Chromogranin A, the Major Catecholamine Storage Vesicle Soluble Protein

MULTIPLE SIZE FORMS, SUBCELLULAR STORAGE, AND REGIONAL DISTRIBUTION IN CHROMAFFIN AND NERVOUS TISSUE ELUCIDATED BY RADIOIMMUNOASSAY

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Chromogranin A (CgA), the major catecholamine storage vesicle (CSV) soluble protein, may index exocytotic sympathoadrenal secretion. To explore CgA in adrenocortical tissues, we developed a radioimmunoassay for bovine CgA. Within adrenal medulla CSV, several minor chromogranins had similar amino acid compositions and peptide maps to that of CgA and also showed partial, cross-reactivity in the CgA radioimmunoassay. CgA immunoactivity represented 7 ± 1% of total adrenal medulla cell protein and was localized to adrenal CSV, representing 46 ± 2% of CSV soluble protein.

In brain, there was 1000-fold less CgA than in adrenal medulla, with a widespread regional distribution (maximal in neocortex) and an unusual subcellular distribution (maximal in cytosol), both of which differ from reported catecholamine distribution. Brain chromogranin immunoactivity also had a lower Stokes radius than adrenal CgA. Sympathetic nerve and serum had 6,000-fold and 30,000-fold less CgA than that in adrenal medulla.

The results suggest a "family" of adrenal medulla chromogranins, similar structurally and immunologically. Adrenal medulla and brain chromogranin differ in concentration, subcellular localization, and molecular size. Finally, CgA in serum may provide a useful tool for sympathoadrenal studies in intact organisms.

MATERIALS AND METHODS

Chemicals

Norepinephrine, epinephrine, leucine enkephalin, methionine enkephalin, adenosine triphosphate, ovalbumin, and N-ethylmaleimide were purchased from Sigma. 125I-Concanavalin A-Sepharose was from Pharmacia Fine Chemicals, Piscataway, NJ. Phenylmethylsulfonyl fluoride was from Calbiochem-Behring. Sulforicinase (ribonuclease-free) and lactoperoxidase-glucose oxidase beads (Enzymobeads) were from Bio-Rad Laboratories. Ultragel ACA-2 was from LKB Produkter, Bromma, Sweden. Na235Cl was from Amersham/Seattle. FITA was from J.T. Baker Chemical Co. Sodium azide was from Mallinckrodt Inc. Ascorbic acid was from Fisher. Sheep anti-rabbit y-globulin (SA, P4 titer) was from Antibodies Incorporated, Davis, CA.

Preparation of Chromogranins

Chromogranins were prepared from bovine adrenal medullary chromaffin granules (13). Chromaffin granules were isolated from the adrenal medulla by centrifugation on sucrose density gradients (3, 14) and then were lysed in 0.001 M sodium phosphate, pH 6.5, and centrifuged at 100,000 × g for 1 h to separate granule membranes from soluble lysate. After extensive dialysis of the soluble lysate against 0.01 M sodium phosphate, pH 6.5, to remove catecholamines, the lysate was then chromatographed on concanavalin A-Sepharose to remove dopamine-β-hydroxylase (15) and then subjected to preparative polyacrylamide gel electrophoresis in 8 M urea to yield pure chromogranin A (13). The quality of the chromogranin A preparation was verified by re-electrophoresis in SDS gels (16), yielding one band (13), as well as by gel filtration in 6 M guanidine HCl and isoelectric focusing (13). Purified bovine chromogranin A was characterized (3) as a 67,000-dalton, acidic (isoelectric point, 4.68-4.81), monomeric protein.

For some experiments, the lysate, after dopamine-β-hydroxylase removal as described, was preparatively electrophoresed on native polyacrylamide gels (17, 18) and not only the major chromogranin (A) band, but also several minor chromogranins (B, C; D; see "Results") bands, were sliced out and eluted from unstained gels, as noted above, using a parallel stained reference gel, as described (13, 17, 18). Chromogranin H was also re-electrophoresed in SDS gels (16).

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‡ The abbreviations used are: RIA, radioimmunoassay; SDS, sodium dodecyl sulfate.
Human chromogranin A was also prepared from the chromaffin vesicle lysate of a pheochromocytoma, exactly as described above and earlier (13), to be used as an immunogen.

Preparation of Antisera
Antisera to chromogranins A (antigen purified from 8 m urea gels, with purity documented by re-electrophoresis on SDS gels) and B (antigen purified on native gels, with purity documented by re-electrophoresis on SDS gels) were prepared in male New Zealand white rabbits by a modification of the schedule of Miras-Portugal and Santos-Ruiz (19). One mg of antigen, emulsified in complete Freund’s adjuvant, was injected in multiple intradermal sites, on three occasions at 2-week intervals. The antisera were harvested 1 month after the last injection, via the central ear artery. The animals received a 500-µg booster injection at 6-month intervals, with antisera harvested 1 month later. Antisera were aliquotted and stored at −70 °C.

Radiodination of Chromogranin A
The purified bovine chromogranin A was radiodinated by the solid phase, immobilized lactoperoxidase-golden method (20) (Enzymobeads, Bio-Rad Laboratories), as previously outlined by us (21). After radiodination, the labeled chromogranin A was separated from iodide and aggregate on an Ultragel ACA-24 gel filtration column (25 × 1.5 cm), equilibrated with 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.4, 0.1% (w/v) ovalbumin, 0.1% (w/v) sodium azide, and eluted at 2 ml/h, collecting 1 ml fractions. This gel filtration resin provided optimal separation of radiodinated chromogranin A (125I-chromogranin A) from both the void volume (V0, determined by blue dextran) and the total internal volume (Vt, determined by Na105I) of the column. 125I-Chromogranin A was detected by counting 10 µl from each fraction for 1 min in a Searle Model 1185 γ counter (Searle Analytic, Inc., Des Plaines, IL).

Antibody Titrations
To determine the immunoprecipitability of the radiodinated antigen and to determine the titer of the first antibody for RIA, immunoprecipitation of 125I-chromogranin A was carried out with serial dilutions of first antibody (rabbit anti-chromogranin A). The incubation mixture contained 5000 cpm of 125I-chromogranin A in 100 µl of buffer PBO-10 (0.15 M NaCl, 0.01 M sodium phosphate, pH 7.4, 1% (w/v) ovalbumin, 0.1% (w/v) sodium azide); 100 µl of 0.1 M EDTA, pH 7.4; 100 µl of various dilutions of first antibody in PBO-10; and 500 µl of PBO-10. After 24 h at 4 °C, the second antibody (in slight excess) and carrier antisera were added: 100 µl of a 1:8 (v/v) dilution of sheep anti-rabbit γ-globulin (SA, P4 titer) in PBO-10 and 100 µl of 2% (v/v) normal rabbit serum diluted in PBO-10. After another 24 h at 4 °C, bound and free antigen were separated by centrifugation at 5000 × g for 20 min at 4 °C. The supernatant was aspirated and the antigen-antibody pellet was washed with 500 µl of PBO-10 and centrifuged at 5000 × g for 20 min at 4 °C. After aspiration of the supernatant, the final antigen-antibody pellet was counted in the tube for 1 min in the γ counter.

Titrations results were graphed as per cent of maximum binding (at low titer of first antibody) versus the log2 of the first antibody dilution.

A time course analysis of incorporation of 125I-chromogranin A into the final antigen-antibody pellet was conducted both for the first antigen-antibody reaction (over 1–3 days, at various dilutions of first antibody), followed by a second antigen-antibody reaction for 24 h, for the second antigen-antibody reaction (over 24 h, at the previously noted final dilution of second antibody used in the assay, after a first antigen-antibody reaction of 24 h at working titer of first antibody).

Radioimmunoassay
The working titer of first antibody was selected to immunoprecipitate 50% (nearest whole number with molecular weight taken as the SDS-gel electrophoretic value) of a blank polyacrylamide preparative gel used in the entire procedure, to correct for amino acid contaminants in the gel buffers or matrix. The eluate from a blank polyacrylamide preparative gel was also carried through this entire procedure, to correct for amino acid contaminants in the gel buffers or matrix.

Peptide Mapping—One hundred- to two hundred-µg portions (1.5–3 nmol) of purified bovine chromogranins A and B were dialyzed against water, lyophilized, oxidized by performic acid (24), diluted, lyophilized, reconstituted in 0.2 M sodium phosphate, pH 7.0, and digested with bovine pancreatic trypsin (diphenylcarbamyl chloride pretreated to inactivate residual chymotrypsin) for 24 h at 25 °C, at a trypsin:substrate mass ratio of 1:50. The digestion was terminated by boiling for 10 min. The digest was lyophilized, resuspended in 10 µl of chromatography buffer (20 µM Tris-HCl, pH 7.5), and 5 µl was spotted onto 5 µl of the α-cyanol, cross-linked, and dried. The dried samples were mounted on a Silanated glass plate and sprayed with a solution of α-cyanol, dried, and placed on a Durrum model D-500 amino acid analyzer (Dionex Corp., Sunnyvale, CA), eluting with a physiological amino acid profile program (25), with data analysis by a Mark II software package (Dionex Corp.). Results are expressed as the mean value (±5 D or coefficient of variation) of two or more determinations, except for serine and threonine, where results are extrapolated to zero time to correct for destructive losses during hydrolysis. Results are expressed both as weight per cent (grams of the particular amino acid/100 g of protein) and as residues/nmol (nearest whole number with molecular weight taken as the SDS-gel electrophoretic value). The eluate from a blank polyacrylamide preparative gel was also carried through this entire procedure, to correct for amino acid contaminants in the gel buffers or matrix.

Ouchterlony Immunodiffusion
Antigenicity and antigenic cross-reactivity among chromogranins was also examined by Ouchterlony double immunodiffusion (22), performed overnight at 4 °C, in a humid chamber with prepunched agar plates (Hyland Laboratories) soaked in phosphate-buffered saline, containing a central antibody well and peripheral antigen wells. After 16 h of incubation, the plates were soaked overnight in phosphate-buffered saline, then stained for 1 h with 1% (w/v) Amido black in 7% (v/v) acetic acid, and destained with 7% (v/v) acetic acid. After photography, plates were soaked in 10% (v/v) glycerol and air-dried for storage.
Chromogranin A in Adrenergic Tissues

3239

parts pyridine, 897 parts H2O, pH 6.5. The buffer was sprayed onto the plate and electrophoresis was performed over a metal cooling plate at 8-10°C, for 50 min at 800 V (cathode on left), whereupon the plate was dried, sprayed with 0.025% (w/v) fluorescein in acetone, then visualized, and photographed under a long wavelength ultraviolet lamp. A control experiment, assessing the peptide map of chromogranin A, in the concentration used in these experiments, incubated without addition of chromogranin substrate, did not result in detectable peptide spots.

Preparation of Other Antigens for Cross-reactivity Studies in the RIA

Bovine adrenal dopamine β-hydroxylase was purified by concanavalin A-Sepharose chromatography on bovine adrenal medulla chromaffin vesicle lysates, as previously described (15).

Chromaffin vesicle soluble lysates from human pheochromocytoma (n = 1), normal dog adrenal medulla (n = 2), normal rat adrenal medulla (n = 250), normal sheep adrenal medulla (n = 5), and normal pig adrenal medulla (n = 2) were prepared by sucrose density step gradient centrifugation (14) as previously described (3).

Bovine Adrenal Medulla Subcellular Fractionations

To localize immunoreactive chromogranin A within the cell, adrenal medulla subcellular fractions were obtained by a modification (3) of the sucrose density step gradient method of Smith and Winkler (14). Fractionation was performed in quadruplicate. The glands were obtained within 20 min of death at the slaughterhouse and transported immediately to the laboratory at 0°C. All steps were conducted in the cold. The medullae were disected out, weighed, minced finely, homogenized at 20% (w/v) in 0.3 M sucrose in a Potter-Elvehjem tissue grinder with glass mortar and Teflon pestle ( Harold H. Thomas) and filtered through cheesecloth. The homogenate was centrifuged at 1000 x g for 10 min (1 x 10^4 x g x min) to sediment nuclei and debris and then at 25,000 x g for 20 min (5 x 10^4 x g x min) to sediment a crude granule fraction from the supernatant, containing cytosol and microsomes. The crude granule fraction was resuspended in 0.3 M sucrose, layered onto step gradients of 1.6 M sucrose, and centrifuged at 20,000 x g for 5 min (6 x 10^5 x g x min) to yield a pink chromaffin granule pellet. Electron microscopy of the granule pellet showed a highly purified chromaffin granule preparation (3). The chromaffin granules were lysed by ressuspension in 0.001 M sodium phosphate, pH 6.5, frozen and thawed, and centrifuged at 100,000 x g for 60 min to separate soluble vesicle lysate from vesicle membranes. The membranes were resuspended in the same buffer, frozen and thawed, and recentrifuged at 100,000 x g for 60 min to wash the membranes free of remaining soluble vesicle lysate. Tissue fractions were frozen at -70°C prior to assay. Tissue fractions were assayed for protein, chromogranin A, dopamine-β-hydroxylase, and catecholamine.

Chromogranin in the Nervous System

Bovine brains were obtained within 30 min of death and transported to the laboratory in ice-cold 0.3 M sucrose. All steps were performed in the cold. For regional distribution, dissections were performed in quintuplicate. The tissue was minced, homogenized in 0.3 M sucrose (at 1:3 ratio of tissue:buffer) in a Potter-Elvehjem homogenizer (glass mortar, Teflon pestle), frozen and thawed, and centrifuged at 1000 x G for 10 min (1 x 10^4 x g x min) to sediment debris, whereupon the supernatant was frozen at -70°C prior to assay.

For subcellular distribution, a crude synaptosomal preparation (28) was obtained in quintuplicate from frontal cortex, since this was a brain region relatively high in chromogranin concentration (see below). The tissue was minced, homogenized in 0.3 M sucrose (at 1:10 ratio of tissue:buffer) in a Potter-Elvehjem homogenizer (glass mortar, Teflon pestle), then centrifuged at 1000 x G for 10 min (1 x 10^4 x g x min) to sediment a nuclear fraction, at 20,000 x G for 20 min (4 x 10^5 x g x min) to sediment a crude synaptosomal fraction, and at 10,000 x G for 10 hr (6 x 10^5 x g x min) to sediment microsomes and membranes, leaving a cell cytosol supernatant. The pellets were resuspended in 0.01 M sodium phosphate, pH 7.4, and then all cell fractions were frozen at -70°C prior to assay. The identity of synaptosomes in the crude synaptosomal pellet was verified in two ways: (a) morphologically, by transmission electron microscopy on a portion of the synaptosomal pellet (see below) and (b) biochemically, by spectrophotometric assay of acetylcholinesterase (27), a synaptosomal marker enzyme localized to the synaptosomal membranes (26). Tissue fractions were assayed for protein, chromogranin A, and acetylcholinesterase.

Sympathetic nerve homogenates were prepared from sympathtic axons dissected from the intrasplenic (distal) portions of splenic neurovascular bundles (28). Homogenizations were performed in a Polytron (Brinkmann Instruments).

Chromogranin in Serum

Whole blood samples were freshly obtained from 10 cows, then allowed to clot in the cold, then centrifuged at 1000 x g for 10 min (10^4 x g x min), whereupon the serum was frozen at -70°C for future assay.

Chromaffin Vesicle Lysate and Brain Homogenate Gel Filtration

One hundred μl of bovine chromaffin vesicle soluble lysate and 100 μl of brain homogenate (frontal cortex, homogenized in a protease inhibitor buffer of 0.01 M EDTA, 0.001 M phenylmethylysulfonyl fluoride, 0.01 M N-ethylmaleimide, 0.01 M sodium phosphate, pH 6.5) were gel-filtered using the same Ultragel ACA-22 column, buffer, and conditions used for isolation of l25I-chromogranin after radioiodination.

Electron Microscopy

Portions (2 x 2 x 2 mm) of selected, freshly prepared subcellular fractions (chromaffin granule pellets, synaptosomal pellets) were fixed, stained, and viewed by transmission electron microscopy as previously described (13).

Assays

Protein was measured by the Coomassie blue dye binding method (29) as recommended for adrenal subcellular fractions (30). Catecholamines were determined separately as epinephrine and norepinephrine, by the fluorimetric method (31). Dopamine β-hydroxylase was assayed spectrophotometrically (32) with inclusion of 30 mM N-ethylmaleimide to neutralize endogenous inhibitors; results were expressed as international units, where 1 unit represents conversion of 1 μmol of tyramine substrate to octopamine product per min at pH 5.0 and 37°C. Acetylcholinesterase (a synaptosomal marker: enzyme) (26) was assayed spectrophotometrically (27). Results are recorded as units of acetylcholinesterase activity per mg of protein, where 1 unit represents the amount of enzyme that liberates 1 μmol of acetic acid from acetylcholine in 20 min at 25°C and pH 7.5, with a lower detection limit of 1 unit in the assay. Assay color blanks were tissue fractions heated to 60°C for 10 min prior to assay, to destroy authentic tissue acetylcholinesterase activity (27).

RESULTS

Chromogranin A was purified to SDS electrophoretic homogeneity from bovine adrenal chromaffin vesicle lysates (Fig. 1, left). At lower protein loads on the gel, chromogranin A gave the appearance of a doublet band, as previously described (3). After solid phase enzymatic radioiodination, the 125I-chromogranin was well separated from aggregated material and Na125I by gel filtration (Fig. 2). The product had a specific activity of 300,000 cpn/μg of protein.

Immunoprecipitation of 125I-bovine chromogranin A (Fig. 3) was carried out with two antisera to bovine chromogranin A and one antiserum to bovine chromogranin B. Each precipitated 70-85% of the total counts/min at excess titters, indicating intact immunoreactivity of the radioiodinated molecule. The 5% immunoprecipitation titers for the antichromogranin A antiserum were approximately 1:1000. The antichromogranin B antiserum also precipitated 125I-chromogranin A, albeit at a somewhat lower titer of approximately 1:200. An antiserum to human chromogranin A also precipitated 125I-labeled bovine chromogranin A, indicating at least some interspecies cross-reactivity. An antiserum directed against rat chromogranin A (#137D-1) did not immunoprecipitate 125I-labeled bovine chromogranin A, at any titer.

Timing of the RIA incubation steps was determined by the time course analysis of the association of first antigen (bovine...
Chromogranin A in Adrenergic Tissues

chromogranin A) with first antibody (rabbit anti-bovine chromogranin A) at various dilutions (Fig. 4, left) and the association of second antigen (rabbit γ-globulin) (Fig. 4, right) with second antibody (sheep anti-rabbit γ-globulin). Both reactions were essentially complete at 24 h; thus an equilibrium RIA was constructed with sequential 24-h incubations at 4 °C. Neither incubation at 25 °C, nor preincubation for 2 h at 37 °C, measurably accelerated the association of first antigen with first antibody.

Unlabeled chromogranin A competitively displaced 125I-chromogranin A from the antigen-antibody pellet, yielding the assay standard curve (Fig. 5). The assay had a sensitivity and working range of 10–100 ng/tube. Repeated measurements on the same unlabeled chromogranin A sample yielded an intra-assay coefficient of variation of 4.8% (n = 20) and an interassay coefficient of variation of 13% (n = 8).

Several other chromogranins, here named B, C, and D, were sliced out of preparative polyacrylamide native gels of chromaffin vesicle lysate (Fig. 1, right). Each of these chromogranins was able to displace 125I-chromogranin A from its antibody in parallel with the pure chromogranin A standard (Fig. 5), suggesting that the antibody recognized the same or a similar site on all the chromogranin antigens. The per cent cross-reactivity, on a weight basis, was 40.7% for chromogranin B, 8.6% for chromogranin C, and 1.2% for chromogranin D (Table I).

The electrophoretic pattern of multiple chromogranins (Fig. 1) was unlikely to be the result of artifactual proteolysis during vesicle preparation, since the same patterns were noted even when the vesicle preparation was conducted in sucrose buffers containing 0.001 M phenylmethylsulfonyl fluoride. Furthermore, the electrophoretic patterns of multiple chromogranins persisted in several vesicle lysate preparations.

Chromogranins A and B were subjected to re-electrophoresis in SDS gels (Fig. 1, left), yielding apparent molecular weights (upon interpolation of relative mobilities on a plot of molecular weight standards’ relative mobility versus log

FIG. 1. Left, SDS-polyacrylamide gel appearance of purified bovine chromogranins A and B. SDS gels were run by the method of Laemmli (16), with migration from top to bottom. The lanes are: 1, bovine chromaffin vesicle soluble lysate; 2, purified and re-electrophoresed bovine chromogranin A at heavy protein load; 3, purified and re-electrophoresed chromogranin A at light protein load; 4, purified and re-electrophoresed chromogranin B at heavy protein load; 5, purified and re-electrophoresed chromogranin B at light protein load; 6, molecular weight standards (α = bovine albumin, 66,000; b = ovalbumin, 45,000; c = pepsin, 34,700; d = trypsinogen, 24,000; e = β-lactoglobulin, 18,400; f = lysozyme, 14,300). DBH = dopamine-β-hydroxylase; CgA = chromogranin A; CgB = chromogranin B. Right, preparative polyacrylamide gel electrophoresis of several chromogranins (Cg). After concanavalin A removal of dopamine-β-hydroxylase from a bovine chromaffin vesicle lysate, the lysate was electrophoresed on 12 native polyacrylamide cylindrical tube gels (17, 18), with migration from top to bottom. A reference gel (shown here) was stained with Coomassie blue G in trichloroacetic acid (26) and destained in 5% (v/v) acetic acid, and regions corresponding to the stained chromogranin bands (CgA through CgD) were sliced out of the remaining 11 gels and eluted as described under "Materials and Methods."
Chromogranin A in Adrenergic Tissues

First antigen-antibody reaction

Counts/minute in antigen-antibody pellet

First antigen-antibody reaction incubation, days

| Dilution of first antibody | Counts/minute |
|---------------------------|---------------|
| 1.000                     | 1500          |
| 1.200                     | 1500          |
| 1.400                     | 1500          |
| 1.600.000                 | 1500          |

Second antigen-antibody reaction

Counts/minute in antigen-antibody pellet

Second antigen-antibody reaction incubation, hours

| Counts/minute |
|---------------|
| 1500          |
| 1500          |
| 1500          |
| 1500          |

FIG. 4. Left, time course of the association of first antigen (131I-chromogranin A) with first antibody (rabbit anti-bovine chromogranin A) at various dilutions of first antibody at 4 °C. At the working dilution of first antibody (1:1000 to 1:1500), the binding is substantially complete by 24 h. Right, time course of association of second antigen (rabbit γ-globulin) with second antibody (sheep anti-rabbit γ-globulin), at the concentrations of both used in the RIA, at 4 °C. The reaction is substantially complete by 24 h.

molecular weight, \( r = -0.999 \) of 67,000 and 52,000, respectively, neither of which changed with inclusion or exclusion of sulphydryl reagents in the electrophoresed sample, precluding intersubunit disulfide links in either. At lighter protein loads on the SDS slab gels, chromogranins A and B each had a doublet appearance (Fig. 1, left), as has been previously described for chromogranin A (13).

Amino acid analysis on chromogranins A, B, and C revealed unusual but similar percent compositions (Table II and Fig. 6)—each was rich in glutamic acid (28.76–30.56%) and proline (7.95–8.03%) and poor in cysteine (0–0.20%).

The tryptic digest peptide maps of chromogranins A and B (Fig. 7) revealed considerable structural homology, with most peptides shared in common.

Ouchterlony immunodiffusion (Fig. 8) also revealed chromogranin cross-reactivity—the precipitin lines for total bovine chromogranins, bovine chromogranin A, and bovine chromogranin B all fused, suggesting that the antibody (rabbit anti-bovine chromogranin A) recognizes a similar antigenic determinant in all three. This antibody does not form a precipitin line with human chromogranin A or B (Fig. 8).

Other chromaffin granule soluble constituents—catecholamines, enkephalins, and dopamine β-hydroxylase—did not cross-react in the RIA with chromogranin A, even at 10,000-fold mass excess in the assay (Table I). The interspecies cross-reactivity of the assay was minimal (Table I).

A chromaffin granule preparation was obtained from bovine adrenal medulla and verified by electron microscopy (Fig. 9) and by biochemical markers (Table III). Chromogranin immunoreactivity was detected in nanoliter quantities of adrenal medullary subcellular fractions. The immunoreactivity paralleled the assay standard curve (Fig. 10) and was not abolished by boiling or treatment with protease inhibitors (0.01 M EDTA, 0.01 M N-ethylmaleimide, and 0.001 M phenylmethylsulfonyl fluoride); thus the immunoreactivity could not be ascribed to proteolytic activity in the adrenal medulla.

Gel filtration of bovine chromaffin vesicle lysate (Fig. 11) yielded a single immunoreactive chromogranin peak that eluted in the same position as 131I-chromogranin A, with an apparent Stokes radius (15) of 80 Å for both. A small trailing shoulder on the peak may represent the minor, lower molecular weight chromogranins.

The subcellular distribution of immunoreactive chromogranin A, catecholamines, and dopamine β-hydroxylase in the adrenal medulla is presented in Table III. All three were largely localized to the catecholamine storage vesicles—chromogranin A and catecholamines were predominantly in the soluble vesicle lysate, while dopamine β-hydroxylase was distributed between soluble vesicle lysate and vesicle membrane. Immunoreactive chromogranin A was almost completely (69%/74% or 93%) released from the vesicles into the soluble lysate during the first in vitro lysis cycle, with an additional 3%/74% or 4% released during the second lysis cycle. Only 2%/74% or 3% of the vesicle's chromogranin remains on the vesicle membrane after two in vitro lysis cycles.

The tissue fraction chromogranin A specific activity (immunoreactive chromogranin A protein/total protein) was highest, within the chromaffin cell, in the catecholamine storage vesicle soluble lysate (Table III), where chromogranin immunoreactivity accounted for 46 ± 2% of the total soluble...
FIG. 5. The chromogranin A radioimmunoassay standard curve. The curve is obtained by displacement of \(^{125}\)I-chromogranin A (5000 cpm total) from first antibody (at 1:1000 working dilution) by increasing concentrations of unlabeled chromogranin A. The results are plotted as B/Bo versus \(\log \_\)concentration of unlabeled chromogranin A, where B = pellet counts/min (minus blank) for a given assay tube and Bo = pellet counts/min (minus blank) for the reaction mixture without added unlabeled antigen. Dose/response cross-reactivities of the minor bovine chromogranins (B, C, D) in the chromogranin A RIA are shown. Various dilutions of each chromogranin were included in the assay. The results are recorded in Table I: chromogranin A (5000 cpm total) from first antibody (at 1:1000 working dilution) were used to calculate percent cross-reactivity.

* <0.01% cross-reactivity indicates no detectable cross-reactivity even at the highest dose of the particular antigen included in the assay.

**TABLE I**

Immunologic cross-reactivities in the bovine chromogranin A radioimmunoassay

| Category | Substance | Immunologic cross-reactivity with bovine chromogranin A in the RIA, % (by mass) |
|----------|-----------|--------------------------------------------------------------------------------|
| Within the bovine chromaffin granule | Other chromogranins | 100 |
| | Chromogranin A | 40.7 |
| | Chromogranin B | 8.6 |
| | Chromogranin C | 1.2 |
| | Dopamine- \(\beta\)-hydroxylase | <0.01* |
| | Catecholamines | <0.01* |
| | Epinephrine | <0.01* |
| | Norepinephrine | <0.01* |
| | Enkephalins | <0.01* |
| | Leucine enkephalin | <0.01* |
| | Methionine enkephalin | <0.01* |
| | Ascorbic acid | <0.01* |
| | Adenosine triphosphate | <0.01* |
| Other species' chromaffin granules; source | Rat adrenal medulla (n = 250) | <0.01* |
| | Dog adrenal medulla (n = 2) | <0.01* |
| | Human pheochromocytoma (n = 1) | <0.01* |
| | Sheep adrenal medulla (n = 5) | Partial, nonparallel |
| | Pig adrenal medulla (n = 2) | Partial, nonparallel |
| Other neuropeptides | Calmodulin | <0.01* |
| | Neurophysin II | <0.01* |

In the initial adrenal medullary cell homogenate, chromogranin immunoreactivity accounted for 7 ± 1% of the cell's total protein.

In the nervous system, chromogranin was detected by parallel titration displacement of \(^{125}\)I-chromogranin A from antibody (Fig. 12). Immunoreactive chromogranin had a widespread distribution in various brain regions (Table IV), being maximal in neocortex and minimal in cerebellum, medulla oblongata, and spinal cord. Even in the neocortex, however, there was 1000-fold less chromogranin in brain than in adrenal medulla. The pituitary gland also contained substantial quantities of chromogranin immunoreactivity (Table IV). Neither boiling nor treatment with several protease inhibitors (0.01 M EDTA, 0.001 M phenylmethylsulfonyl fluoride, 0.01 M N-ethylmaleimide) abolished brain tissue chromogranin immunoreactivity, suggesting that brain proteases were not contributing to apparent tissue immunoreactivity.

Subcellular distribution studies included preparation of a crude brain synaptosomal fraction (Table V, Fig. 13). Electron microscopy of the synaptosomal pellet revealed typical synaptic vesicles (Fig. 13). The synaptosomal fraction also contained acetylcholinesterase activity (4.14 ± 0.97 units/mg of protein, range 2.98 to 6.22 units/mg of protein), while the other cell fractions (nuclear, cytosol, microsomal, and membrane) did not contain detectable acetylcholinesterase (<1 unit/100 \(\mu\)l of cell fraction). Only 18 ± 2% of the cell's immunoreactive chromogranin was found in the synaptosomes, at a specific activity of only 10.4 ± 1.4 ng of chromogranin/mg of protein. By contrast, the cytosol contained the majority (69 ± 6%) of the cell's chromogranin, at a higher specific activity of 123 ± 4.5 ng of chromogranin/mg of protein. In fact, chromogranin specific activity in the cell increased with each successive centrifugation (Fig. 13), culminating in a maximal specific activity in the very high speed supernatant (cytosol). This is in marked contrast to the results in the adrenal medulla, where per cent chromogranin localization and specific activity were both maximal in the sedimentable vesicle fraction (Table III). Similar chromogranin subcellular distribution results in brain frontal cortex were noted (n = 10) when another buffer (0.3 M Tris, pH 7.0) was used for homogenization and centrifugation. A similar chromogranin subcellular distribution (maximal per cent of total and specific activity in the cytosol) was
Chromogranin A in Adrenergic Tissues

The values are presented as weight per cent (grams of that amino acid/100 g of protein) and as residues/mol (to the nearest integral number of residues), taking molecular weights from SDS gel relative electrophoretic mobility (chromogranin A, $M_r = 67,000$; chromogranin B, $M_r = 52,000$). Numbers shown are the mean value for 3 hydrolysates at 24, 48, and 72 h, with coefficient of variation ((standard deviation/mean) (100)), except for serine and threonine, which were extrapolated back to time zero to correct for destructive losses during acid hydrolysis. The amino acid composition of bovine chromogranin A has been reported previously (13).

| Amino acid | Weight per cent g/100 g | Chromogranin A | Chromogranin B | Chromogranin C | CgA* | CgB | CgC | Coefficient of variation % | residues/mol |
|------------|-------------------------|----------------|----------------|----------------|------|-----|-----|----------------------------|--------------|
| Glutamic acid | 28.76 | 30.56 | 28.82 | 0.2 | 1.8 | 2.5 | 149 | 123 |
| Arginine | 9.64 | 10.63 | 10.09 | 1.7 | 3.5 | 5.9 | 41 | 35 |
| Lysine | 9.62 | 9.68 | 9.21 | 0.1 | 1.9 | 3.8 | 50 | 39 |
| Aspartic acid | 8.41 | 8.82 | 8.86 | 1.2 | 0.1 | 4.6 | 49 | 40 |
| Leucine | 8.06 | 7.34 | 7.81 | 2.4 | 3.4 | 4.7 | 48 | 34 |
| Proline | 7.95 | 7.79 | 8.03 | 3.7 | 8.0 | 4.4 | 55 | 42 |
| Alanine | 5.51 | 6.48 | 5.84 | 0.2 | 0.3 | 3.6 | 52 | 48 |
| Glycine | 4.36 | 5.00 | 4.98 | 1.5 | 5.5 | 2.6 | 51 | 45 |
| Valine | 4.51 | 3.14 | 3.37 | 1.4 | 1.3 | 2.5 | 31 | 17 |
| Serine | 2.78 | 3.02 | 2.85 | 1.5 | 1.9 | 3.8 | 50 | 39 |
| Phenylalanine | 2.03 | 2.12 | 2.32 | 0.1 | 3.6 | 4.1 | 9 | 8 |
| Histidine | 1.74 | 1.17 | 1.26 | 0.9 | 1.8 | 3.0 | 9 | 4 |
| Methionine | 1.57 | 1.63 | 1.35 | 1.6 | 2.9 | 15.2 | 8 | 4 |
| Threonine | 1.57 | 1.15 | 1.42 | 1.4 | 2.5 | 15.2 | 10 | 6 |
| Isoleucine | 1.40 | 0.84 | 1.33 | 2.4 | 10.0 | 5.1 | 8 | 4 |
| Tyrosine | 1.10 | 1.13 | 1.20 | 25.5 | 25.3 | 47.7 | 5 | 3 |
| Cysteine | 0 | 0 | 0.20 | 48.8 | 48.8 | 0 | 0 | 0 |

*CgA, chromogranin A; CgB, chromogranin B; CgC, chromogranin C.

Fig. 7. Thin layer peptide maps of bovine chromogranin A (left) and bovine chromogranin B (right). The trypsin-digested peptides were applied to the origin (lower left-hand portion of each panel) and then subjected to chromatography (dimension 1, from bottom to top) followed by electrophoresis (dimension 2, from left to right). The separated peptides were visualized by long wave ultraviolet irradiation after fluorescamine spraying. The peptide map for chromogranin A (left) has been reported previously (13).

also found in the hypothalamus ($n = 5$).

Brain chromogranin also differed from adrenal medullary chromogranin on gel filtration. While adrenal medullary chromogranin immunoreactivity co-eluted with purified $^{131}$I-chromogranin A (Fig. 11), brain (frontal cortex) chromogranin immunoreactivity eluted from the calibrated column consistently later than purified $^{131}$I-chromogranin A (Fig. 14), with an apparent Stokes radius (15) of 54 Å. The result could not easily be ascribed to proteolytic degradation of chromogranin during homogenization and preparation of the brain tissue sample for gel filtration, since these steps were carried out in a buffer containing several protease inhibitors: 0.01 M EDTA, 0.001 M phenylmethylsulfonyl fluoride, and 0.01 M N-ethylmaleimide. Similar results were obtained ($n = 2$) when brain homogenates were prepared and gel-filtered in the absence of protease inhibitors.

Chromogranin immunoreactivity was also detected in sympathetic nerve and in serum (Fig. 12, Table IV), although the relative amounts were 6,000-fold and 30,000-fold, respectively, less than that found in the adrenal medulla. Equivalent results were obtained in bovine serum, EDTA plasma, or heparinized plasma. Immunoreactive chromogranin was detectable with this assay in bovine and sheep sera, although not in sera from pig, rat, rabbit, or man.

**DISCUSSION**

We sought to develop an immunoassay for chromogranin A because it is a major soluble component of catecholamine storage vesicles and may be a useful probe of catecholamine storage and exocytotic release (9-12). Prior attempts to measure chromogranin A used microcomplement fixation; while useful information emerged, the technique was insensitive and prone to large errors (−50% to +200%) because the results emerge from an analysis of degree of antigen dilution required to fix complement (9-12).
Chromogranin A in Adrenergic Tissues

Our assay is sensitive (working range, 10–100 ng/tube) and reproducible (intra- and interassay coefficients of variation, 4.8% and 13%). Within the chromaffin vesicle, the minor chromogranins (B, C, D) cross-react to some extent (1.2%–40.7%) (Fig. 5; Table I), while other vesicle constituents do not cross-react (Table I). The parallel displacement of $^{125}$I-chromogranin A from antibody by all four chromogranins suggests that the antibody recognizes a similar antigenic determinant on all four chromogranins. Further evidence for immunologic relatedness is the ability of anti-chromogranin B to precipitate chromogranin A (Fig. 3). In addition, anti-chromogranin A forms fused precipitin lines with both chromogranin A and chromogranin B during double immunodiffusion (Fig. 8). Antigenic cross-reactivity among the chromogranins has also been reported by Hörtnagl et al. (17), who found that anti-chromogranin A also recognized minor chromogranins with faster electrophoretic mobility than chromogranin A.

These immunologically cross-reacting chromogranins also possess considerable structural homology, as judged by similar amino acid compositions (Table II, Fig. 6) and similar trypptic digest peptide maps (Fig. 7). Thus, there seems to be a "family" of chromogranins, structurally and immunologically related, with a spectrum of sizes. The similarities of chromogranins A and B are consistent with Smith and Kirshner's report (2) that chromaffin granule proteins $S_1$ and $S_2$ (analogous to our chromogranins A and B) have similar amino acid compositions and peptide maps.

It should be noted that the biosynthetic relationship of the minor chromogranins to chromogranin A has not been established. The minor, lower molecular weight chromogranins could be either separate gene products from chromogranin A or post-translational (e.g. proteolytic) modifications of chromogranin A, even though they are present when vesicles are prepared in the presence of 0.001 M phenylmethylsulfonyl fluoride to inhibit serine proteases. This issue could perhaps be addressed by in vitro translation of adrenal medullary messenger ribonucleic acid, with immunoprecipitation of chromogranin-like translation products prior to in vivo post-translational modifications. Also, we have not entirely excluded the possibility that some of the minor chromogranins might have been contaminated with fragments of chromogranin A. In particular, we have not shown that all of the chromogranin B, C, and D preparations can be quantitatively precipitated with antisera to chromogranin A. However, chromogranin B's homogeneous appearance on re-electrophoresis in SDS (Fig. 1), coupled with its substantial post-translational modifications. Also, the assay recognizes only 46% of the soluble vesicle proteins (Table III) and does not recognize enkephal-

![Image](http://www.jbc.org/)

**Fig. 9.** Electron microscopy of the bovine chromaffin granule preparation. The electron dense cored structures are chromaffin granules. There is no visible mitochondrial contamination. The magnification is 5000 diameters.

**Table III**

*Subcellular distribution of chromogranin A, epinephrine, dopamine-β-hydroxylase activity, and total protein in the bovine adrenal medulla*

Results are recorded as mean ± S.E. for the four subcellular preparations. The subcellular fractions were prepared by differential sucrose gradient centrifugation, as described under "Materials and Methods." The supernatant over the crude chromaffin granule pellet constituted the cytosol plus microsomes. Chromogranin A was quantitated by radioimmunoassay, epinephrine was measured fluorimetrically, and dopamine-β-hydroxylase was measured spectrophotometrically. $n = 4$ for all determinations.

| Subcellular fraction | Total protein | | Chromogranin A | | Epinephrine | | Dopamine-β-hydroxylase activity |
|----------------------|--------------|----------------|----------------|----------------|-----------------|-----------------|------------------|
|                      | mg           | % total protein | mg            | % total immunoreactive chromogranin A | mg            | % total epinephrine | mg               | % total dopamine-β-hydroxylase activity |
| Homogenate (total cell) | 802 ± 66.9 | 100 | 58.9 | 100 | 0.074 | 114 | 100 | 410 | 100 |
| Chromaffin granules | | | | | | | | | |
| Soluble lysate | 86.5 | 11 | 40.4 | 69 | 0.460 | 62.8 | 55 | 57.4 | 14 |
| Membranes | 4.0 ± 0.5 | 1.3 | 0.24 | 0.4 | 0.045 | 0.46 | 0.4 | 86.7 | 21 |
| Membrane washings | 28.1 | 4 | 1.8 | 3 | 0.198 | 1.97 | 2 | 1.51 | 0.4 |
| Cytosol + microsomes | 408 | 51 | 16.5 | 28 | 0.041 | 27.9 | 24 | 83.2 | 20 |
| | ± 27.0 | ± 3 | ± 2.1 | ± 4 | ± 0.006 | ± 4.8 | ± 4 | ± 14.5 | ± 4 |
done according to Fig. 5. Both total cell homogenate and chromaffin vesicle soluble lysate displace $^{125}$I-chromogranin A from its antibody in parallel with displacement by unlabeled chromogranin A, suggesting that the antibody recognizes a similar, if not identical, antigenic determinant in pure chromogranin A and in the tissue fractions.

ins, opioid peptides recently detected in catecholamine storage vesicle lysates (33, 34).

$^{125}$I-labeled bovine chromogranin A can be immunoprecipitated by anti-human chromogranin A (Fig. 3), albeit at a low titer of approximately 1:100. However, unlabeled human chromogranins (pseudochromocytoma chromaffin vesicle lysate) do not cross-react in the bovine chromogranin A RIA, even at 10,000-fold mass excess (Table I). This suggests that, while bovine chromogranin A possesses antigenic determinants reacting with both the anti-bovine chromogranin A and the anti-human chromogranin A sera, at least some of the bovine chromogranin A antigenic determinants interact with a site on anti-bovine chromogranin A antibodies that does not recognize human chromogranin A. This limits the interspecies applicability of the bovine RIA. Partial immunologic cross-reactivity among the chromogranins from several species (cow, sheep, horse, pig, deer, elk) has been observed by Helle (35, 36) via immunodiffusion of the antigens against anti-bovine chromogranin.

The RIA apparently detects authentic chromogranin A antigenic determinants in adrenal medullary subcellular fractions, as evidenced by assay standard curve parallelism (Fig. 10), and gel filtration co-elution of pure $^{125}$I-chromogranin A and immunoreactive chromaffin vesicle chromogranin A (Fig. 11). The apparent Stokes radius (15) of both $^{125}$I-chromogranin A and immunoreactive vesicle chromogranin was 80 Å, very close to the figure of 77 Å for bovine chromogranin A reported by Smith and Winkler (1). Within the adrenal me-
TABLE V
Chromogranin A in the nervous system: subcellular distribution of chromogranin immunoreactivity in bovine brain (frontal cortex)
The subcellular fractions were prepared by differential centrifugation as detailed under "Materials and Methods"

| Subcellular fraction | Total protein | % cell total | Specific activity |
|----------------------|---------------|--------------|------------------|
|                      | mg           | µg          | mg chromogranin/ mg protein |
| Initial homogenate   | 634 ± 42     | 15.4 ± 1.81 | 100              |
| Nuclei               | 151 ± 23     | 1.72 ± 0.219| 11 ± 1           |
| Synaptosomes         | 269 ± 21     | 2.73 ± 0.314| 18 ± 2           |
| Microsomes, membranes| 86 ± 7      | 0.74 ± 0.066| 3 ± 0.4          |
| Cytosol              | 29 ± 2       | 10.6 ± 0.887| 69 ± 6           |

FIG. 13. Electron microscopy of the crude synaptosomal pellet after differential centrifugation of brain (frontal cortex) homogenate. Typical synaptosomal elements are identified, including the presynaptic cell of the synaptosome (Sy), synaptic vesicles (SV), and the postsynaptic density (PSD). The magnification is 10,000 diameters.

FIG. 14. Gel filtration of brain (frontal cortex) chromogranin immunoreactivity. The column, elution buffer, and conditions were the same as those in Figs. 2 and 11. Eluted fractions were tested for chromogranin by incubating 200 µl from each fraction in the RIA. The chromogranin immunoreactive peak eluted consistently later than the peak for purified I25I-chromogranin A, suggesting an effective hydrodynamic (Stokes) radius of 54 Å for brain chromogranin. Vo = void volume; Vt = total internal volume; I25I-chromogranin A = elution position for purified I25I-chromogranin A.

as an indicator of exocytotic release of protein along with neurotransmitter from the vesicles; that is, unlike dopamine β-hydroxylase, a major fraction of chromogranin does not remain behind, bound to the vesicle membrane, during discharge of vesicle contents to the extracellular space.

Chromogranin was present in the nervous system, although it apparently differed from adrenal medullary chromogranin in several fashions. First, there was far less chromogranin immunoreactivity in nervous tissue than in the adrenal medulla (1000-fold less in frontal cortex; 6000-fold less in sympathetic nerve). Second, the regional distribution of immunoreactive chromogranin in brain (maximal in neocortex, minimal in cerebellum, medulla oblongata, and spinal cord) is quite different from the regional distribution of norepinephrine (37), suggesting that chromogranin in brain may have a function independent of catecholamines. This dissociation is reinforced by the subcellular finding that chromogranin immunoreactivity is most prominent in the brain cell cytosol (Table V), rather than the synaptosomes, the usual storage site for neurotransmitters, including catecholamines (26). Finally, the molecular size of immunoreactive chromogranin, estimated by gel filtration (Figs. 11 and 14), was smaller in brain (at 54 Å) than in adrenal medulla (at 80 Å). All of these observations suggest that chromogranin in brain may have a very different role from adrenal chromogranin, although no function has yet been postulated for brain chromogranin.
Also of note, substantial amounts of chromogranin were detected in the pituitary gland and the pineal gland (Table 4). In the pituitary gland, there is a 30-fold more than that in the adrenal medulla, but 30-fold more than that in any other brain region. This further suggests a distribution of chromogranin independent of catecholamines and opens the possibility that chromogranin may be associated with polypeptide hormone producing tissues other than the adrenal medulla alone, as may also be the case in the parathyroid gland (38).

Easily detectable chromogranin in serum (Fig. 12, Table IV) provides a means of studying chromogranin's release and significance as a potential sympathoadrenal index molecule in intact animals via this accessible tissue.

In summary, we have used a chromogranin RIA, coupled with electrophoretic, amino acid, and peptide studies, to elucidate several structurally and immunologically related chromogranins. Application of the RIA to the adrenal medulla revealed a soluble phase, vesicular localization of chromogranin along with catecholamines. Analysis of brain, however, revealed an unusual chromogranin distribution (regional and subcellular) and molecular size. Measurement of chromogranin in serum may provide a tool for assessing chromogranin release in vivo and hence for investigation of exocytotic sympathoadrenal catecholamine release.

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