Volume 239, number 2, 203–206 FEB 06457 November 1988

Calcium-dependence of chromogranin A-catecholamine interaction

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Received 15 September 1988

Major components of the secretory organelle of bovine adrenal medullary cells, the chromaffin vesicles, are the acidic protein chromogranin A, catecholamines and Ca^{2+}. The binding of Ca^{2+} to chromogranin A has been established. To study the interaction between chromogranin A and catecholamines and its dependence on Ca^{2+} we immobilized chromogranin A to a newly raised monoclonal antibody. It is shown that chromogranin A can bind (i) about 0.5 mol catecholamines per mol in a non-calcium-dependent manner and (ii) about 5 mol per mol in the presence of calcium. These results further support the notion that chromogranin A may act as a secretory granule-condensing protein.

Chromaffin granule; Chromogranin A; Catecholamine; Monoclonal antibody; Ca^{2+}

1. INTRODUCTION

Chromogranins (CHR) are acidic, soluble proteins of the catecholamine (CA) storing vesicles in adrenal medullary cells [1,2] which are secreted together with CA. Despite the fact that their biochemical properties are well characterized [3–5], their biological function(s) is (are) unknown. It has been proposed that they may act as hormone-specific proteases, as hormone precursors, and, because of their Ca^{2+}-binding [6,7] and aggregating properties, as secretory vesicle-condensing proteins [8,9]. The high abundance and co-storage of CHR, CA and Ca^{2+} in chromaffin granules prompted us to analyze the putative interaction between these three groups of molecules. We raised a monoclonal antibody against bovine CHR-A which was used for both CHR-A purification and as a solid support for CHR-A in the binding studies.

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2. MATERIALS AND METHODS

2.1. *Materials*

Cell culture medium (Iscove's medium) was obtained from Seromed (Berlin). Myoclone fetal calf serum was from Gibco (Karlsruhe, FRG); cell culture plastic material from Falcon (Becton and Dickinson, Heidelberg, FRG); and Sepharose 4B from Pharmacia (Freiburg, FRG). All other chemicals were of analytical grade and obtained from Sigma, Hoechst or Merck (Munich, Frankfurt, Darmstadt, FRG).

2.2. *Methods*

Bovine chromaffin cell vesicles were isolated according to *Winkler and Smith [10] with the exception that the last centrifugation step was performed with a 1.7 M instead of 1.6 M sucrose cushion. After freezing and thawing, vesicles were lysed* in 5 mM phosphate, pH 6.5, and centrifuged for 30 min at 100000 × *g*. The supernatant was extensively dialysed against *lysis buffer and subsequently against deionized water and lyophilized or directly loaded onto the anti-CHR-A affinity column*. Determination of protein [11] and CA [12] as well as the preparation of immunoadsorbents [13] were performed as *described. For the production of monoclonal antibodies*, female Balb/C mice were injected with purified bovine chromaffin cells [14] in biweekly intervals. 4 days after an intravenously applied booster injection isolated spleen cells were fused to myeloma cells (ratio 2:1) using PEG 4000 [15]. Culture supernatants were screened by enzyme-linked immunosorbent assays (ELISA) [16], immunofluorescence and immunoblotting [17,18]. After stringent recloning and mass culture one hybridoma line (7A1/H3) was injected into pristane-primed mice for ascites production.

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00145793/88/$3.50

3. RESULTS AND DISCUSSION

3.1. *Characterization of the monoclonal antibody against bovine chromogranin A*

The antibody (MAB 7A1/H3) which belongs to the IgG1 subclass was identified to be specific to CHR-A and its degradation products based on the following lines of evidence: (i) in one- and two-dimensional immunoblots the molecular mass and p*I* of the main band (75 kDa, 5.0, fig.1) are similar to CHR-A (75 kDa, 5.0) but different from CHR-B (100 kDa, 5.2) and secretogranin II (= CHR-C, 86 kDa, 5.0) [3,4]; (ii) in two-dimensional immunoblots the pattern is identical to that obtained with a polyclonal antiserum monospecific for CHR-A (fig.1b); but, (iii) clearly different from anti-CHR-B and anti-secretogranin II patterns (fig.1c,d); (iv) proteins obtained by affinity chromatography with MAB 7A1/H3 are reactive with the polyclonal anti-CHR-A antiserum (fig.2a) and show a main band of 75 kDa (fig.2b); (v) these proteins are also different from prominent enzymes of chromaffin cells (dopamine-β-hydroxylase and phenylethanolamine-*N*-methyltransferase) as revealed by enzyme- and immunoassays (Seidl, K., personal communication); and, (vi) immunohistochemical label on bovine chromaffin cells is restricted to vesicles (fig.3). Since no cross-reactivity with human or rat CHR-A was detectable (fig.4), MAB 7A1/H3 appears to be specific to bovine CHR-A. For quantitative determination a competitive ELISA with a detection limit of about 1 ng was established (fig.5).

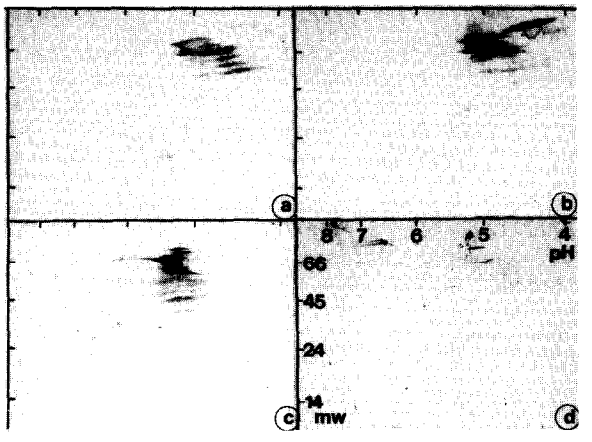

Fig.1. Comparison of 2D immunoblots of soluble bovine chromaffin granule proteins incubated with (a) MAB 7A1/H3, (b) anti CHR-A, (c) anti CHR-B, or (d) secretogranin II (= CHR-C) shows similar patterns for MAB 7A1/H3 and anti CHR-A.

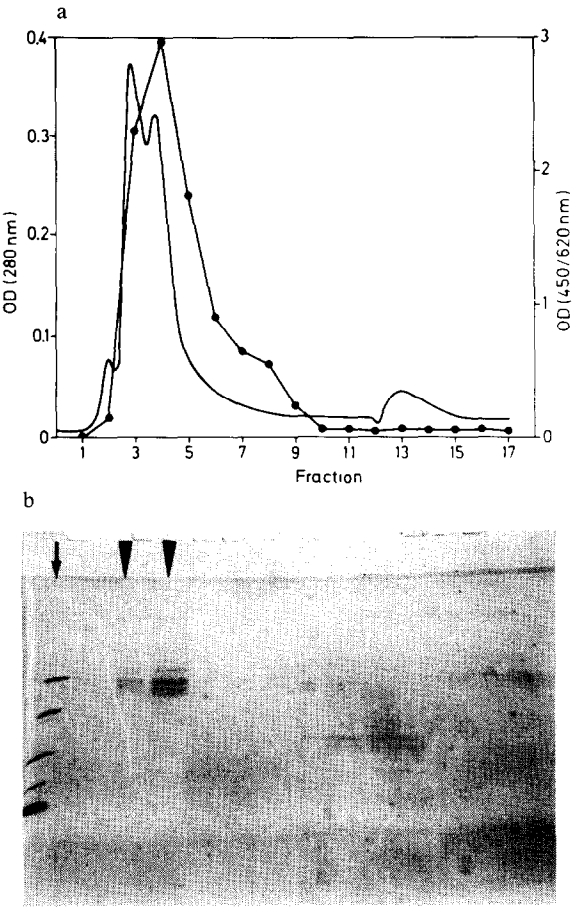

Fig.2. Characterization of proteins obtained by affinity chromatography of soluble chromaffin vesicle content on a MAB 7A1/H3 column. Dialyzed vesicle content was passed over the antibody column. The bound proteins were eluted and further separated by gel filtration on an FPLC-Superose column (a, ——). An ELISA using the polyclonal anti-CHR-A antiserum shows strong immunoreactivity for FPLC fractions 3–5 (a, ●——●). Separation by SDS-electrophoresis of FPLC fractions 2–15 (b, left to right) reveals the presence of 4 proteins of 75, 63, 57, and 53 kDa in fractions 3 and 4 (arrowheads) indicating that most, if not all protein bound to the MAB 7A1/H3 column corresponds to CHR-A and its degradation products (arrow in b, molecular mass marker proteins of 66, 45, 34, 20.1, and 12.4 kDa).

3.2. *Catecholamine binding to immobilized chromogranin A*

The protein CHR-A is one of the most prominent constituents of bovine chromaffin vesicles

Fig.3. Immunohistochemistry of MAB 7A1/H3 on cultured bovine chromaffin cells shows a granular intracellular distribution of the antigen.

[1,2,10]. It was proposed that CHR-A is a secretory granule-condensing protein because of its ability to bind Ca^{2+} [6–9]. Besides CHR-A and Ca^{2+}, CA are the most abundant molecules present in bovine chromaffin vesicles. We therefore first investigated the CA-binding capacity of immobilized CHR-A in the absence of Ca^{2+}. The MAB was coupled to BrCN-activated Sepharose 4B and loaded in succession with (i) saturating amounts of immunoaffinity-purified CHR-A and (ii) a pulse of 600 μg adrenaline. Elution of CA-

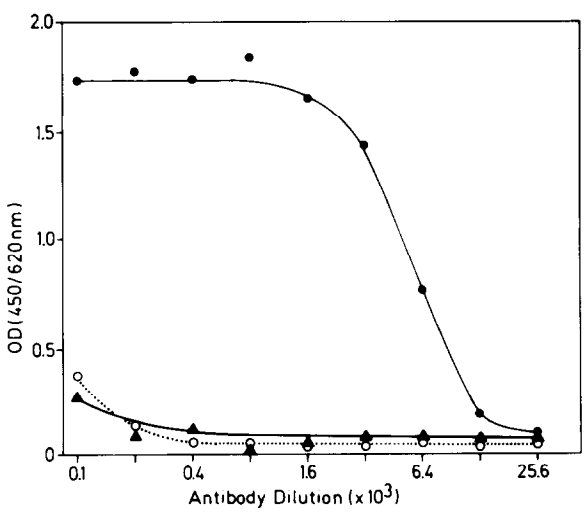

Fig.4. Species specificity of MAB 7A1/H3 for bovine CHR-A is evident from an ELISA with soluble bovine chromaffin vesicle proteins (1 μg/well, ●—●) and extracts from adrenal glands (70 μg/well) of rat (○---○) and man (▲—▲).

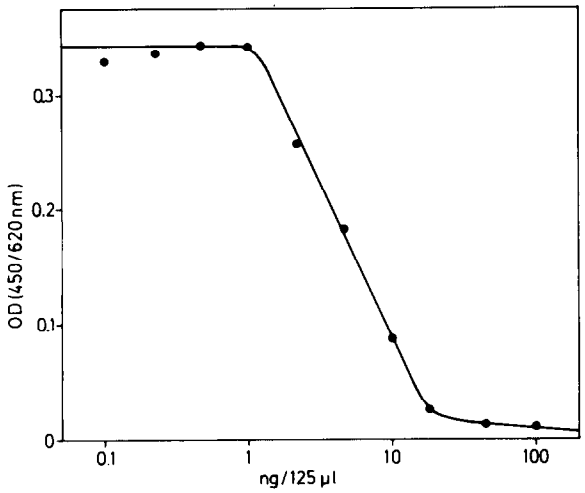

Fig.5. Typical standard curve obtained by the competitive ELISA for CHR-A with MAB 7A1/H3. Antibody was diluted 1:50000 in phosphate buffered saline with the indicated amounts of affinity-purified CHR-A and incubated overnight at 4°C. Following centrifugation the supernatant was transferred to CHR-A-precoated (10 ng/well) microtiter plates, incubated for 20 min at room temperature and further processed as in normal ELISA. Data represent mean values of a triple estimation.

CHR-A complexes with 50 mM glycine-HCl, pH 2.0, showed a distinct peak containing CHR-A as well as CA (fig.6). Quantitation by the CHR-A competitive ELISA and CA-HPLC revealed a molar ratio of 1:0.5 for CHR-A to adrenaline. These results clearly demonstrate the ability of CHR-A to bind low, but significant amounts of CA.

3.3. *Catecholamine binding in the presence of Ca^{2+}*

To analyze the effects of Ca^{2+} on CA binding, the immunoaffinity column was equilibrated overnight with undialyzed vesicle content, i.e. in the presence of physiological relations of CHR-A, CA, and Ca^{2+}. Elution with a Ca^{2+} gradient from 0.05 to 1.0 M resulted in a rapid release of only CA, while during removal of Ca^{2+} by EDTA and elution of CHR-A with glycine-HCl distinct peaks containing CHR-A and CA were observed (fig.7). The total amount of bound CA to CHR-A corresponded to a molar ratio of about 5:1. Results demonstrate that bovine CHR-A not only binds Ca^{2+} but also CA. Attached via a high-affinity

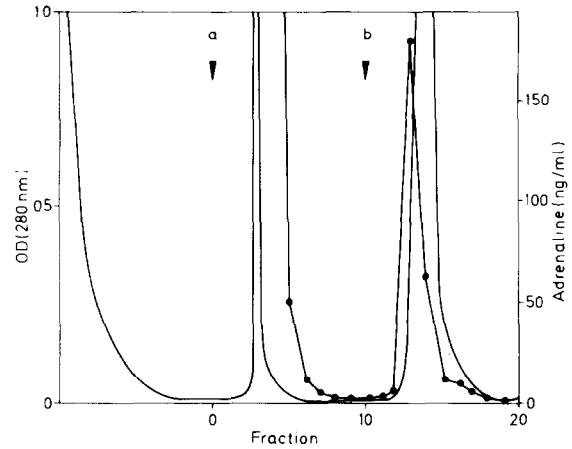

Fig.6. Elution profiles of CHR-A and CA in the absence of Ca^{2+}. Bovine CHR-A was bound to the MAB 7A1/H3 immunoaffinity column and unbound material washed off with 10 mM Tris-HCl, pH 7.0. Then a pulse of 600 μg adrenaline/ml (arrowhead a) was given and unbound adrenaline washed off with Tris buffer. The CHR-A-CA complexes were eluted with 50 mM glycine-HCl, pH 2.0 (arrowhead b), and CHR-A as well as adrenaline quantified by competitive ELISA or HPLC, respectively. A molar ratio of 0.5:1 for CA/CHR-A was observed. (——) Absorbance at 280 nm; (●——●) adrenaline concentration.

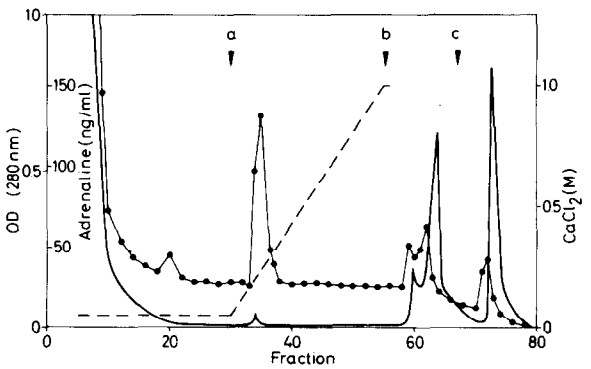

Fig.7. Elution profiles of CHR-A and CA in the presence of Ca^{2+}. The MAB 7A1/H3 immunoaffinity column was equilibrated overnight with total, undialyzed soluble chromaffin vesicle content. Unbound material was removed by washing with 10 mM phosphate buffer, pH 6.6, containing CA (25 ng adrenaline/ml) and Ca^{2+} (50 μM). Immediately after starting a linear Ca^{2+} gradient (arrowhead a) a CA peak was observed, while nearly no CHR-A was detectable. Coelution of CHR-A and CA occurred during removal of Ca^{2+} with 0.1 M EDTA (arrowhead b) and elution with 50 mM glycine-HCl, pH 2.0 (arrowhead c). Quantification of CHR-A as well as CA showed a total ratio of 5:1 for CA/CHR-A. (——) Absorbance at 280 nm; (●——●) adrenaline concentration.

monoclonal antibody to a solid phase, CHR-A can bind only minor quantities of CA in the absence of Ca^{2+} (0.5 mol CA per mol CHR-A, assuming a molecular mass of 75 kDa). In the presence of Ca^{2+} (up to 100 μm, see fig.7) the binding ratio increased by a factor of 10 (5 mol CA per mol CHR-A). These findings support the hypothesis that CHR-A may act as a secretory granule condensing-protein to reduce osmotic pressure during storage of small molecules like CA [8,9].

Acknowledgements: The technical assistance of Mrs C. Giesen-Konrad and Mrs M. Johannsen is gratefully acknowledged. Monospecific antisera against CHR-A,B,C were a generous gift from H. Winkler, Innsbruck. This work was supported by grants from the Deutsche Forschungsgemeinschaft.

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