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Phosphatidylethanolamine-binding protein (PEBP), alternatively named Raf-1 kinase inhibitor protein, is the precursor of the hippocampal cholinergic neurostimulating peptide (HCNP) corresponding to its natural N-terminal fragment, previously described to be released by hippocampal neurons. PEBP is a soluble cytoplasmic protein, also associated with plasma and reticulum membranes of numerous cell types. In the present report, using biochemistry and cell biology techniques, we report for the first time the presence of PEBP in bovine chromaffin cell, a well described secretion model. We have examined its presence at the subcellular level and characterized this protein on both secretory granule membranes and intragranular matrix. In addition, its presence in bovine chromaffin cell and platelet exocytotic medium, as well as in serum, was reported showing that it is secreted. Like many other proteins that lack signal sequence, PEBP may be secreted through non-classic signal secretory mechanisms, which could be due to interactions with granule membrane lipids and lipid rafts. By two-dimensional liquid chromatography-tandem mass spectrometry, HCNP was detected among the intragranular matrix components. The observation that PEBP and HCNP were secreted with catecholamines into the circulation prompted us to investigate endocrine effects of this peptide on cardiovascular system. By using as bioassay an isolated and perfused frog (*Rana esculenta*) heart preparation, we show here that HCNP acts on the cardiac mechanical performance exerting a negative inotropism and counteracting the adrenergic stimulation of isoproterenol. All together, these data suggest that PEBP and HCNP might be considered as new endocrine factors involved in cardiac physiology.

Chromaffin cells are derived from the neural crest and constitute the adrenal medulla. In the adrenal medullary chromaffin cell, secretory granules contain a complex mixture of proteins and peptides that are co-released with catecholamines into the circulation in response to splanchnic nerve stimulation (1). Among the high molecular mass water-soluble proteins, proenkephalin-A and the chromogranins family constitute the major constituents of chromaffin granules with other neuropeptides (neuropeptide Y, vasointestinal peptide, and others). These proteins are actively processed in the intragranular matrix to peptides with various molecular weight (1–3). Recently, using highly sensitive proteomic techniques we have characterized the maturation products of proenkephalin-A (4) and established the presence of other unexpected proteins (5).

Phosphatidylethanolamine-binding protein (PEBP)[1] (6, 7)/Raf-1 kinase inhibitor protein (8) has previously been described in brain and adrenal gland (9, 10). PEBP is a 21-kDa protein initially described as a cytoplasmic protein and later found to be associated with plasma or reticulum membranes (11). This protein has been found in numerous tissues of many species, including rat testis, liver, kidney, and human platelets (6, 9, 10), indicating its ubiquitous occurrence. Several reports have also described the presence of PEBP immunoreactivity in biological fluids, including rat haploid testicular germ cell secretions (12), testicular interstitial fluid (13), the culture media of transfected Rat-1 fibroblasts (14), and more recently in the conditioned medium from adult rat hippocampal progenitors (15). PEBP and its N-terminal-derived peptide named hippocampal cholinergic neurostimulating peptide (HCNP), corresponding to the first eleven N-terminal amino acid residues ($PEBP_{1–11}$), were also found in synaptic vesicles after subcellular fractionation and were detected by immunohistochemistry in nerve cell terminals of neurons in rat brain (10) and small intestine (16). Interestingly, HCNP has also been detected in the cerebrospinal fluid of Alzheimer patients (17) and shown to be released from rat hippocampal neurons (10), as well as by murine adipocytes (18). Because PEBP lacks signal

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[1] The abbreviations used are: PEBP, phosphatidylethanolamine-binding protein; CGA, chromogranin A; CGG, chromaffin granule ghost; DBH, dopamine-β-hydroxylase; HCNP, hippocampal cholinergic neurostimulating peptide; HPLC, high performance liquid chromatography; PNMT, phenylethanolamine *N*-methyl transferase; PVDF, polyvinylidene difluoride; TRAP, thrombin receptor-activating peptide; LC/MS/MS, Liquid chromatography-tandem mass spectrometry; Q-TOF, quadrupole time-of-flight; ES/MS, electrospray mass spectrometry.

sequence, this protein might follow a non-classic secretion pathway as many others proteins (19).

Because PEBP has been previously immunodetected in adrenal gland, in this report we looked for the localization of PEBP and HCNP in chromaffin cells using confocal laser and electronic microscopy, subcellular fractionation, Western blot analysis, purification by reverse-phase HPLC, sequencing, and mass spectrometry analysis techniques. We found them to be present in chromaffin secretory granules. They were also detectable in platelet secretions and in serum. Because upon a stimulation catecholamines display a positive inotropic effect on heart physiology (for review, see Ref. 20) and on the basis of our recent data showing the negative inotropic effect on frog heart (21) of a natural intragranular N-terminal chromogranin A (CGA)-secreted peptide, vasostatin-I, we have examined the effect of HCNP, the natural N-terminal PEBP-derived fragment on cardiac mechanical performance. The experimental model chosen was the avascular frog heart (*Rana esculenta*), isolated and working at physiological loads that represent an ideal system to explore the specific role of various cardioactive molecules on heart physiology (22). We have shown that HCNP acts on the cardiac physiology, indicating that it may be considered, with its precursor, as new endocrine factors.

EXPERIMENTAL PROCEDURES

Immunocytochemistry and Confocal Microscopy Analysis—Primary bovine chromaffin cells were seeded at a density of 2×10^5 cells per well on glass coverslips (1-cm diameter) coated with fibronectin and treated as previously described (23). Cells were incubated for 2 h at 37 °C with either monoclonal mouse anti-human $CGA_{367–379}$ (dilution 1:2) or affinity repurified rabbit polyclonal antibody against bovine PEBP (dilution 1:100) in NaCl/P_i buffer (0.9% NaCl (w/w), 25 mM sodium phosphate at pH 7.5) containing 3% (m/v) bovine serum albumin. Cells were then washed (6×5 min) with NaCl/P_i buffer and incubated with secondary antibodies (Cy-3-conjugate goat anti-mouse IgG or Cy-5-conjugate goat anti-rabbit IgG at both the dilution 1:2,000) in NaCl/P_i buffer containing 3% (m/v) bovine serum albumin for 45 min at 37 °C. Finally, glass coverslips were washed twice in NaCl/P_i buffer and deionized water and mounted upside down on a glass slide with a drop of Mowiol 4-88. Various controls were carried out to assess antibody specificity and nonspecific labeling (omission of first antibody and absorption of the first antibody with the respective antigen prior to cell immunolabeling). Immunofluorescence staining was monitored with a Zeiss laser scanning microscope (LSM 510 inverted; Gottingen, Germany) equipped with a planapo oil immersion objective (63×, numerical aperture 1.4). Cy-5 was excited using the He/Ne laser 643-nm line. A long pass filter of 665 nm was used for Cy-5 fluorescence and was represented in red. Cells were subjected to optical serial sectioning to obtain images in the *x-y* plane. Each optical section was scanned eight times to obtain an average image. Images were recorded digitally at 768×576 pixels.

Immunoelectron Microscopy—For electron microscopy, 2- to 3-mm-thick transverse slices of fresh bovine adrenal gland were fixed by immersion for 2 h at 4 °C with freshly prepared NaCl/P_i buffered 4% (v/v) paraformaldehyde. The tissue was stored overnight at 4 °C in NaCl/P_i buffer and we have prepared vibratome tissue sections at 60 μm. Immunohistochemistry was performed as previously described (24). Briefly, after blocking nonspecific binding sites, free-floating vibratome sections were incubated successively in rabbit anti-RKIP/PEBP antibody (diluted 1:300 in NaCl/P_i buffer) and, after washing in phosphate-buffered saline, in biotinylated donkey anti-rabbit IgG (diluted 1:200 in NaCl/P_i buffer). Sections were then incubated with avidin-biotin-peroxidase complex (Vectastain ABC kit, Vector, CA), and peroxidase activity was detected with 3,3′-diaminobenzidine tetrahydrochloride and H_2O_2. Vibratome sections were then post-fixed in 0.1 M sodium cacodylate-buffered 1% osmium tetroxide (pH 7.2) for 90 min, rinsed in the same buffer, dehydrated in increasing concentrations of ethanol, and flat embedded in Spurr's resin. Thin sections, cut parallel to the surface of the original vibratome sections, were viewed without counterstaining at 60 kV with an Hitachi 7500 electron microscope (Hitachi, Tokyo, Japan), and images were obtained with an AMT digital camera system (Advanced Microscopic Techniques Corp., Danvers, MA). Controls were performed in parallel omitting the primary antibody.

Isolation of the Subcellular Fractions of Chromaffin Cells—Subcellular fractions (*i.e.* plasma membrane, cytoplasm, soluble granule matrix, and granule membranes) of chromaffin cells were isolated from bovine adrenal medulla dissected from 20 glands, according to a procedure first described by Smith and Winkler (25) and previously modified by our group (5). The soluble intragranular matrix was separated from granule membrane by centrifugation at $100{,}000 \times g$ (30 min at 4 °C), collected, acidified with trifluoroacetic acid up to 0.1% to prevent proteolytic degradation, and stored at −20 °C (4). Pellets corresponding to granule membranes were suspended in NaCl/P_i buffer and centrifuged at $100{,}000 \times g$ during 30 min. To avoid possible contamination with intragranular matrix components, this procedure was repeated three times. Proteic material present in granule membranes was extracted by incubation during 2 h at 4 °C in 1 mM EDTA, 150 mM NaCl, 0.2 mg/ml aprotinin, 50 mM octyl-β-D-glucopyranoside, 10 mM Hepes, pH 7.4. After centrifugation for 15 min at $100{,}000 \times g$, the supernatant containing granule membrane proteins was diluted twice in NaCl/P_i buffer. Samples were then loaded onto a Sep-Pak Plus C-18 cartridge (Waters, Milford, MA), previously activated with 100% acetonitrile, and washed with 0.1% trifluoroacetic acid in water. Proteins were eluted with 60% aqueous acetonitrile solution containing 0.1% of trifluoroacetic acid, and the collected fractions were concentrated with a Speed-Vac (Savant Instruments, New York) and stored at −20 °C.

To investigate in more detail the presence of PEBP in granule membranes, chromaffin granule ghost fraction (CGG) was prepared by subcellular fractionation of bovine adrenal medulla as previously described (26). The membranes were separately treated for 1 h at 4 °C with an equal volume of buffer A containing 20 mM sodium Hepes, 1 mM EDTA, and 50 mM NaCl pH 7; buffer B, similar to buffer A, with 1% (v/v) Triton X-100; and buffer C, as in buffer A, with 0.1% (v/v) saponin. Each incubation was followed by a centrifugation for 40 min at $100{,}000 \times g$. The supernatant was collected, and the pellet washed twice with buffer A before resuspending in this same buffer.

Isolation of Proteins Released from Stimulated Chromaffin Cells—Chromaffin cells were isolated from fresh bovine adrenal glands, cultured as previously reported (2), and plated in plastic Petri dishes. After 3 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 (2). The extracellular medium of 2.5×10^6 non-stimulated cells (control) was collected, centrifuged at $800 \times g$ for 10 min at 4 °C to remove cells that might be present in secretions and acidified up to 0.1% trifluoroacetic acid to prevent proteolytic degradation (5).

In another series of experiments we have studied the secretion time course of CGA and PEBP. Cells (20×10^6 cells) were subsequently stimulated at 37 °C for 0 s, 30 s, 60 s, 90 s, 2 min, 3 min, 5 min, and 10 min with 4 ml of 0, 1, 10, or 10 μM nicotine in Locke's solution. The resulting fractions were acidified up to 0.1% (v/v) trifluoroacetic acid/water to prevent proteolytic degradation (5) and concentrated 10 times. 400 μl of each fraction was dotted using a Bio-Dot (Bio-Rad, Ivry-sur-Seine, France) on a nitrocellulose membrane (Amersham Biosciences, Uppsala, Sweden). PEBP immunoreactivity present in the secretion was assayed using a specific affinity repurified rabbit polyclonal antibodies raised against bovine PEBP (dilution 1:3000). Secretion efficiency was controlled with an intragranular granule matrix marker (CGA), by using a specific polyclonal anti-bovine CGA antibody (dilution 1:1000). The nitrocellulose sheet was treated as previously described (5), and immunodetection was performed with the SuperSignal West Dura Extended Duration Substrate kit (Pierce, Perbio Science, Brebières, France) according to the manufacturer's instructions. PEBP and CGA immunoresponses were evaluated by immunoreactivity of standard amounts of the human recombinant PEBP and human recombinant CGA1–76 and using the software Gene tools (SynGene Bioimaging, Ozyme, Paris, France). The amount of PEBP and CGA were expressed as the percentage of total PEBP or CGA secretions obtained for 100 μM nicotine and 600 s.

Isolation of Proteins from Platelet Secretions—Platelet-rich plasma from 240 ml of hemachromatosis blood from healthy volunteers collected in acid citrate-dextrose (courtesy of the Blood Bank at Haukeland Hospital, Bergen, Norway) was passed through a Purecell PL filter (capacity 500 ml) to remove leukocytes. Platelets were pelleted in the presence of 0.15 M NaCl, 5 mM EDTA, 10 mM Hepes buffer, pH 7.4, and washed two times (0.15 M NaCl buffer containing 1 mM EDTA, 5 mM glucose, 10 mM Hepes, pH 7.3). Platelets were suspended in the same buffer at 3.5×10^8 platelets/ml. This suspension (15 ml) was treated with control saline buffer or with 50 μM thrombin receptor-activating peptide (TRAP) (27–29) at 37 °C for 5 min. Platelets were pelleted, and supernatants were collected and concentrated to dryness. Pellets and supernatants from control- and TRAP-treated platelets were resus-

pended in 1 ml of 0.05 M HCl, filtered through 0.22-μm filters and loaded onto a fast desalting column (PC 3.2/10, Sephadex G-25, Amersham Biosciences) using 0.05 M HCl as a mobile phase (SMART System, Amersham Biosciences). Desalted fractions were dried using a Speed-Vac and resuspended in loading buffer before Western blot analysis. In these experiments, ADP and ATP release from the platelet dense granules was routinely monitored as a positive control for secretion, and over 50% of platelet adenine nucleotides (nearly all of the secretable pool) was released.

Preparation of Bovine Serum—Bovine blood was obtained from the Strasbourg slaughterhouse just after animal death. Serum was recovered after centrifugation (800 × *g*, 10 min, 4 °C) and passed through 0.22-μm filters to remove red blood cells. An equal volume of glacial acetic acid was added to the serum (v/v), and, after a 15-min centrifugation (7000 × *g*, 4 °C), the supernatant was collected and used for further analysis.

Western Blot Analysis—Proteins from cytoplasm, granule matrix, plasma, and granule membrane proteins, as well as proteins secreted from chromaffin cells were separated on SDS-PAGE containing 17% acrylamide (30). Secretion control corresponding to the medium recovered without stimulating agent was also loaded. Proteins were electrotransferred (45 min, 75V (31)) onto polyvinylidene difluoride membrane (PVDF; Amersham Biosciences) and immunodetected with specific affinity repurified rabbit polyclonal antibodies raised against bovine PEBP (21 kDa, dilution 1:3000); a cytosolic marker, bovine phenylethanolamine *N*-methyl transferase (PNMT, 31 kDa, dilution 1:4000); a granule membrane marker, bovine dopamine-β-hydroxylase (DBH, 70 kDa for each subunit, dilution 1:2500); and a granule matrix marker, bovine CGA (66 kDa, dilution 1:1000). The PVDF sheet was treated as previously described (5), and immunodetection was performed with the ECL-Plus kit (Amersham Biosciences) according to the manufacturer's instructions.

In human platelet secretion experiments, the secondary antibody was a goat anti-rabbit IgG horseradish peroxidase-conjugated immunoglobulin (1:10,000; Sigma-Aldrich, France), and the detection was directly observed using a SuperSignal West Dura Extended Duration Substrate kit (Pierce, Perbio Science). Furthermore, in these experiments a polyclonal anti-human ubiquitin antibody (dilution 1:700; Sigma Aldrich, Paris, France) was used to control the absence of cytoplasmic contamination and to check the absence of lysis of TRAP-treated platelets.

The amount of PEBP present in each fraction was evaluated by immunoreactivity of standard amounts of bovine brain PEBP or the human recombinant protein using the software Gene tools (SynGene Bioimaging, Ozyme). The amount of PEBP was expressed as the percentage of total protein present in each fraction.

To study the presence of PEBP in the chromaffin granule membrane fraction, CGG was applied onto nitrocellulose membranes for dot blot analysis. After treatment as described for Western blot analysis, the nitrocellulose membrane was incubated with antibodies against PEBP, bovine chromogranin $A_{17–39}$, and DBH. The blots were incubated with secondary antibody (goat anti-rabbit protein IgG-horseradish peroxidase conjugate) and developed by using standard ECL reagents. The relative amounts of each protein in the fractions were estimated with Fluor-S™ Multimager and software QuantityOne (Bio-Rad, Illkirch, France).

Purification of PEBP by Reverse-phase HPLC—PEBP in the soluble material from granules, cytoplasm, and secreted material from chromaffin cells, as well as in bovine serum extracts was purified using an Δkta Purifier HPLC system (Amersham Biosciences) and a Nucleosil reverse-phase 300–5C18 column (4 × 250 mm; particle size 5 μm, porosity 300 Å; Macherey-Nagel, Hoerdt, France). Absorbance was monitored at 214 nm, and the solvent system consisted of 0.1% (v/v) trifluoroacetic acid in water (v/v) (solvent A) and 0.09% (v/v) trifluoroacetic acid in 70% acetonitrile in water (solvent B). Elutions were performed at a flow rate of 700 μl/min using the gradient indicated on chromatograms.

N-terminal Sequence Analysis—The sequence of purified protein was determined by automatic Edman degradation on an Applied Biosystems 473A sequencer (Applied Biosystems/PerkinElmer Life Sciences, Boston, MA). Samples purified by HPLC were loaded on Polybrene-treated and precycled glass fiber filters (2). Phenylthiohydantoin-amino acids were identified by chromatography on a C18 column (phenylthiohydantoin C18, 2.1 × 200 mm).

Mass Spectra Analysis—Mass analyses were performed using ES/MS on a Q-TOF II (Bio-Tech, Manchester, UK) in positive mode. Scanning was performed from *m/z* = 500 Da to *m/z* = 1500 Da in 1 s. Calibration was performed using the multicharged ions produced by a separate introduction of horse heart myoglobin (16,951.4 Da).

Two-dimensional LC/MS/MS—Intragranular extract from bovine chromaffin granules was digested with sequencing grade trypsin previously chemically modified to eliminate chymotrypsin activity (ratio E:S = 1:20; Promega, Charbonnieres, France (32)) during 12 h at 4 °C and then analyzed using a ProteomeX system based on the LCQ Deca XP Plus (ThermoFinnigan, Les Ulis, France) combined with a two-pump Surveyor LC system. The ion exchange chromatography was performed on a Thermo Hypersil Biobasic SCX column (0.32 × 100 mm). Mobil phase was constituted with 0.1% formic acid (v/v) in 5% acetonitrile (v/v) in water, and eight elution steps were successively performed with 0, 20, 50, 100, 150, 200, 250, and 500 mM ammonium chloride in 0.1% formic acid (v/v), 5% acetonitrile (v/v) in water. The reverse-phase separation was performed on Biobasic C18 column (0.18 × 100 mm; Thermo Hypersil). Solvent system consisted of 0.1% formic acid (v/v) in water (solvent A) and 0.1% formic acid (v/v) in acetonitrile (solvent B). Elutions were performed at a flow rate of 130 μl/min, which was then split to 2 μl/min onto the reverse phase column. Elution was performed using a linear gradient from 5% to 65% of B solvent. Data were collected in data-dependent MS/MS mode with dynamic exclusion on. In this mode both full scan and MS-MS data were acquired continuously throughout the acquisition. The full scan mass range was *m/z* 350–1800 Da. Spectra were then analyzed using the research algorithm SEQUEST and bovine Protein Data Bank.

Isolated and Perfused Working Heart Preparation—Frog hearts were isolated from specimens of both sexes of *Rana esculenta* (weighting 22.0 ± 1.2 g; mean value ± S.E.) and connected to a perfusion apparatus as previously described (33). Experiments were done at room temperature (18–21 °C) from autumn to spring. A Grass S44 stimulator was used (single pulses of 20 V, 0.1 s) to electrically pace the heart preparations; the stimulation rate was identical to the control (unpaced) rate. The saline buffer composition was: 115 mM NaCl, 2.5 mM KCl, 1.0 mM $CaCl_2$, 5.6 mM anhydrous glucose, 2.15 mM Na_2HPO_4, 0.85 mM NaH_2PO_4, pH 7.2 (33), equilibrated with air. Mean input pressure and minimal output pressure (diastolic afterload) were regulated by moving the reservoirs up or down with reference to the level of the atrium and the aortic trunk, respectively.

Hemodynamic Measurements—The heart were stabilized at basal conditions (see below) for 15–20 min before being treated with drugs. Pressure was measured through T-tubes placed immediately before the input cannula and after the output cannula, using two MP-20D pressure transducers (Micron Instruments, Simi Valley, CA) and connected to a Unirecord 7050 (Ugo Basile, Comerio, Italy). Pressure measurements were expressed in kilopascals and corrected for cannula resistance. The heart rate was calculated from pressure-recording curves. The cardiac output was collected over 1 min and weighted. Values were corrected for temperature and fluid density by calculation and expressed as volume measurements. The afterload (mean aortic pressure) was calculated as 2/3 diastolic pressure plus 1/3 maximum pressure. Cardiac output and stroke volume (SV = cardiac output/heart rate) were normalized per kilogram of wet body weight. Stroke volume at constant pre- and afterload in paced hearts was used as measure of ventricular performance. Changes in stroke volume under these conditions were considered inotropic effects.

Ventricular stroke work (SW), an index of systolic functionality, was calculated as mJ/g (afterload − preload) × stroke volume/ventricle weight. The duration of the systolic phase and the height of peak pressure were determined from recording traces. Cardiac output, heart rate and aortic pressure were measured simultaneously during the experiments. Hearts that did not stabilize within 10 min from the onset of perfusion were discarded. The basal condition parameters of cardiac performance were measured after a 20 min perfusion (33).

After the 20-min control period, the treated hearts were perfused for 20 min without or with HCNP-enriched saline. Other experiments were repeated in presence or absence of isoproterenol in the saline buffer. Each heart was tested for one concentration of HCNP. These values were not significantly different when the second perfusion step lasted as much as 40 min.

RESULTS

Confocal Laser and Electronic Microscopy Analysis of the PEBP in Chromaffin Cells—The localization of PEBP in cultured cells isolated from bovine adrenal medulla was first investigated by immunocytochemistry coupled with confocal laser microscopy. Dual labeling was performed with CGA (the major bovine chromaffin granule protein; in *red*) and PEBP (in *green*). Whereas CGA localization (Fig. 1*A*, *CGA*) was fully

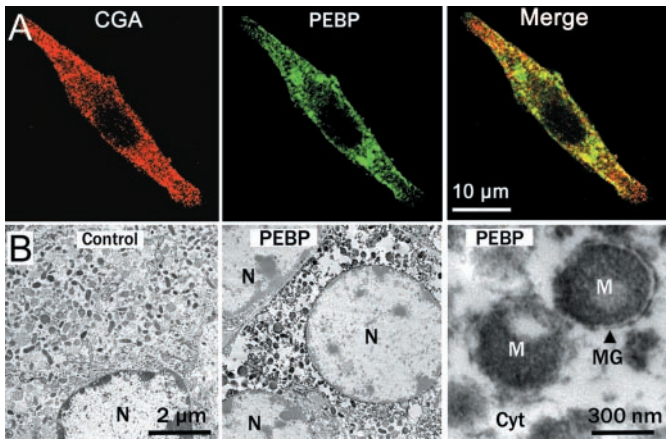

FIG. 1. **Immunolabeling of PEBP in cultured chromaffin cells.** *A*, double immunofluorescence confocal micrographs for anti-CGA antibodies (mouse monoclonal anti-human $CGA_{367–379}$) detected with Cy-3-conjugated anti-mouse antibodies (showed in *red*) and anti-PEBP antibodies revealed with Cy-5-conjugated anti-rabbit antibodies (showed in *green*). Optical sections were taken through the center of the nucleus. Colocalized proteins were revealed by *yellow labeling* (*Merge* image), showing a double labeled pixel in images of CGA- and PEBP-labeling recorded simultaneously in the same optical section. *B*, immunoelectron microscopy of bovine sections of adrenal medullary chromaffin cells. Vibratome sections immunoperoxidase labeled for PEBP protein as described under "Experimental Procedures." *Left panel*, control section; no labeling is observed. *Middle panel*, general view of a chromaffin cells illustrating labeling of cytoplasm, plasma and nuclear (*N*) membranes, and granules. *Right panel*, higher magnification of an anti-PEBP-labeled granule showing both granule matrix (*M*) and granule membrane (*MG*) labeling.

restricted to secretory granules (1), PEBP labeling was observed in granules and other compartments (Fig. 1*A*, *PEBP*). *Yellow labeling* (Fig. 1*A*, *Merge*) that corresponds to the colocalized Cy-3 and Cy-5 pixels in images of CGA- and PEBP-label recorded simultaneously in the same optical section was detected in some areas, whereas in others, *red* and *green colors* were visible as such.

Immunoelectron microscopy experiments performed on adrenal gland sections have shown that several subcellular compartments of adrenal medullary chromaffin cells were labeled for PEBP (Fig. 1*B*, *middle panel*). Immunoperoxidase reaction product was associated with the cytoplasmic surfaces of the plasma membrane, the endoplasmic reticulum membrane, and the nuclear membrane (Fig. 1*B*, *middle panel*). Immunoperoxidase labeling, in the form of a floccular precipitate, was also present in the cytoplasm not apparently associated with organelles. In addition, both the matrix and the membrane of chromaffin granules were labeled (Fig. 1*B*, *right panel*). In control sections (Fig. 1*B*, *left panel*), no electron-dense precipitate was observed in the cytoplasm of chromaffin cells or associated with plasma, nuclear membranes, or chromaffin granules, and the density of the intragranular matrix, which is osmiophilic, was considerably less than in PEBP-immunolabeled sections (Fig. 1*B*, *middle panel*). To characterize more in detail the presence of PEBP in chromaffin cells, an analysis of different subcellular fractions was performed.

Western Blot Analysis of PEBP in Chromaffin Cell Subcellular Compartments—A reliable and accurate approach to demonstrate the subcellular localization of PEBP in chromaffin cells was carried out by comparing its distribution with protein markers of different cell compartments separated by density gradient centrifugation. Subcellular fractions were characterized by immunoblotting with anti-PNMT (cytoplasm), anti-DBH (granule matrix and membrane in a ratio 1:20), and anti-CGA (intragranular matrix and membrane in a ratio 1:10), whereas PEBP extracted from bovine brain was used as a standard (Fig. 2*A*). In bovine chromaffin granule DBH is localized both in soluble intragranular matrix and membrane compartments and represents 5% of the soluble granule proteins as measured by immunological methods and is a major component of the membrane (34). CGA is found both in the soluble granule matrix and in the membrane (35). As a control of the purity of the resulting subcellular fraction, the detection of subcellular protein markers has shown the absence of contamination between the subcellular fractions. Western blot analysis revealed an unique immunoreactive PEBP band in all chromaffin cell fractions (Fig. 2*A*, *lanes 2–5*) with an apparent molecular mass of 21 kDa similar to bovine brain PEBP (Fig. 2*A*, *lane 1*). Amounts corresponding to 0.05% of total protein present in cytoplasm and intragranular fraction was loaded. The amount of PEBP present in these two fractions was obtained by comparison with the immunoreactivity observed with standard amounts of bovine brain PEBP and expressed in the percentage of PEBP per total protein. PEBP represents 4.3% and 0.41% of total protein in the cytoplasmic fraction and the intragranular matrix fraction respectively, thus revealing a 10-fold difference.

The observation that PEBP is localized in secretory granules prompted us to investigate its possible presence in material released from nicotine- and K^+-stimulated bovine primary chromaffin cells. We found a 21-kDa immunoreactive band, and its labeling intensity indicated a similar concentration (0.05 μg/2.5 $\times$ 10^6 cells by comparison to standard concentrations of bovine brain PEBP) in the material recovered in the medium upon nicotine (Fig. 2*A*, *lane 6*) and K^+ (data not shown) stimulation of chromaffin cells, but not in the medium released from cells treated only with Locke's solution (Fig. 2, *lane 7*), suggesting that PEBP is released by exocytosis.

To determine if the PEBP follow a similar exocytotic pattern as other secreted proteins (*i.e.* CGA), secretion kinetic experiments were performed on bovine primary chromaffin cells (Fig. 2*B*). These experiments have shown that the PEBP follows a secretion pattern similar to the one obtained for CGA (Fig. 2*B*), indicating that, as for the CGA, almost all of the PEBP is secreted after a short time (*i.e.* before 300 s), as previously described (36). In addition, we observed that the amount of PEBP and CGA increase with the nicotine concentration used for the stimulation. Thus, if we expressed the relative value of 100% for the total PEBP (0.21 μg) recovered after a 100 μM nicotine treatment, we observe that 82% (0.17 μg) and 56% (0.12 μg) of PEBP are secreted after a 10 μM and a 1 μM nicotine stimulation, respectively (Fig. 2*B*). For the CGA, the same consideration lead us to a 100% CGA secretion level (20 μg) for a 100 μM nicotine treatment, whereas only 56% (11.2 μg) and 33% (6.6 μg) of CGA release was observed for a 10 μM and a 1 μM nicotine stimulation, respectively (Fig. 2*B*). The PEBP immunoreactivity was further examined in the cytoplasm, intragranular matrix, and secretions of chromaffin cells using HPLC reverse phase chromatography, sequencing, and mass spectrometry analysis techniques.

Characterization of Intragranular PEBP—An initial chromatography of intragranular material was performed using a C18 column with the gradient indicated in Fig. 3*A*. An aliquot of each collected fraction (1/5) was analyzed on 17% SDS-PAGE (Fig. 3*A*, *inset*). After electroblotting onto PVDF membrane using polyclonal anti-PEBP antibodies, only one 21-kDa band immunostaining was detected in the fraction indicated by an *arrow* (Fig. 3*A*). PEBP human recombinant protein was used as a standard (21 kDa; Fig. 3*A*, *inset*, *last right lane*). The higher apparent molecular weight band observed in the control PEBP corresponded to a dimer (42 kDa) of protein as previously

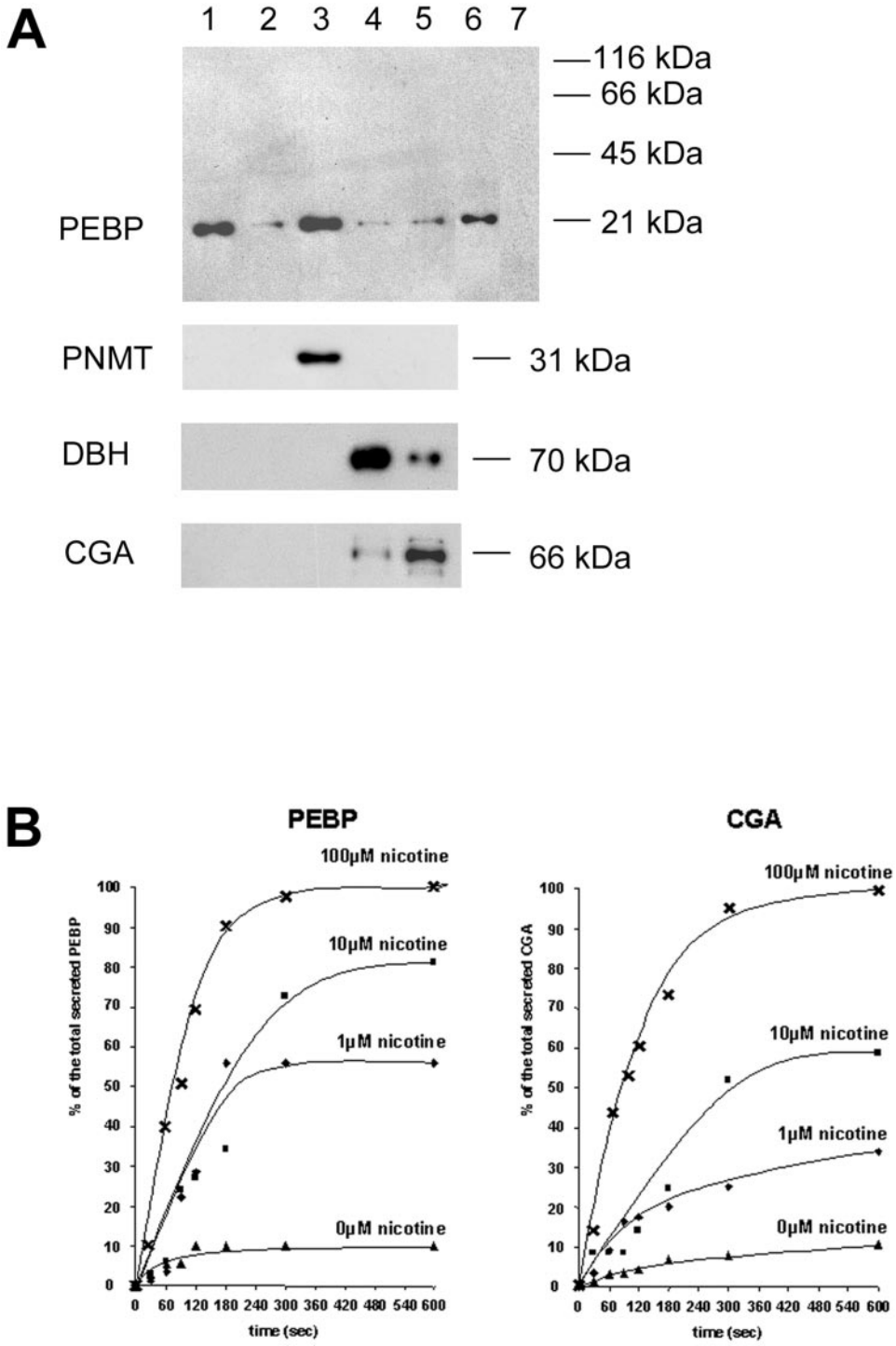

FIG. 2. *A*, immunodetection of PEBP by Western blot. Protein material from subcellular chromaffin cell fractions was quantified by Bradford protein assay and aliquots were separated by 17% acrylamide SDS-PAGE and electrotransferred onto PVDF sheet. Immunodetection was carried out with affinity purified polyclonal anti-bovine PEBP (diluted 1:3,000). The immunoreactivity at 21 kDa corresponds to whole PEBP purified from bovine brain (*lane 1*, 0.1 μg). *Lane 2*, 0.5 μg of plasma membrane proteins. *Lane 3*, 4.6 μg of cytoplasm proteins. *Lane 4*, 6 μg of granule membrane proteins. *Lane 5*, 4.8 μg of intragranular matrix proteins. *Lane 6*, protein material secreted from 2.5×10^6 bovine primary chromaffin cells after 10 μM nicotine stimulation. *Lane 7*, protein material secreted from 2.5×10^6 bovine primary chromaffin cells without nicotine stimulation (control). Immunodetections were performed with antibodies specific to protein markers: PNMT (31 kDa) for cytoplasmic fraction, DBH (70 kDa) for granule membrane, and CGA (66 kDa) for intragranular matrix. *B*, comparative kinetic of the secretory process of PEBP and CGA. Secreted material from primary bovine chromaffin cells (20×10^6 cells) recovered at different times and nicotine concentrations were doted onto nitrocellulose sheet. Immunodetection was carried out on (10×10^6 cells) with affinity-purified polyclonal anti-bovine PEBP (diluted 1:3000) and anti-bovine CGA (1:1000). Immunolabeling was quantified and expressed as the percentage of the total immunoreactivity recovered with secretions after a 100 μM nicotine stimulation.

described (37). This form was only visible in fractions containing a high amount of this protein.

This PEBP-containing fraction was then repurified on a C18 column with a new elution gradient (Fig. 3*B*). Automated Edman degradation of the fraction marked by an *arrow* (Fig. 3*B*) revealed the presence of the sequence PVDLSKWSGP corresponding to the N-terminal fragment of bovine brain PEBP (7). Mass spectrometry analysis (Fig. 4) indicated a molecular mass

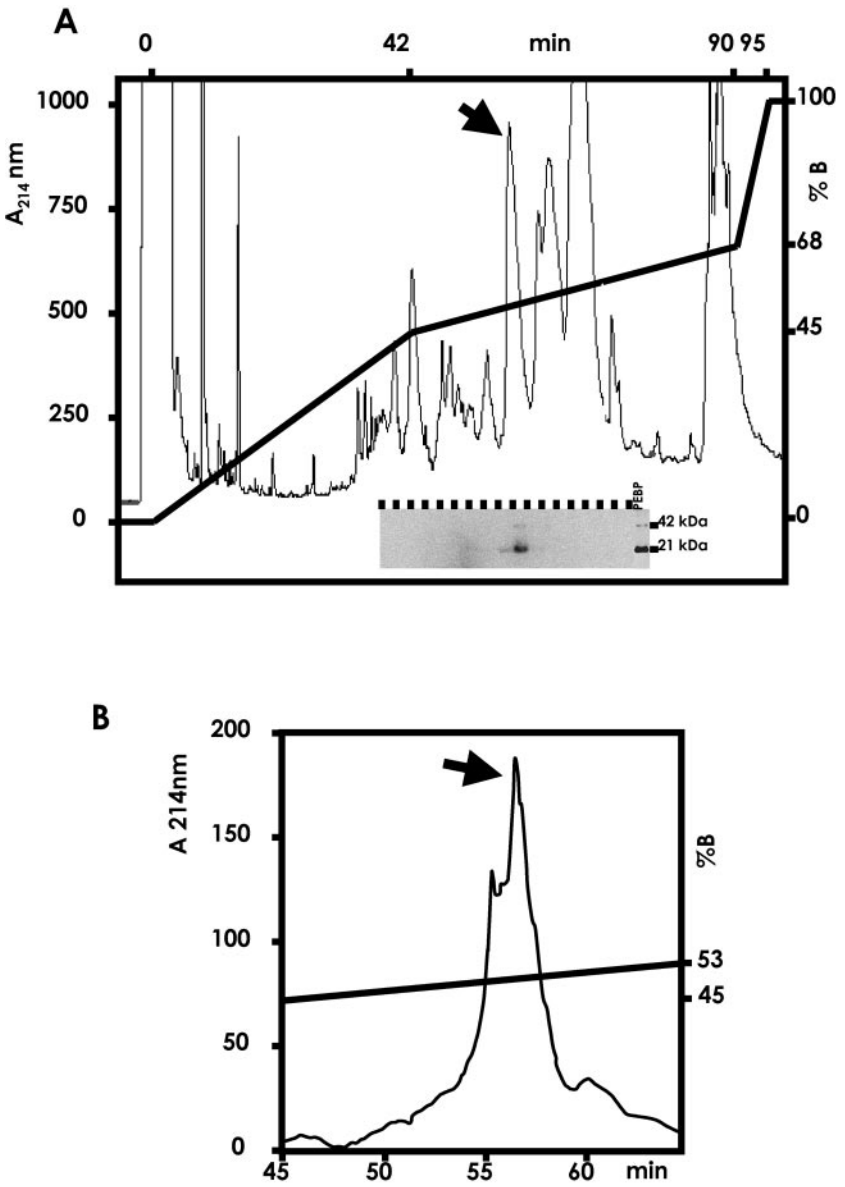

FIG. 3. **HPLC purification of PEBP from bovine chromaffin granules.** *A*, 2.9 mg of intragranular proteins from bovine chromaffin cells were separated on a Macherey-Nagel reverse-phase Nucleosil 300–5C-18 column (4 × 250 mm). Absorbance was monitored at 214 nm, and elution was performed at a flow rate of 700 μl/min with the gradient indicated on the chromatogram. Aliquots of each resulting HPLC fractions were then separated by 17% acrylamide SDS-PAGE and electrotransferred onto a PVDF sheet. Immunodetection was carried out with affinity-purified polyclonal anti-bovine PEBP (diluted 1:3000). 0.02 μg of human recombinant PEBP was used as a control (*last right lane*). The immunoreactivity at 21 kDa corresponds to whole PEBP, whereas the higher band corresponds to previously described dimers (42 kDa) of PEBP, which are only visible for a high amount of this protein (37). The *arrow* on the chromatogram indicates the fraction immunolabeled with the anti-PEBP antibody (*inset*). *B*, HPLC repurification of the PEBP-containing fraction. The *arrow* indicates the fraction containing PEBP.

of 20,856 Da ($M+H^+$) in agreement with the theoretical and experimental molecular mass of the bovine brain cytosolic protein (Swiss-Prot P13696; 20,854 Da). PEBP was also purified from cytoplasmic fraction and nicotine-stimulated resulting secretions using the same procedure (data not shown). PEBP present in each of these fractions was found to be identical to the protein found in the intragranular matrix of chromaffin granules and isolated from bovine brain (7).

Since a signal sequence is lacking on PEBP sequence, a non-classic secretion pathway should be involved. According to its high affinity for lipids and our present results showing the presence of PEBP in granule membrane fraction by Western blot, we analyzed in detail its interaction with granule membranes of chromaffin cells.

Analysis of the Presence of PEBP in Granule Membranes—The absence of a signal sequence on PEBP sequence prompted us to characterize the mechanism by which PEBP enters into chromaffin granules. Thus, we investigated its presence in granule membranes. By Western blot analysis, we showed that non-ionic detergents, like Triton X-100 or octyl-β-D-glucopyranoside, were able to solubilize bound PEBP (Fig. 2, *lane 4*). Experiments were set up to investigate whether membrane-bound PEBP may also be associated with the detergent-insoluble fraction of CGG, containing cholesterol- and sphingolipid-enriched membrane compartments and described as lipid rafts (38–40). The relative amount of PEBP present in supernatant and pellet, after treatment of the membranes with either Triton X-100 or saponin, were estimated and compared with the distribution of two other membrane-associated chromaffin granule proteins, CGA (41, 42) and DBH (43). As shown in Table I, a significant fraction of PEBP immunoreactivity (43%) remains in the pellet after treatment of membranes with Triton X-100. In comparison only 13% of CGA- and 22% of DBH-immunoreactivity remained in the pellet after Triton X-100 treatment. Treatment with saponin, which extracts cholesterol from membranes, was found to remove 55% of PEBP immunoreactivity, leaving 45% in the pellet. As much as 74% of DBH immunoreactivity remained in the pellet after saponin treatment, whereas almost all CGA was removed (12% in the pellet). In addition to these results, we also showed that DBH does not interact with cholesterol in granule membrane, because DBH immunoreactivity was not modified in comparison to the value obtained for the control buffer treatment (Table I). From our results showing the secretion of PEBP from chromaffin cells

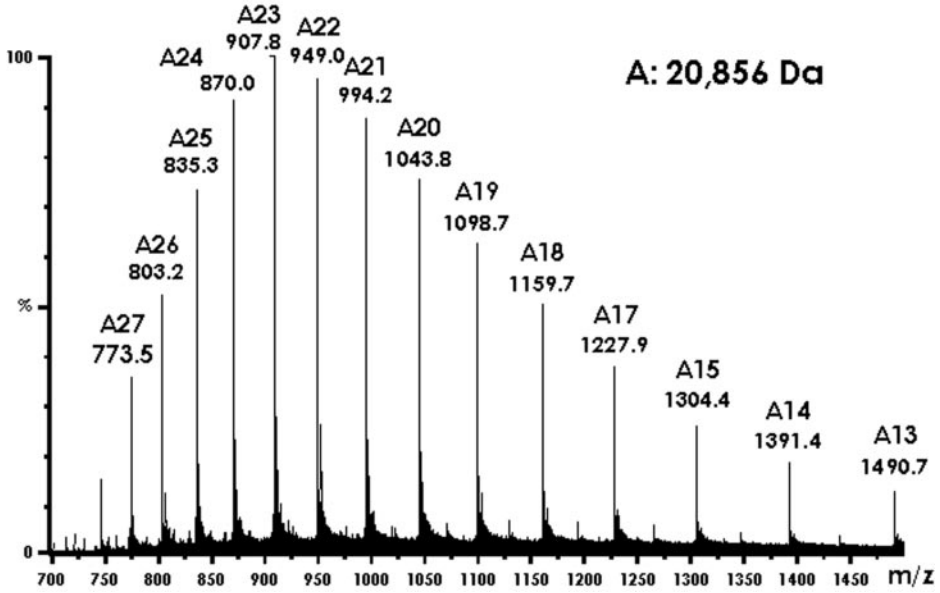

FIG. 4. **Mass spectrometry analysis of PEBP purified from intragranular proteins.** Mass analyses were performed using the electrospray mass spectrometry technique (ES/MS) on a Q-TOF II (Bio-Tech, Manchester, UK) in positive mode. Scanning was performed from *m/z* = 500 Da to *m/z* = 1500 Da in 1 s. Calibration was performed using the multicharged ions produced by separate introduction of horse heart myoglobin (169,551.4 Da). PEBP was found to have a molecular mass of 20,856 Da ($M+H^+$).

TABLE I
Detergent solubility of PEBP present in granular membranes

	Control (buffer)			1% Triton X-100			0.1% saponin		
	PEBP	CGA	DBH	PEBP	CGA	DBH	PEBP	CGA	DBH
					%				
Percentage in supernatant	14	57	26	57	87	78	55	88	26
Percentage in pellet	86	43	74	43	13	22	45	12	74

and in the light of its previous description in whole human platelets (6), we investigated whether this protein is secreted by platelets stimulated by TRAP.

Immunodetection of PEBP in Secretions of TRAP-treated Platelets—Purified platelets prepared as described under "Experimental Procedures" were treated with or without 50 μM TRAP during 5min. After desalting, platelet pellets and secretions were analyzed on 17% SDS-PAGE (Fig. 5). Immunoreactivity for PEBP was detected after electroblotting onto PVDF membrane using polyclonal anti-PEBP antibodies. PEBP human recombinant protein was used as a standard (21 kDa; Fig. 5, *upper panel*, *lane 1*). Western blot analysis of the pellets from control and TRAP-treated platelets (Fig. 5, *upper panel*, *lanes 2* and *3*, respectively) shows the presence of a high amount of PEBP immunoreactivity corresponding to the 21-kDa cytoplasm form. The higher apparent molecular weight bands correspond to the dimer (42 kDa) and trimer (66 kDa) forms of PEBP as previously described (37). These forms were only visible in fractions containing a high amount of this protein. Western blot analysis of secretions from control and TRAP-treated platelets revealed the presence of immunoreactive PEBP material in TRAP-treated platelets and not in the control (Fig. 5, *upper panel*, *lanes 5* and *4*, respectively). Quantification using standard amounts of human recombinant PEBP indicated that 2.3 μg of PEBP was present in pellets of 10^9 control- and TRAP-treated platelets, whereas 0.035 μg/10^9 platelets representing 1.5% of total PEBP was recovered in platelet secretion.

To check if PEBP found in platelet secretion was not due to cell lysis, the presence of a specific cytoplasmic marker (*i.e.* ubiquitin) was looked for in control- and TRAP-treated platelet secretion, as well as in corresponding pellets. Immunoreactivity at an apparent molecular mass of 8 kDa for standard ubiq-

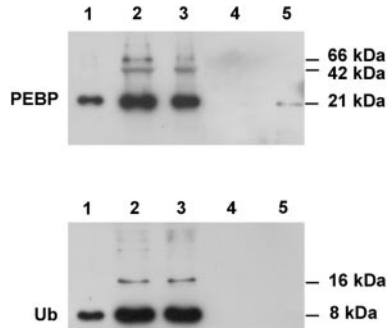

FIG. 5. **Immunodetection by Western blot of PEBP in platelets stimulated with TRAP.** Protein material from unstimulated and TRAP-treated platelets (pellets and secretions) was quantified by Bradford protein assay and aliquots were separated by 17% acrylamide SDS-PAGE and electrotransferred onto a PVDF sheet. *Upper panel*, immunodetection of PEBP carried out with an affinity-purified polyclonal anti-bovine PEBP (diluted 1:3000) recognizing the human PEBP. *Lane 1*, 0.005 μg of human recombinant PEBP. *Lane 2*, control platelet pellet proteins (0.21×10^8 platelets). *Lane 3*, platelet TRAP-treated pellet proteins (0.21×10^8 platelets). *Lane 4*, control of platelet secretion (2.62×10^8 platelets). *Lane 5*, secretion of platelet treated with TRAP (2.62×10^8 platelets). Immunolabeled band at 21 kDa corresponds to whole human PEBP, whereas the higher bands correspond to previously described dimers (42 kDa) and trimers (66 kDa) of PEBP, which are only visible for fractions containing a high amount of this protein (37). *Lower panel*, immunodetections were also performed with antibody specific to ubiquitin (*Ub*, 8 kDa, *lane 1*), a specific protein marker of the cytoplasm. *Lanes 2–5* were loaded as in the *upper panel*. Ubiquitin immunolabeling was only observed in platelet pellets and not in platelet secretion, even after 12 h of overexposure (data not shown), showing that the presence of PEBP in the secretion of TRAP-treated platelets did not derive from cytoplasmic contamination due to cell lysis. Higher apparent molecular masses correspond to dimers of ubiquitin (16 kDa).

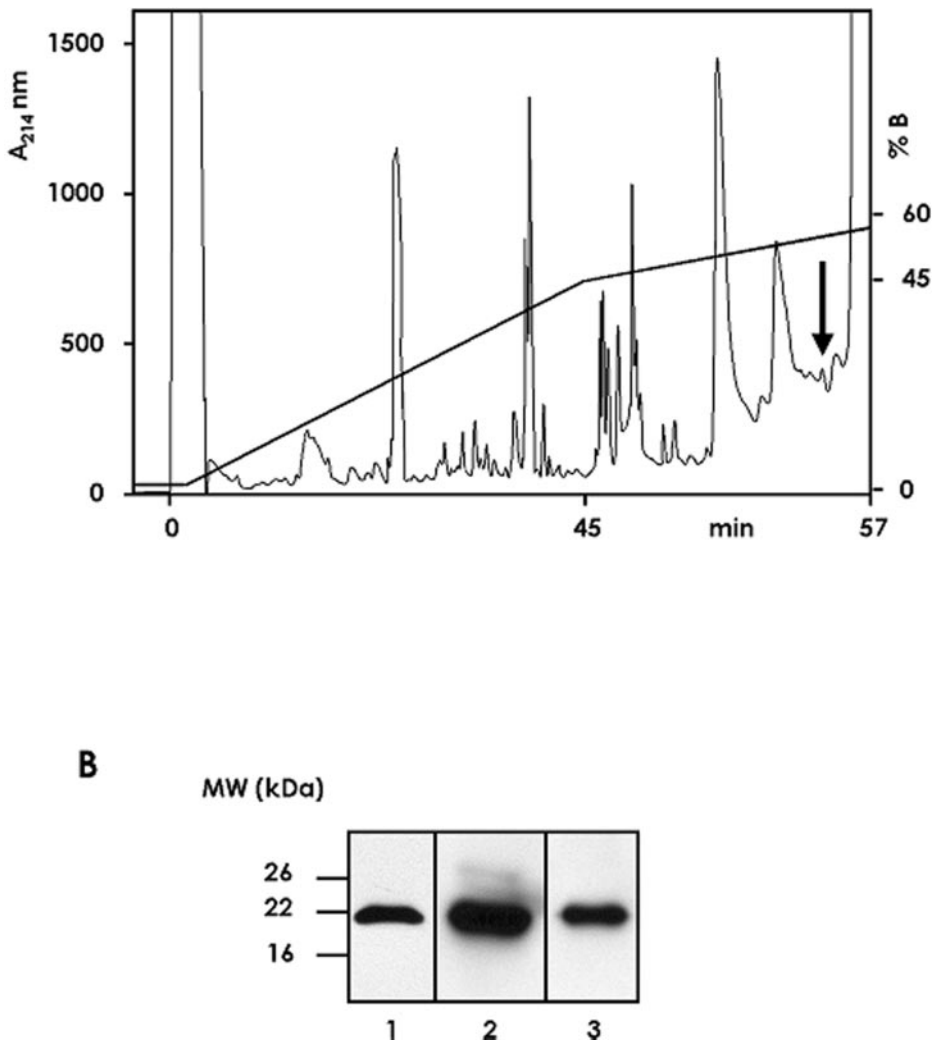

FIG. 6. **HPLC purification and Western blot analysis of PEBP in bovine serum.** *A*, 400 μl of serum was purified on a Macherey-Nagel reverse-phase Nucleosil 300-5 C18 column (4 × 250 mm). Absorbance was monitored at 214 nm, and elution was performed at a flow rate of 700 μl/min with the gradient as indicated on the chromatogram. The *arrow* indicates the fraction in which PEBP was detected. *B*, Western blot analysis using anti-PEBP antibody was performed on a serum fraction eluted from HPLC. *Lane 1*, 0.1 μg of PEBP purified from bovine brain; *lane 2*, 10 μg of intragranular proteins from bovine chromaffin granules; *lane 3*, serum HPLC fraction (1/4) indicated by the *arrow* in *A*. The immunoreactive band at 21 kDa corresponds to whole PEBP.

uitin (Fig. 5, *lower panel*, *lane 1*) was observed only in platelet pellets (Fig. 5, *lower panel*, *lanes 2* and *3*) and not in control or TRAP-evoked secretion medium (Fig. 5, *lower panel*, *lanes 4* and *5*) even after a 12-h overexposure, indicating that PEBP recovered in secretion medium was not due to cytoplasmic contamination. Because proteins released from chromaffin cells and platelets upon stimulation are recovered in the circulation upon stress situations (1), we examined the presence of PEBP in bovine serum.

Isolation and Immunodetection of PEBP in Bovine Serum—Acid-extracted serum (400 μl) was submitted to HPLC purification on a reverse-phase C18 column (Fig. 6*A*). Each peak was collected, and the protein profile was analyzed on 17% SDS-PAGE. Immunoreactivity for PEBP was detected after electroblotting onto PVDF membrane. Western blot analysis of the fraction indicated by the *arrow* (Fig. 6*A*) revealed the presence of an immunoreactive band of 21 kDa (Fig. 6*B*, *lane 3*) corresponding to PEBP label observed for bovine brain PEBP standard (Fig. 6*B*, *lane 1*) and PEBP present in intragranular matrix of the chromaffin cells (Fig. 6*B*, *lane 2*). An estimation of the concentration of PEBP in bovine serum was obtained after protein sequencing which indicated a value of 35 nM (± 5%). Because we have found the PEBP in intragranular matrix and in secretion of chromaffin cells, we further investigated if HCNP, the natural N-terminal peptide derived from PEBP that has been identified in various biological fluids (10, 12), is present among the granule constituents and thus may be released upon stress stimulation.

Characterization of Intragranular HCNP—To characterize the HCNP present in intragranular chromaffin matrix, we have used a new high sensitive technique, the two-dimensional LC/MS/MS that is composed by two successive liquid chromatographies (LC) coupled with an ion-trap mass spectrometry (MS) analyzer. Synthetic bovine HCNP was used as a standard to determine its elution profile and its MS-MS spectrum (data not shown). Analysis of the tryptic digest of the intragranular proteic material and comparison with control experiments allowed us to characterize the natural HCNP that displays an identical elution patterns and the same MS-MS profile as the synthetic HCNP (data not shown). Using the algorithm SEQUEST on the MS-MS parent ions, we unambiguously identify the bovine HCNP and its presence was not due to tryptic proteolysis, as indicated by the PEBP sequence.

Because PEBP and HCNP are released from endocrine cells and recovered in the blood in the same time as the catecholamines, we assumed that they could display endocrine effects. It has been previously found that PEBP is expressed in a large variety of neuronal and non-neuronal tissues, including testis, epididymis, intestine, kidney, liver, and heart (9). Based on catecholamine functions (32) and CGA-derived vasostatin-I (21), which possess, respectively, positive and negative inotropic effects on cardiac physiology, we investigated the HCNP potential on heart contractions.

Effects of HCNP on Frog Heart Physiology—The isolated avascular frog heart was used to examine the role of HCNP on heart physiology (22, 33). Experiments performed on perfused

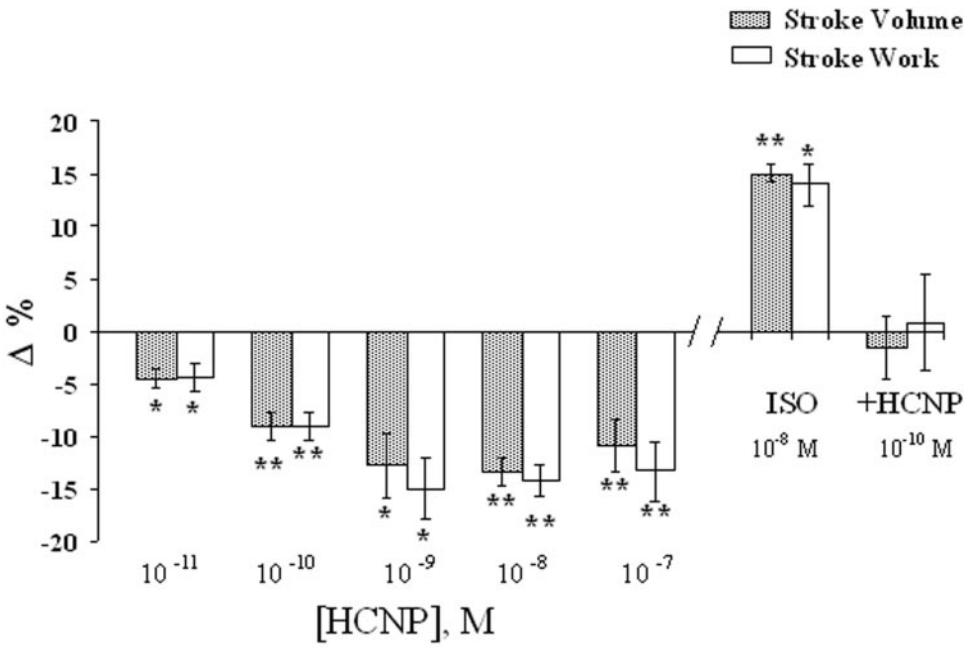

FIG. 7. **Inotropic effect of HCNP on frog heart.** Effects of HCNP on stroke volume and stroke work in paced frog heart. Concentration-dependent responses of the peptide alone (*left panel*) and in the presence of isoproterenol (*ISO*, *right panel*). Percent changes (Δ%) were evaluated as means ± S.E. of the experiments: concentration-dependent curve $n = 6$ or 7 hearts; ISO $n = 3$. Significance of difference from control values: *, $p < 0.05$; **, $p < 0.01$.

isolated frog heart have indicated that HCNP exerts a significant inotropic negative action from 10^{-11} to 10^{-7} M (Fig. 7). Control experiments with either bovine serum albumin (1–15 nM; data not shown) or the frog CGA N-terminal fragment (CGA4–16) synthesized with the same method as for the HCNP (10–200 nM (21)) did not affect the hemodynamic parameters (SV and SW). Because HCNP can be released together with catecholamines, we have examined its effect during the stimulation of cardiac β-adrenergic receptors with isoproterenol, the β-adrenergic agonist known to produce a positive inotropic effect (22, 33). As shown in Fig. 7 (*right panel*), 10^{-10} M HCNP was found to counteract the positive inotropic effect of isoproterenol (10^{-8} M).

DISCUSSION

PEBP is an ubiquitous protein expressed in various of cell types, with a level of expression varying markedly. Interestingly, a highly regional expression of PEBP in the fully differentiated rat brain indicated immunoreactivity in the arcuate nucleus and A2/A1 regions, suggesting a possible association with catecholamine transmission (9).

Subcellular Localization of PEBP—In the present study, we report the presence of PEBP in chromaffin cells, platelets, and serum. As in other cells (11), this protein was found to be localized in plasma membrane and cytoplasm. However, we show for the first time the presence of PEBP in the matrix of secretory granules of chromaffin cells. In addition, the N-terminal PEBP-derived peptide, named HCNP, was characterized in the intragranular matrix using two-dimensional LC/MS/MS, indicating the proteolysis of the precursor probably by a granular chymotrypsin-like enzyme (10, 44). Despite the lack of a known consensus signal peptide, PEBP is secreted like numerous proteins secreted without apparent signal sequences such as interleukin-1, fibroblast growth factor, transglutaminases, coagulation factor XIIIa (for review see Ref. 19). Furthermore, PEBP is not only localized in the matrix as a soluble protein, but also present to a minor extent in granule membrane. Previously, other proteins were found to exist in both cytosolic and membrane associated forms. Thus, proteins, including annexin 2 (45) and tyrosine hydroxylase (46), have been retrieved in the membrane fraction of chromaffin granules, and both have been found in detergent-insoluble fractions. Exocytosis is a late stage of a broader mechanism involved in general membrane trafficking, which includes membrane transport in the endoplasmic reticulum/Golgi apparatus and endocytosis (1). In the case of PEBP, which is synthesized in the cytoplasm and present mainly in this compartment, the most probable mechanism for its translocation into the granule matrix could be the binding of PEBP to the granule membrane, as a first step prior to its transfer within the granule. Our experiments indicate that, in chromaffin cells, PEBP follows a kinetic pattern of secretion close to the one observed for CGA.

The binding of PEBP to chromaffin granule membrane is compatible with its phospholipid composition and charge. Indeed, chromaffin granule is composed of numerous lipids including cholesterol and free fatty acids (47). Furthermore, PEBP is able to bind to *in vitro* negative membranes containing phosphatidylglycerol (48), which is also present in chromaffin granule membranes (49). The detergent-insoluble fractions are also known to contain cholesterol- and sphingolipid-enriched functional membrane domains known as lipid rafts (39, 40). We show here that a significant amount (43%) of the total PEBP immunoreactivity in chromaffin granule membrane is also found in the detergent-insoluble fraction after treatment of CGG with Triton X-100. Extraction with saponin removed 55% of total immunoreactivity, suggesting its association with cholesterol-enriched lipid rafts, whereas 45% could be associated with other membrane components. The physiological significance of the association of PEBP with lipid rafts in CGG is currently not known. Interaction with lipid-rafts has been found to be important for the sorting of several proteins, such as carboxypeptidase E and prohormone convertases (PC2, PC1/3) from the *trans*-Golgi network to the secretory granules (40, 50). The lipid-rafts are suggested to form a sorting platform in which prohormones may aggregate and immature granules form (51). However, several cytosolic protein, like PEBP, TH (46), and annexin 2 (45), are also observed in the

detergent-insoluble lipid-rafts containing fraction of CGG. Thus, it is possible that lipid-rafts may also play a role in the recruitment of certain cytosolic proteins to the CG membranes. However, the mechanism of PEBP transport across the membrane and the possible involvement of lipid-rafts in this process are still unknown. Thus, several proteins were described to be sorted to the regular secretory pathway by interacting with lipid rafts (40). These proteins include enzymes such as carboxypeptidase E and prohormone convertases (PC2, PC1/3) also present in the matrix of secretory chromaffin granules (for review, see Ref. 50). Nevertheless, until now, no apparent consensus sequence for directing proteins into rafts seems to exist (50).

Previous biochemical studies on the organization of the secretory granules from adrenal medulla have shown that bovine chromaffin granules contain, in addition to small compounds (catecholamines, nucleotides, and others), several prohormones (chromogranin family and proenkephalin-A), which are the precursors of many bioactive peptides (for review, see Ref. 52) and are co-stored with numerous proteolytic enzymes (4, 53). The chromaffin granule, which has been used as a model for the characterization of proteases and enzymes involved in neuropeptide maturation (53), contains the subtilisin-like prohormone-convertase family, prohormone-thiol-protease (cysteine protease), adrenorphin-Gly-generating enzyme, proopiomelanocortin-converting enzyme (or 70-kDa aspartic acid protease (54)) and serine proteases with trypsin-like activity (4, 55–58). More recently, Parmer and colleagues (59) have shown that the plasmin-plasminogen system, which is present in the intragranular chromaffin matrix, seems to be implicated in chromogranin maturation. Interestingly, it has been reported that the yeast member of the PEBP family displays carboxypeptidase-Y inhibitor activity with a K_i of 0.1 nM (60, 61) and that mouse PEBP inhibits serine protease-like enzymes such as thrombin (K_i = 380 nM), chymotrypsin (K_i = 1.8 μM), and neuropsin, but not plasmin, trypsin, and elastase (14). Taken together, these observations indicate that PEBP, as serpins (62), may modulate intragranular serine protease activities within chromaffin granules and thus control intragranular prohormone maturation.

Secretion of PEBP by the Platelets—Previously, PEBP has been shown to be present in platelets (6). We demonstrate here that PEBP is secreted from platelets upon stimulation with 50 μM TRAP. *In vivo*, the platelet secretory process occurs during stress, *i.e.* endothelium injury or activation of endothelium in response to chemicals, cytokines, or inflammatory processes (63, 64). Thus, damage to arterial or venous endothelium renders the subendothelial matrix proteins (*e.g.* collagen and Willebrand Factor) accessible to platelets that then become adherent to the site (63). At the same time, thrombin (a platelet agonist) is produced, thus recruiting additional platelets to the injury site. Thrombin converts fibrinogen to fibrin to stabilize the platelet aggregate. The presence of PEBP in such a secretion at an amount of 0.035 $\mu g/10^9$ platelets fits well with previous reports that described the secretion of protease inhibitors such as α_2-antiplasmin (0.06 $\mu g/10^9$ platelets), α_1-antitrypsin (0.4 $\mu g/10^9$ platelets), or the C1 inhibitor (0.6 $\mu g/10^9$ platelets) by platelets at similar concentrations (for review, see Ref. 65). Thus, PEBP could act as an inhibitor of thrombin at the platelet secretion site, thus regulating platelet aggregation.

Presence of PEBP in the Serum—The presence of PEBP and HCNP in the chromaffin granule matrix indicates that this PEBP-derived peptide will be released at the same time as its precursor. These experimental data agree with several previous studies that have described the presence of PEBP and HCNP in different biological fluids (*i.e.* rat haploid testicular germ cell secretions, testicular interstitial fluid (12, 13, 66), secretion of murine adipocytes (18), and the culture media of transfected Rat-1 fibroblast cells (14)). We have shown here that PEBP is present in bovine serum, although the protein has never been previously detected in human serum, lymph, or plasma probably because of low detection sensitivity (66). The quantification of PEBP present in blood was estimated to 35 nM, and this value fits well with the K_i described for PEBP-evoked carboxypeptidase-Y inhibition at 0.1 nM (60, 61). In addition, because this peptide has been described to result from the activity of a granular chymotrypsin-like enzyme (10, 44), it could also result from the activity of a circulating chymotrypsin-like enzyme after its secretion into the blood (67).

HCNP and Heart Physiology—Upon stimulation, catecholamines and intragranular protein and derived peptides present in chromaffin granule matrix (chromogranins, PEBP, HCNP, and others) are released into the circulation. Catecholamines are known to display a direct positive inotropic effect on heart (68), whereas our laboratory has recently described the negative inotropic effect of the N-terminal CGA-derived peptide named vasostatin-I on avascular frog heart (21). The heart of the frog is devoid of coronary vessels, being only supplied through the intertrabecular spaces (lacunae) (33). This provides a suitable model for evaluating specific myocardial effects of cardioactive agents without the confounding intracardiac vascular influences. Thus, the frog endocardial endothelium is unique in being the only endothelial barrier between blood and myocardial microenvironment. Only substances from blood cross this barrier and interact with the myocardium (33). Based on our results concerning vasostatin-I inotropic effect, we investigated the HCNP effect in the isolated and perfused working heart of the frog bioassay system. Our data have shown that HCNP, the most conserved domain of PEBP (residues 1–11) (37), exerts dramatic effects on the mechanical cardiac performance. In fact, this peptide induces a strong dose-dependent negative inotropic effect from 10^{-11} to 10^{-7} M and is able to counteract the positive inotropic effect of β-adrenergic stimulation (isoproterenol). Experiments performed on the same experimental model with the human recombinant PEBP display no significant effects (data not shown), probably due to a less conservation of the sequence following the 11th residue in the frog and human proteins. In addition, preliminary experiments on a mammalian model, the Langendorff isolated and perfused rat heart, have shown a HCNP-negative induced inotropism under basal cardiac condition.[2] In the light of the secretion of PEBP and HCNP into the blood by chromaffin cells together with catecholamines (20), our data suggest that HCNP might have a potential role as a cardiac modulator.

CONCLUSIONS

Finally, we have shown that PEBP and its derived peptide HCNP are present in secretory granules of chromaffin cells. In the matrix of chromaffin granules, PEBP can be involved in prohormone maturation mechanisms, by inhibiting granule serine proteases and thus affecting the generation of neuropeptides involved in homeostasis. We also demonstrated that PEBP is secreted and recovered into the circulation in which it could be processed into HCNP by chymotrypsin-like enzyme. PEBP is secreted from TRAP-stimulated platelets, acting locally as an inhibitor of thrombin and other serine proteases, to modulate platelet aggregation. In addition, we show here for the first time that HCNP displays dramatic negative inotropic

[2] Y. Goumon, T. Angelone, F. Schoentgen, S. Chasserot-Golaz, B. Almas, M. M. Fukami, K. Langley, I. D. Welters, B. Tota, D. Aunis, and M.-H. Metz-Boutigue, unpublished data.

effect and is able to inhibit isoproterenol stimulation of the heart. Taken together, our results suggest that PEBP and HCNP represent new important endocrine factors able to modulate the physiological responses of heart.

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