Chromogranins, widespread in endocrine and nervous tissue, bind Ca^{2+}

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The proteinaceous components of the secretory vesicle contents isolated from bovine adrenal medulla bind Ca^{2+} (number of binding sites, 152 ± 52 nmol Ca^{2+} per mg protein; dissociation constant, 54 ± 8 μM ($n = 5$)). SDS-polyacrylamide gel electrophoresis and $^{45}Ca^{2+}$ binding of the proteins following their separation and blotting on nitrocellulose revealed that Ca^{2+} binds to chromogranins. Moreover, it was shown that the chromogranins, like other known Ca^{2+}-binding proteins, can be specifically stained with a cationic carbocyanine dye. The Ca^{2+}-binding function of the chromogranins described here, in conjunction with recent findings concerning Ca^{2+} transport across chromaffin vesicle membranes and the widespread distribution of chromogranins in many different endocrine and nerve cells, points to the general importance of these proteins in the metabolism of Ca^{2+}.

Chromogranin *Ca^{2+} binding* *Metalloprotein* *Secretory vesicle*

1. INTRODUCTION

The chromogranins, an acidic family of soluble proteins present within chromaffin vesicles, are secreted together with catecholamines from the adrenal medulla (cf. [1]). Recent investigations support the idea that a membrane-bound fraction of chromogranins also exists within chromaffin vesicles [2]. In the last few years data have accumulated on the ubiquitous occurrence of chromogranin A, the major member of the chromogranin family [3], in a variety of endocrine cells secreting by exocytosis. Chromogranin A has been found not only in chromaffin cells but also in the hypophysis, islet cells of the pancreas, parafollicular C-cells of the thyroid gland, the chief cells of the parathyroid gland, and enteroendocrine cells [4–10]. In addition, chromogranins have been detected in several regions of the brain within aminergic and peptidergic neurons [11]. However, despite the widespread distribution of this protein, no function has been conclusively established.

2. MATERIALS AND METHODS

The proteins of the chromaffin vesicle contents were isolated in the following way. The adrenal medullae were homogenized in a medium containing 340 mM sucrose and 20 mM Mops/KOH, pH 7.3. After low-speed centrifugation at 2200 × g_{av} for 10 min, crude secretory vesicles were collected from the supernatant by a second centrifugation at 12500 × g_{av} for 20 min. The pellet was resuspended in homogenisation buffer and the fraction put on a sucrose step gradient consisting of 2.4/2.0/1.8/1.7 M sucrose in 20 mM Mops/KOH, pH 7.0, and centrifuged for 1 h at 146000 × g_{av} in a Beckmann L8-M ultracentrifuge using a 50.1 Ti rotor. Mitochondria and lysosomes (as determined with the marker enzymes glutamate dehydrogenase and arylsulfatase) remained on top of the gradient, whereas secretory vesicles were concentrated around the 1.8/2.0 M sucrose interface. To lower the sucrose concentration, the collected secretory vesicles were dialysed for 60 min against 340 mM buffered sucrose and concentrated by centrifuga-

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tion (146000 × g_{av}, 30 min). Afterwards they were lysed in 20 mM Mops/KOH, pH 7.0 (1 vol. vesicle fraction to 40 vols buffer). The secretory vesicle membranes were removed by centrifugation (146000 × g_{av}, 30 min). The supernatant was lyophilised and then dialysed twice for 24 h in 20 mM Mops/KOH, pH 7.0, to remove low-M_r components present within the secretory vesicles and for a further 15 h in the same buffer plus 0.25% Chelex 100 to remove all the residual Ca^{2+}.

The chromaffin vesicle content proteins were suspended in extraction medium containing 5% mercaptoethanol and 2% SDS in 0.0625 M Tris-HCl, pH 6.8, and put on a 1 mm slab gel (separation gel, 10% acrylamide; stacking gel, 4% acrylamide [12]). After electrophoresis the gel was cut into separate lanes to perform nitrocellulose blotting and staining. The proteins were transferred (1 h, 60 V) to nitrocellulose (Schleicher and Schüll) in a buffer containing 192 mM glycine/25 mM Tris/20% methanol, pH 8.3 [13].

3. RESULTS AND DISCUSSION

Here, we document the Ca^{2+}-binding property of chromogranins. Ca^{2+} binding by secretory vesicle content proteins, determined with the aid of a Ca^{2+}-specific electrode [14], increases as a function of the Ca^{2+} concentration (fig.1A). Using the experimental values obtained below 60 μM free Ca^{2+}, the line extrapolated to the abscissa and the ordinate by linear regression analysis, in a Scatchard plot (fig.1B) gave a binding capacity of 152 ± 52 nmol Ca^{2+} per mg protein and a dissociation constant of the Ca^{2+}-protein complex of 54 ± 8 μM (mean ± SD, n = 5). The deviation of the experimental values from the calculated line at higher Ca^{2+} concentrations indicates further binding sites with lower affinity.

To identify the proteins which are able to bind Ca^{2+}, SDS-polyacrylamide gel electrophoresis [12] was performed. The separated proteins were blotted onto nitrocellulose and incubated with ^{45}Ca. On subsequent autoradiography (fig.2, lane C) 2 labeled bands, which correspond to M_r 74000 and 60000, were obtained. The protein of M_r 74000 represents the dominant protein of the protein pattern obtained after staining with amido black (lane B) and is known as chromogranin A ([3], cf. [1]). Staining the gel with a cationic carbocyanine dye,

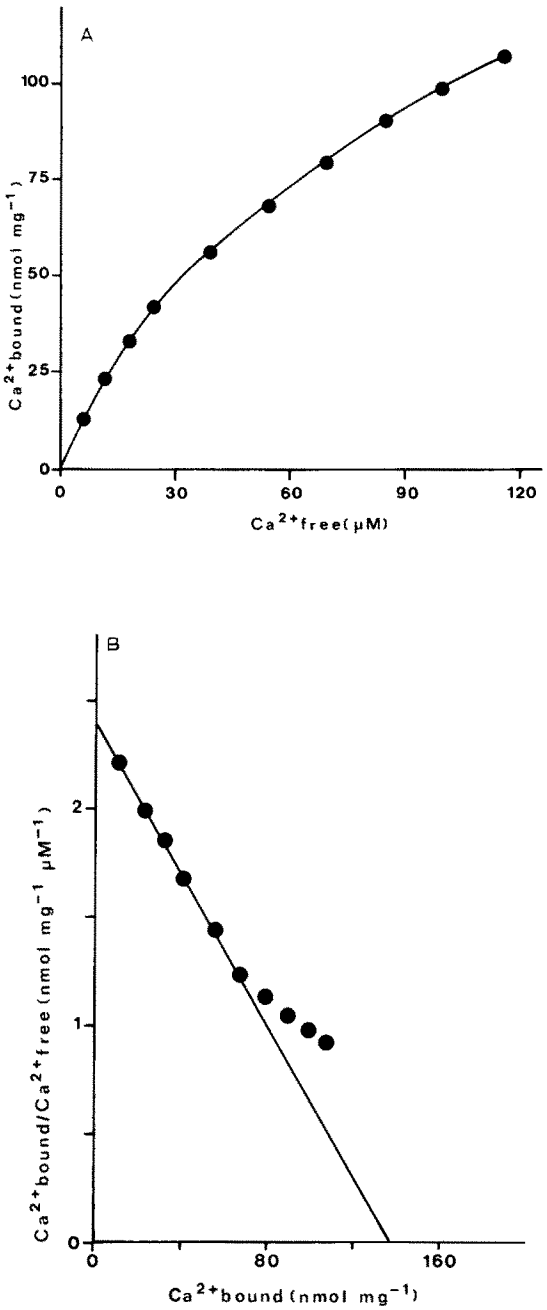

Fig.1. Ca^{2+} binding to chromaffin vesicle contents. 300–400 μg chromaffin vesicle content proteins (see section 2) per ml 20 mM Mops/KOH, pH 7.0, were titrated with 5 mM Ca^{2+} solution in the same buffer using a Ca^{2+}-specific electrode [14]. Bound Ca^{2+} was estimated from the difference to the buffer control values. The data of a representative titration are given in a direct plot (A), as well as in a Scatchard plot (B).

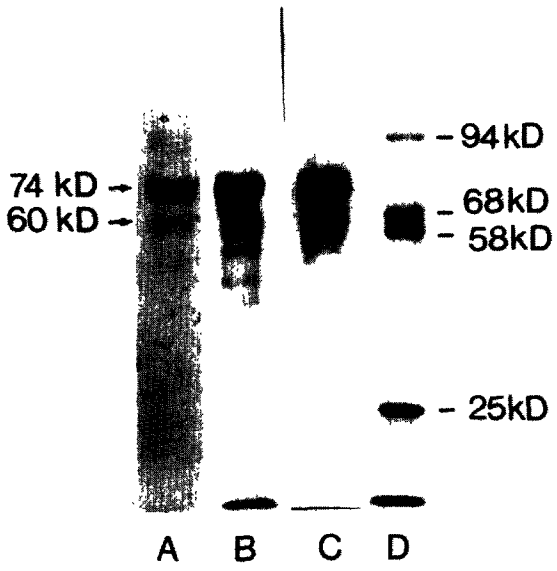

Fig.2. Characterization of the Ca^{2+}-binding proteins present in chromaffin vesicle contents. Staining with the cationic carbocyanine dye (stains-all) [15] was performed in the original gel (A). The gel was first washed 4 times with 25% isopropanol. Afterwards it was stained for 24 h in the dark in a solution containing 0.0025% stains-all/25% isopropanol/7.5% formamide/ 30 mM Tris, pH 8.8. The proteins (B) on the nitrocellulose sheet were stained using amido black (0.1% amido black/45% methanol/10% acetic acid) and destained in methanol/acetic acid/H_2O (12:1:28). Incubation with radioactive Ca^{2+} was performed as follows: The nitrocellulose sheet was washed 3 times for 20 min in 20 mM Mops/KOH, pH 7.0. Afterwards it was incubated in 15 ml of the same buffer containing 15 μCi $^{45}Ca^{2+}$ (spec. act. 13.66 mCi/mg) for 10 min under constant shaking. Then it was washed twice for 5 min in 20 ml distilled water and dried between filter papers [22]. The autoradiography is shown in lane C. The molecular mass standards (D) used were phosphorylase *b* (rabbit muscle, 94 kDa), albumin (bovine, 68 kDa), catalase (bovine liver, 58 kDa), and α-chymotrypsinogen A (bovine pancreas, 25 kDa).

known to interact specifically with Ca^{2+}-binding proteins [15], resulted in the appearance of the same 2 bands already identified using ^{45}Ca (cf. lane A and C).

To confirm the selectivity of ^{45}Ca binding to separated proteins on nitrocellulose sheets we used a bovine brain S-100 protein preparation (fig.3, lane A). S-100 is a well known Ca^{2+}-binding protein [16]. Only the major S-100 band exhibits ^{45}Ca

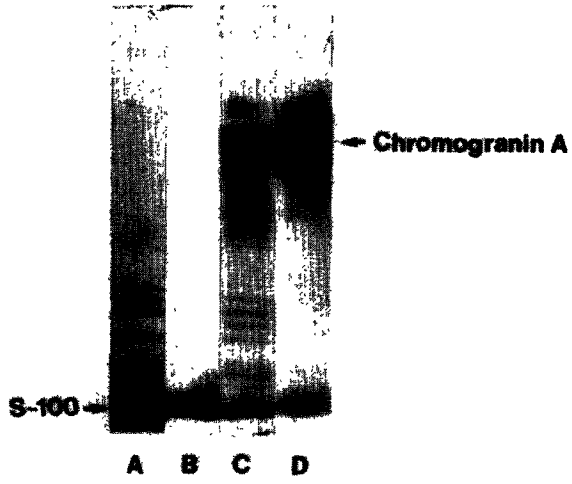

Fig.3. Selectivity of ^{45}Ca binding. Lanes: A, protein stain of the S-100 protein preparation; B, ^{45}Ca binding of the proteins of lane A; C, protein stain of a mixture of chromaffin vesicle content proteins and the S-100 protein preparation; D, ^{45}Ca binding of the proteins of lane C.

binding, as shown in the autoradiography of lane B.

A mixture of the S-100 protein preparation and chromaffin vesicle proteins (see protein stain, lane C) exhibits 2 ^{45}Ca-binding bands; one can be attributed to S-100 and the other to chromogranin A (lane D).

In many histochemical studies it has been shown that secretory vesicles contain Ca^{2+}. This has been confirmed by direct determination of the Ca^{2+} content of isolated secretory vesicles. The binding capacity for Ca^{2+} of the proteins present within chromaffin vesicles reported here is of the same order of magnitude as the amount of Ca^{2+} found in the chromaffin vesicle (cf. [1]).

The essential role of Ca^{2+} during exocytosis has led to many investigations of the Ca^{2+} metabolism of secretory cells. These studies were mostly concerned with the participation of the cell membrane, endoplasmic reticulum and mitochondria in the regulation of the low (between 10^{-6} and 10^{-7} M) free intracellular concentration of Ca^{2+}. More recently, interest has also focussed on Ca^{2+} transport across the chromaffin secretory vesicle membrane, which is endowed with an Na^+/Ca^{2+} exchange system [17,18]. The energy requirement for this transport system (i.e. the number of Na^+

necessary for the uphill transport of Ca^{2+}) would be enormous if the total Ca^{2+} present within the vesicles were in the free state. (In this case the apparent concentration of Ca^{2+} within the vesicles would be about 20–40 mM.) However, in mitochondria and secretory vesicles, most of the Ca^{2+} has been found to be bound [19,20]. The systems involved in this process have not been identified, although a participation of proteins like calsequestrin, the Ca^{2+}-binding protein present in sarcoplasmic reticulum [21], is conceivable. The affinity as well as the capacity of Ca^{2+} binding to the proteins present in chromaffin vesicles would be sufficient to account for the maintenance of low intravesicular Ca^{2+} in these subcellular organelles.

Recent investigations on the distribution of chromogranins in different tissues [4–11] support the idea that the Ca^{2+}-binding function of these proteins is of paramount importance in the regulation of intracellular free Ca^{2+} in many endocrine cells and neurons.

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