.

For 20 years, cerebral endogenous morphine has been isolated and characterized and its structure shown to be identical to morphine from poppy (1,2). In the 1990s, few groups have succeeded in characterizing endogenous morphine from organs and fluids of vertebrates, including brain and adrenal gland from bovine, heart and adrenal gland from rat, human heart and urine (for review (1-3)), invertebrates (4-6) and parasites (7,8). In mammals, endogenous morphine synthesis pathway is associated with the biosynthesis pathway of dopamine and catecholamines (for review (1,3,9)). Then, very recently, several crucial steps were reached since Poeaknapo et al. demonstrated that morphine can be formed by a multi-step biosynthetic route (10,11), and Zhu et al. have shown that human primary polymorphonuclear cells are able to synthesize morphine (12). These authors have showed morphine de novo synthesis in human and animal primary and cancer cell cultures.

Chromaffin cells are neuroendocrine cells originating from the neural crest and are the predominant cell type in the adrenal medulla (see review (13)). These cells possess the catecholamine biosynthetic pathway leading to dopamine and adrenaline/noradrenaline synthesis (13). Chromaffin secretory granules contain a complex mixture of peptides and proteins that are
co-released with catecholamines into circulation in response to splanchnic nerve stimulation (13). Based on the morphine biosynthetic pathway and on the presence of morphine in bovine and rat adrenal gland (14,15), rat adrenal pheochromocytoma PC-12 cells (10,16) and eel chromaffin cells (17), we hypothesized that mammalian chromaffin cells might have the capacity to synthesize morphine and that their secretory granules could potentially release this alkaloid into the blood.

Several molecular blood transporters of clinically administrated morphine have been identified (e.g., serum albumin, gamma globulin (18) or alpha 1-acid glycoprotein (19)). In addition, it has been reported that the PhosphatidylEthenolamine-Binding Protein (PEBP) (20), alternatively named Raf-1 kinase inhibitor protein (RKIP) (21), is also able to bind morphine (22). We have recently reported that PEBP is present in secretory granules, as well as in the exocytotic medium of stimulated bovine primary cultured chromaffin cells and in bovine serum (23).

Exogenously administered morphine is catabolized in the liver by the UDP-glucuronosyltransferase 2B enzyme family (UGT2B) (24), leading to the formation of morphine 3-glucuronide (M3G) and morphine 6-glucuronide (M6G). M3G is totally inactive whereas M6G appears to display stronger analgesic activity than morphine (50 to 600 times depending on animal model) (25) as well as exhibiting much longer half-life (26).

Using immunochemical, biochemical and proteomic strategies, the present study reveals that bovine chromaffin cell secretory granules store M6G-PEBP complex. We were able to show that M6G is secreted from bovine chromaffin cells upon nicotinic stimulation. Co-immunoprecipitation and affinity chromatography experiments also revealed the interaction between the alkaloid carrier (i.e., PEBP) and an UGT2B-like enzyme necessary for the conversion of morphine into M6G. Finally, M6G de novo synthesis was demonstrated by using the UGT2B-like enzyme present in chromaffin granules.

**MATERIALS AND METHODS**

**Immunocytochemistry and confocal microscopy analysis**- Primary bovine chromaffin cells, cultured and fixed as previously described (23) were incubated for 2h at 37°C with a polyclonal rabbit anti-bovine chromogranin A (CGA124-143, dilution 1:200; ref. 25) and a commercial sheep polyclonal antibody against morphine-like compounds (8) (i.e., morphine, M3G, M6G and with minimal cross-reactivity to codeine as declared by the supplying company; dilution 1:1,000; Biogenesis, Bournemouth, U.K.) in NaCl/Pi buffer (0.9% NaCl (w/v), 25mM sodium phosphate pH 7.5) containing 5% bovine serum albumine (BSA). Cells were then washed (6 x 5min) with NaCl/Pi buffer and incubated with secondary antibodies (Cy-5-conjugate donkey anti-rabbit IgG at a dilution 1:2,000 and Alexa 488-conjugate donkey anti-sheep IgG at a dilution 1:2,000; Molecular Probes, Eugene, Oregon, USA) in NaCl/Pi buffer containing 5% BSA for 45min at 37°C.

UGT2B was immunodetected using a commercial goat polyclonal antibody against human UGT2B family (dilution 1:500; Santa Cruz Biotechnology, Santa Cruz, USA) and donkey Alexa 488-conjugated antibodies (dilution 1:2,000). Chromogranin A (CGA) was detected with rabbit polyclonal anti-bovine CGA124-143 (dilution 1:200, see above) and Cy-5-conjugate donkey anti-rabbit IgG (1:2,000).

All glass cover slips were mounted on a glass slide with a drop of Mowiol 4-88.

Various controls were carried out to assess antibody specificity: (i) cells without antibody; (ii) second antibody incubated alone; (iii) first antibody incubated with the respective antigen prior to immunolabelling. UGT2B-antibody was blocked with the commercial peptide (12h, 4°C, ratio 1:5 (w:w); Santa Cruz Biotechnology) and anti-morphine antibody with M6G (12h, 4°C, ratio 1:50, mol:mol).

Immunofluorescence staining was monitored with a laser scanning microscope (LSM 510, Zeiss, Gottingen, Germany) equipped with a planapo oil immersion objective (63X, numerical aperture 1.4) (23). Colocalization with CGA was quantified using Zeiss CLSM instrument software 2.8.
Isolation of chromaffin cell subcellular fractions- Chromaffin cell plasma membrane, cytoplasm, intragranular matrix and granule membrane were prepared as described by Smith and Winkler (27) and modified by us (23,28). After protein quantification using Bradford technique, the purity of subcellular fractions was assayed as previously described by using subcellular markers (23). Total microsomal fraction was purified according to Levesque et al. (29).

Isolation of proteins exocytotically released from stimulated chromaffin cells- Chromaffin cells (2.5x10^6) cultured for 3 days were washed 4 times 5min with 10 ml of Locke’s solution at 37°C in order to get rid of the culture medium and floating dead cells. Cells were subsequently stimulated for 10 min with 10 μM nicotine in Locke’s solution as previously described (28).

The extracellular medium of unstimulated cells and Locke’s solution were treated as controls. Secreted medium and controls were centrifuged at 800 g for 10 min at 4°C and filtered on a 0.22 µm syringe filter to remove cells that might be present in secretions. Samples were acidified up to 0.1% trifluoroacetic acid in order to prevent degradation (23).

Purification of alkaloids by reverse phase HPLC- Subcellular fractions (1 ml of the corresponding sucrose gradient fraction) were deproteinized and prepared as previously described (16). Alkaloids were purified using an Äkta Purifier HPLC system (Amersham Pharmacia Biotech, Uppsala, Sweden) and a Nucleosil reverse phase (RP) 300-5C18-column (4 x 250 mm; particle size 5 µm, porosity 300 Å ; Macherey Nagel, Hoerdt, France) as described (23). Gradients are indicated on chromatograms.

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/acetonitrile 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. The absence of M6G prior to sample application was systematically controlled using MS and MS-MS mode.

Affinity chromatography- Purified anti-bovine PEBP1-11 antibody (3 mg prepared as described in (30)) or commercial anti-human UGT2B antibody (200 μg) were coupled to a HiTrap NHS-activated HP 1ml column (Amersham Pharmacia Biotech) according to manufacturer’s instructions. Antibody-coupled column was first washed with 5 volumes of buffer A (75 mM Tris-HCl pH 8.0). Samples (i.e., cytoplasm, microsome, lysosome, mitochondria, intragranular matrix) resuspended in buffer A (2 mg in 1 ml final volume) were loaded on the column and let stand for 10 min at room temperature. The column was then washed with 10 volumes of buffer A prior to elution performed with 1 ml of buffer B (150 mM NaCl, 100 mM glycine, pH 3.0). The eluate was immediately neutralized with 20 µl of buffer C (1.5M Tris-HCl, pH 8.0) prior to RP-HPLC analysis or desalting.

Coimmunoprecipitation assays- 500 μg proteins isolated from chromaffin granule matrix was treated as previously described using anti-human UGT2B antibody (31). Immunoprecipitates were analysed by SDS-PAGE and Western blotting using the rabbit polyclonal antibody against bovine PEBP1-11 (1:2,000). A semi-quantitative approach, using different amounts of granule matrix protein was used to quantify the amount of PEBP bound to the UGT2B-like enzyme.

Gel electrophoresis and Western blot analysis- For mono-dimensional electrophoresis analysis, proteins were separated on SDS-PAGE containing 10% or 12% acrylamide and electrotransferred as described (23). For two-dimensional gel electrophoresis analysis, isoelectric focusing (IEF) was performed using the Protean IEF cell™ Isoelectric Focusing System (Bio-Rad) equipped with dry polyacrylamide gel strips (IPG strip, 11cm) using an immobilised pH gradient 4-7 (32).
Proteins were immunodetected with goat polyclonal antibodies raised against human UGT2B family (60 kDa, dilution 1:500) and immunorevealed with an HRP-conjugate anti-goat antisera. Control experiments on the antibodies were performed as described above. For gel silver staining, gel was incubated in ethanol : acetic acid : water solution (40% : 10% : 50%) and treated with Dodeca Silver Stain Kit (Bio-Rad, Marnes-la-Coquette, France) according to manufacturer’s instructions.

Tryptic digestion and mass spectrometry. Silver stained bands were excised and washed with 100 µl of 25 mM NH₄HCO₃, dehydrated twice with 100 µl of acetonitrile and dried with a Speed Vac evaporator before reduction (10 mM DTT in 25 mM NH₄HCO₃) and alkylation (55 mM iodoacetamide in 25 mM NH₄HCO₃) (33). For tryptic digestion, gel pieces were resuspended in three gel volumes of trypsin (12.5 ng/µl) freshly diluted in 25 mM NH₄HCO₃ and incubated overnight at 35°C. The digested peptides were then extracted from gel in a buffer containing 25% H₂O, 70% acetonitrile, 5% formic acid and analyzed by matrix assisted laser desorption/ionization time-of-flight (MALDI TOF) and LC/MS/MS. For nano-HPLC, a CapLC system (Micromass Ltd., Manchester, UK) was used. Samples were concentrated on a precolumn and peptides were separated on a 15 cm x 75 µm i.d. column packed with 3 µm 100 Å C18 PepMap (LC-Packings). The MS and MS/MS analyses were performed with a Q-TOF 2 hybrid quadrupole/time-of-flight mass spectrometer (Micromass Ltd., Manchester, UK). Data analysis was performed with Global Server (MicroMass, Ltd., Manchester, UK) software and Mascote (Matrix Science Ltd., London, UK) against NCBI (The National Center for Biotechnology Information) database.

M6G de novo synthesis- UGT2B was purified by affinity chromatography on an Äkta Purifier HPLC system. The eluted material was desalted on Sep Pak cartridge and analyzed by Western blot using an anti-UGT2B antibody in order to confirm the presence of UGT2B-like material. Eluted fraction was also submitted to gel migration followed by silver staining. Silver stained bands were excised and treated as described above. Affinity-purified UGT2B-like enzyme was dried and incubated 3 h at 37°C in a reaction volume of 100 µl (100 µM UDP-glucuronic acid, 2 mM saccharolactone, 0.9 µM morphine, 50 mM Tris HCl, pH 5.5). The resulting sample was deproteinized and desalted prior to Q-TOF MS-MS analysis to examine the presence of M6G. Controls experiments were performed with the boiled affinity column eluate, the reaction buffer alone and morphine used for the assay.

Protein deglycosylation- Intragranular UGT2B-like enzyme was deglycosylated using an Enzymatic Protein Deglycosylation Kit (Sigma Aldrich) in presence of Triton X100. Total deglycosylation was carried on using PNGase F, O-glycosidase, α-(3,6,8,9)-neuraminidase, β-1,4-galactosidase and β-N-acetylglucosaminidase. The non-deglycosylated control and the resulting deglycosylated products were loaded on a 12% SDS-PAGE gel prior to electrotransfer. Deglycosylation method efficiency was tested on a control glycosylated protein (fetuin, 10 µg) provided in the manufacturer’s kit.

RESULTS

Immunolabelling of morphine-like components in chromaffin cells
We extend our previous observations related to the presence of morphine and its derivatives in adrenal medulla and PC-12 (10,14-16) by investigating the subcellular localization of morphine and its derivatives in chromaffin cells by laser confocal microscopy. Morphine localization was compared with CGA immunoreactivity, a specific marker of the intragranular matrix of secretory granules (23). Morphine-like immunolabelling (Fig. 1A, green) was observed as bright dots in the cytoplasm, similar to that obtained with CGA (Fig. 1A, red). The superimposition of the two labelled materials may suggest a partial intragranular colocalization in chromaffin granules (Fig. 1A and B, Merge and arrows). Control experiments using anti-morphine antibody blocked with M6G prior to immunocytochemistry experiments or secondary antibody alone (Fig. 1C) were carried out to determine the specificity of labelling.
Characterization of M6G in chromaffin intragranular matrix

In order to clearly demonstrate the presence of M6G in chromaffin granules, the intragranular matrix of bovine (33 mg) was isolated and loaded on a RP-HPLC column to purify and characterize the alkaloids present. The HPLC gradient was specifically designed to separate M3G, morphine, M6G, COD and MA (500 pmoles ; Fig. 2A). Comparison of the chromatography profile of the deproteinized intragranular material with the elution profile of standards indicated the presence of a peak with the same retention time as M6G. Q-TOF MS-MS analysis of this peak allowed its unambiguous identification as M6G (462.15 Da) according to its fragmentation profile generating morphine (i.e., 286.13 Da ; Fig. 2B).

Identification of M6G secreted from nicotine-stimulated cultured chromaffin cells

In order to characterize M6G as a secretory product from stimulated chromaffin cells, alkaloid standards (390 pmoles ; Fig. 3A1) and extracellular medium were analysed by RP-HPLC. After stimulation with 10 µM nicotine, the secreted material was submitted to 1N HCl and chloroform/isopropanol extractions. Alkaloids present in the aqueous phase were separated by HPLC on a RP C18 column (Fig. 3A2). A major peak corresponding to the M6G standard was observed.

In extracellular media corresponding to basal secretions, M6G could not be detected (Fig. 3A3) demonstrating the genuine release of M6G upon nicotine stimulation.

Q-TOF MS-MS analysis of M6G-corresponding peak unambiguously demonstrated the presence of M6G (462.15 Da) according to its fragmentation profile whereby morphine is generated (i.e., 286.13 Da ; Fig. 3B). Similar analysis revealed the absence of M6G in secretions from unstimulated cells (Fig. 3C).

Evidence for interaction between PEBP and M6G

We recently reported that PEBP is present in chromaffin granules and able to translocate from cytoplasm to the intragranular space probably via a granule membrane raft-binding mechanism (23). Based on this property, we then examined the capacity of PEBP to bind intragranular morphine-like components using immunoaffinity experiments carried out on intragranular matrix, cytoplasm and microsome, mitochondria, as well as on lysosome fractions. Immunoaffinity experiments using the purified antibody directed against PEBP$_{1-11}$ were carried out to isolate the putative intragranular PEBP-morphine-like complex(es). Subcellular fraction extracts (20 mg each) were loaded on a HighTrap-NHS column coupled with the anti-PEBP$_{1-11}$ antibody. The eluted PEBP binding-molecules were concentrated and then purified by RP-HPLC. The chromatograms on figure 4A represent the elution profile of a mixture of 500 pmol of standards (Fig. 4A1 ; M3G, morphine, M6G, COD and MA), as well as the purification of immunoaffinity eluate from 20 mg of intragranular matrix (Fig. 4A2), cytoplasm and microsome (Fig. 4A3), mitochondria and lysosomal fractions (Fig. 4A4 and 4A5). Peaks marked with arrows on chromatograms representing the affinity elution of granule matrix, cytoplasm and microsomes (Fig. 4A2 and 4A3), correspond to the elution time of the M6G standard. Only the intragranular matrix and, at a lower level, the fraction corresponding to the cytoplasm and microsomes, contain M6G-like molecules, but not morphine. MS-MS Q-TOF mass spectrometry analysis of the M6G-corresponding peak of the elution from the granule matrix (Fig. 4A2) detected M6G (462.15 Da) and its fragmentation-derived fragment (i.e., morphine 286.13 Da ; Fig. 4B). Control experiments using MS and MS-MS mode on the elution buffer confirmed that the eluted M6G did not result from contamination (Fig. 4C).

Subcellular localization of UGT2B-like enzyme in chromaffin cells

Given the presence of M6G in secretory granules, the presence of an enzyme able to convert morphine to M6G was investigated. It has been reported that UGT2B family enzymes which act for this specific enzymatic reaction are present in the microsomal fraction of several organs including in liver (34). Immunocytochemical experiments were performed on primary cultured bovine chromaffin cells using antibodies specific to UGT2B enzymes. Confocal laser microscopy revealed UGT2B immunoreactivity as dispersed dotted pattern in cytoplasm (Fig. 5A, green
labelling), that was found to colocalize partially with CGA immunoreactivity (Fig. 5A, red). At higher magnification, the colocalization of UGT2B with the granular marker CGA was further suggested particularly in cell extensions (Fig. 5B).

UGT2B labelling specificity was assessed by absorbing antibody with the commercial UGT2B-derived peptide or by incubating the second fluorescent antibody alone (Fig. 5C).

In order to confirm UGT2B-like enzyme localization, SDS-PAGE followed by Western blot analysis was performed on the intragranular matrix and microsomal fractions. In this experiment, 10 µg of each fraction was loaded on gel and immunodetection was carried on with the anti-UGT2B antibody. Analysis of the chromaffin granule matrix extract showed a strong labelling as a unique band with an apparent molecular mass of 55-60 kDa, as expected for UGT2B enzymes (34) (Fig. 6A, lane 1). One band with same molecular mass was also detected in the microsomal fraction (Fig. 6A, lane 2). Control experiments using adsorbed anti-UGT2B antibody and commercial blocking peptide confirmed antibody specificity (Fig. 6A, lane 5).

Immunoprecipitation experiments using anti-UGT2B antibodies were performed to investigate if a putative complex involving UGT2B and PEBP exist in the granule matrix. These data have indicated that a complex between UGT2B-like enzyme and PEBP exist in chromaffin granules since PEBP is co-precipitated by the antibody directed against UGT2B family (data not shown).

**Evidence for glycosylation of UGT2B-like enzyme**

The UGT2B family has been described to be N-glycosylated, glycosylation being involved in their functional properties (35). On this basis, deglycosylation experiments were designed on the UGT2B-like enzyme present in chromaffin granules (Fig. 6, lane 3). Granular extract was treated with PNGase F, O-glycosidase, α-2(3,6,8,9)-neuraminidase, β-1,4-galactosidase and β-N-acetylglucosaminidase. Total deglycosylation (Fig. 6A, lane 4) was accompanied by band shift in comparison with that of the intragranular material (Fig. 6A, lanes 1 and 3). Control deglycosylation assayed on fetuin showed the effective deglycosylation (data not shown).

The physico-chemical characteristics of the UGT2B-like enzyme were examined on 2D-gel electrophoresis using 300 µg of intragranular protein material. A single board spot at 55-60 kDa and pI of 5 was observed (data not shown) suggesting the structural heterogeneity of the protein.

**Evidence for M6G de novo synthesis by UGT2B-like enzyme**

De novo synthesis experiment was designed in order to determine whether the UGT2B-like enzyme present in intragranular matrix is able to transform morphine into M6G. The UGT2B-like enzyme was first purified from intragranular matrix using an anti-UGT2B affinity column. The resulting eluate was then separated by SDS-PAGE.

A Western blotting analysis using anti-UGT2B antibody showed an immunoreactive band at a molecular mass of 55 kDa (Fig. 7A). This immunoreactive band was identical to the one obtained with 5 µg of recombinant human UGT2B7 (Fig. 7A; E.C.#2.4.1.17, Sigma Aldrich).

To complete this work, a duplicate gel was silver stained. A band at 55 kDa corresponding to the putative UGT2B-like enzyme, as well as other bands were observed (Fig. 7B). Thus, four additional bands were observed at 24, 26, 28 and 60 kDa. Proteomic analysis indicated the presence in the 24, 26, and 28 kDa bands of PEA1-165, PEA1-196 and PEA1-209 fragments, respectively (36) (data not shown). The proteomic analysis of the 55 kDa silver stained band did not show a similarity to a known protein. UGT2B enzyme was not identified because of the absence of bovine UGT2B in data protein data banks and the poor conservation UGT2B proteins that was described to be a barrier to identify orthologues across species (for review: (37)).

M6G de novo synthesis experiments were carried out at the intragranular pH of 5.5 (13) using the affinity eluate. After incubation, the reaction medium was deproteinized and desalted in order to analyse the presence of newly synthesized M6G. Q-TOF MS-MS analysis unambiguously demonstrated the presence of M6G (462.15 Da) and its characteristic fragmentation profile (286.13 Da; Fig. 7C). Q-TOF MS and MS-MS analyses performed on the reaction buffer (UDP-glucuronic acid, saccharolactone and Tris HCl), the commercial morphine and the affinity eluate prior...
to incubation indicated the absence of M6G (data not shown). An additional control was performed by incubating with boiled affinity-eluted UGT2B-like enzyme (Fig. 7D). A 464.16 Da molecular mass component was visible that corresponded to an unidentified molecule; it did not display the specific M6G fragmentation pattern at 286.13 Da corresponding to morphine. This last control demonstrated that the formation of M6G was totally abolished upon enzyme heat inactivation.

**DISCUSSION**

**Presence of M6G into chromaffin granules**

In the present work we demonstrate for the first time that M6G (and not morphine) is present in secretory chromaffin granules of bovine adrenal medulla. Since P450 enzymes are localized in the ER membrane of these cells, we surmise that morphine is synthesized in the ER-Golgi apparatus by salutaridine synthase (i.e., cytochrome P450 reductase) (38). Using together immunocytochemical experiments and biochemical techniques, M6G was identified within chromaffin granules. Some diffuse non-granular labelling can be attributed to morphine-like components present in the ER.

**Presence of a PEBP-M6G complex into chromaffin granules**

Recently the cytoplasmic PEBP, also named RKIP (20) has been identified in bovine adrenal gland and chromaffin cells (23,39). In addition, its presence in chromaffin intragranular matrix, secretion materials and serum has been shown by our group (23). PEBP has been described to bind various molecules, including indocyanine, phosphatidylethanolamine, bromosulfophtaleine and hormones, such as oestradiol-17β and dehydroepiandrosterone (40). It was thus postulated that PEBP might represent an organic anion transporter (OAT) with the same affinity as bovine serum albumin (40). In the present study we further demonstrate that in the chromaffin intragranular matrix, M6G is a novel endogenous ligand to PEBP. This binding data is in agreement with previous reports that described PEBP as a morphine-binding protein (22).

In our experimental conditions, it is important to point out that the quantity of M6G could not be determined precisely because a part of the M6G bound to PEBP has been lost probably during the deproteinsation step (i.e., precipitation of a part of the PEBP-M6G complex).

**Presence of a UGT2B-like enzyme within chromaffin granules**

Once synthesized, we surmise that morphine could bind to PEBP in the early granule stage, before its presentation to a conversion enzyme (i.e., UGT2B–like enzyme), producing the final active M6G material. Our data suggest that such a complex, comprising UGT2B-like enzyme and PEBP exists in these experimental conditions.

UDP-glucuronosyltransferases (UGTs) represent a superfamily of glycosylated enzymes (35) localized into the endoplasmic reticulum (29,41) which catalyses the glucuronidation reaction of several drugs and steroid hormones. The glucuronidated products are more polar, less toxic and easier to excrete through bile or urine. According to their sequence homologies, UGTs include two classes : UGT1 and UGT2, the later subdivided in 2A and 2B components (41). To date, the UGT2B subfamily catabolize morphine into M3G and M6G (24) and amongst these, the UGT2B7 (42,43) appears to be the only one able to produce M6G from morphine. Using immunochemical techniques, we have shown the presence of UGT2B-like immunoreactivity in chromaffin granules. Our data also demonstrate the presence of a glycosylation on the intragranular UGT2B, a posttranslational modification reported to occur on other UGT2B family members (35). The affinity-purified chromaffin granule UGT2B has been shown to transform morphine into M6G, suggesting for the first time its implication in a metabolic process. Up to now, this property of UGT2B has never been reported.

In our experiments, the proteomic analysis of the 55 kDa silver stained band (Fig. 7B) did not show similarity to any protein because of the lack of bovine UGT2B sequences in the protein databases or the presence of a new UGT2B variant. Indeed, 12 different UGT2B genes exist in humans (7 genes and 5 pseudogenes) and many remnant genes are also present (for review: (37)), whereas no UGT2B gene or protein were described in cow. UGT2B proteins display with 70% of similar sequences, the higher conservation
being present in their C-ter domain. This low conservation was described to be a barrier to identify orthologues across species (37).

**Relevance of PEBP-M6G complex upon stress situation**

Since PEBP and M6G are bound together within granule matrix and are cosecreted from bovine chromaffin cells upon stimulation (see Fig. 3) (23), it is likely that the PEBP-M6G complex exists after secretion and is present into blood. A large variety of proteolytic enzymes are present in chromaffin secretory granules, acting to process precursor proteins such as PEA and chromogranins/secretogranins (36). In contrast, intragranular PEBP is highly resistant to proteolytic degradation (23). PEBP may also be unaffected by proteolysis in circulation, thus protecting M6G for bodily clearance (i.e., excretion into urine and/or bile).

Furthermore, our data suggest that the analgesia observed upon acute stress (often related, but never totally explained by enkephalin and corticoid release (44)) might also be due, totally or in part, to M6G secretion, with PEBP acting as a transporter and molecular shield. After secretion, M6G may mediate several systemic actions based on its affinity for mu opioid receptors present on the cell surface of neurons, neuroendocrine cells, endothelium and immune cells (2,45-47).

Interestingly, data from literature support a separate and select mu opiate receptor for M6G. Thus, Rossi et al., using an antisense probe targeting Gi alpha 1 in mammals found that both heroin- and M6G-evoked analgesia but not that induced by morphine were blocked (48,49). These results show that heroin, 6-acetylmorphine, fentanyl and etonitazine can all produce analgesia through a novel mu analgesic system which is similar to that activated by M6G (48). Antisense mapping studies on exons 1, 2 and 3 of MOR-1 (i.e., mu1 opioid receptor) in mice suggested the presence of a novel mu receptor subtype responsible for M6G analgesia that may represent a splice variant of MOR-1 (50,51). In addition we previously demonstrated that in three murine macrophage cell lines (J774.2; RAW 264.7; BAC1.2F5), the mu opiate receptor subtype is mu3 because it binds only opiate alkaloids (i.e., M6G), excluding M3G or any of the opioid peptides tested (52).

**Clinical relevance of M6G for pain control**

It is known that M6G may have a greater affinity to mu1 opioid receptor (responsible for analgesia) than to mu2 (responsible for respiratory depression), suggesting that M6G can offer benefit as systemic analgesic (53,54). M6G is presently under Phase III trial in post-operative pain (CeNeS pharmaceutical, Cambridge, U.K.) highlighting its biological potential.

Interestingly, since 20 years, cellular therapy using adrenal chromaffin cell transplants was tested for pain management in both acute and chronic pain models (55,56). The resulting antinociceptive effect was related to the secretion of opioid peptides and catecholamines from the transplants. Taking into account the present findings and the highly potent analgesic activity of M6G, we suggest that this alkaloid also participates in the analgesia observed in those early experiments.

Taken together, the present data may represent important evidence in cell biology and physiology suggesting a new role of M6G in response to stress.

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**FOOTNOTES**

This work was funded by Inserm, the University Louis-Pasteur at Strasbourg, the Région Alsace (Ph.D grant to C.M.), the French Ministère délégué à la Recherche et à l'Enseignement Supérieur (Ph.D grant to E.G. and A.M.), the Fondation pour la Recherche Médicale (M.H.M.B.), the Ligue Contre le Cancer (DA), the Hôpital Universitaire de Strasbourg PHRC 3150 (M.H.M.B.). We thank N. Aslan for his technical help.
ABBREVIATIONS

BSA, bovine serum albumine; CGA, chromogranin A; COD, codein; DBH, dopamine-β-hydroxylase; HPLC, high performance liquid chromatography; M3G, morphine-3-glucuronide; M6G, morphine-6-glucuronide; MA, morphine acetate; MS, mass spectrometry; PEA, proenkephalin-A; PEBP, phosphatidylethanolamine-binding protein; PNMT, phenylethanolamine N-methyl transferase; PVDF, polyvinylidifluorene; RP, reverse phase; TFA, trifluoroacetic acid; UGT2B, UDP-glucuronosyltransferase 2B.

FIGURE LEGENDS

Fig. 1. Immunolabelling of morphine-like components in bovine primary cultured chromaffin cells. A, Double immunofluorescence confocal micrographs with anti-CGA antibody detected with Cy-5-conjugate IgG (red) and with antibody against morphine-like molecules revealed with Alexa 488-conjugate IgG (green). Colocalized immunolabellings (arrows and merge window) were revealed by yellow staining, showing double labelled pixel in images of CGA- and morphine-labelling recorded simultaneously in the same optical section. B, Higher resolution showing the immunolabelling in a chromaffin cell neurite-like extension showing visible individual granules. C, Control experiments performed with M6G-adsorbed anti-morpine antibody or with secondary antibody alone.

Fig. 2. HPLC-purification and mass spectrometry analysis of morphine-derivatives present in bovine chromaffin granules. A, Top panel, RP-HPLC purification of standards (M3G, morphine, M6G, COD and MA). Lower panel, purification of alkaloid extracted from soluble bovine intragranular material. B, Q-TOF MS-MS analysis of the HPLC fraction corresponding to M6G present in the intragranular material (marked with arrow in Fig. 2A, granule matrix).

Fig. 3. Identification of morphine-derivatives secreted from primary bovine chromaffin cells. A, (1) RP-HPLC purification of alkaloid standards; (2) extracted material present in secretory fluid from primary chromaffin cells in culture stimulated with 10 mM nicotine; (3) extracted material present in the medium of unstimulated chromaffin cells. B, Q-TOF MS-MS analysis of the HPLC fraction corresponding to M6G present in the material secreted from chromaffin cells (A2, arrow). C, Q-TOF MS analysis of the corresponding fraction in control (basal secretion; A3).

Fig. 4. Characterization of morphine-derivatives bound to PEBP. A, RP-HPLC of components eluted from the anti-PEBP<sub>1-11</sub> affinity chromatography. (1) Chromatography of M3G, morphine, M6G, COD and MA standards. RP-HPLC of affinity elution of 20mg of (2) intragranular, (3) cytoplasm and microsomes, (4) mitochondria and (5) lysosomal subcellular fractions. B, Q-TOF MS-MS spectra analysis of the M6G-like fraction from affinity-eluted intragranular fraction. C, Q-TOF MS spectra analysis of the control elution medium.

Fig. 5. Immunolabelling of UGT2B in bovine primary cultured chromaffin cells. A, Double immunofluorescence was performed on chromaffin cells with the antibody against human UGT2B family revealed with Alexa 488-conjugated antibodies (green) and with the anti-CGA<sub>124-143</sub> antibody detected with Cy-5-conjugate IgG (red). Colocalized immunolabellings were revealed as yellow staining (arrows and merge window) showing a double labelled pixel in images of CGA- and UGT2B-labelling recorded simultaneously in the same optical section. B, Immunolabelling of a chromaffin cell extension showing individual granules. C, Control experiments performed using UGT2B-adsorbed anti-UGT2B antibody and secondary antibody Alexa 488 alone are shown.

Fig. 6. Characterization of an UGT2B-like enzyme in chromaffin granule matrix by Western blot analysis. Immunodetection was carried out with the antibody directed against the UGT2B family. The
immunoreactivity at 55-60 kDa corresponds to UGT2B present in intragranular matrix (lane 1, 10 µg) and microsomal proteins (lane 2, 10 µg). Lane 3, UGT2B-like enzyme purified from 3 mg granule matrix extract. Lane 4, total deglycosylation of purified UGT2B-like enzyme from 3 mg granule matrix extract. Lane 5, immunodetection performed on intragranular matrix extract using UGT2B antibody adsorbed with the commercial corresponding blocking peptide.

Fig. 7. M6G de novo synthesis experiment. A, Western blot analysis of the UGT2B-like affinity purified from 4 mg intragranular matrix and of commercial recombinant human UGT2B7 (5 µg) using the anti-UGT2B antibody recognizing the 55 kDa form. B, Silver staining of 5 µg intragranular matrix and of the affinity purified UGT2B-like enzyme resulting from UGT2B-affinity purification performed on 4 mg intragranular matrix. Silver stained bands analyzed by mass spectrometry after digestion corresponded to PEA or CGA fragments. C, Q-TOF MS-MS analysis of de novo M6G synthesis performed on affinity-purified UGT2B-like enzyme collected from an assay on 13 mg intragranular proteins. D, Q-TOF MS analysis of de novo M6G synthesis using boiled purified UGT2B-like enzyme showing the absence of M6G and of morphine.
Figure 1

A

Morphine

CGA

Merge

10 μm

B

Morphine

CGA

Merge

2 μm

C

Control

M6G-adsorbed

Control

Alexa 488
Figure 2
Figure 4
Figure 5
Figure 7
Identification of morphine-6-glucuronide in chromaffin cell secretory granules
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J. Biol. Chem. published online January 24, 2006

Access the most updated version of this article at doi: 10.1074/jbc.M502298200

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