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Calmodulin-binding proteins in granule and plasma membranes from bovine chromaffin cells

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Calmodulin-binding proteins in chromaffin granule membrane and chromaffin cell plasma membranes have been investigated and compared. Chromaffin granules were purified by centrifugation over a 1.7 M sucrose layer. Plasma membranes were obtained in a highly purified form by differential and isopycnic centrifugation. Enzymatic determinations of 5′-nucleotidase, a generally accepted plasma membrane marker, showed a 40–50-fold enrichment as compared to the cell homogenate. Marker enzyme studies demonstrated only minimal contamination by other subcellular organelles. After solubilization with Triton X-100, calmodulin-binding proteins were isolated from chromaffin granule membranes and plasma membranes by affinity chromatography on a calmodulin / Sepharose 4B column. On two-dimensional polyacrylamide gelelectrophoresis a prominent protein (M_r = 65000, p*I* ranging from 5.1 to 6) consisting of multiple spots, was present in the calmodulin-binding fraction from chromaffin granule membranes as well as from plasma membranes. Besides this 65 kDa protein both fractions had at least four groups of proteins in common. Also, proteins typical for either preparation were observed. In the calmodulin-binding protein preparations from chromaffin granule membranes a prominent spot with M_r = 80000 and a pH ranging from 5.0 to 5.7 was present. This protein was enzymatically and immunologically identified as dopamine-β-monooxygenase.

Introduction

Calcium ions play a central role in the 'excitation-secretion coupling'-process in several cell types, including the adrenal medullary chromaffin cells. Although it is clear that an increase in cytosolic free Ca^{2+} is necessary for catecholamine release, the precise molecular mechanism by which Ca^{2+} is involved in exocytosis is poorly understood. One attractive hypothesis is that the action of Ca^{2+} is mediated by calmodulin, an intracellular Ca^{2+}-binding protein ubiquitous to the eukaryotes. Calmodulin has been shown to regulate a large spectrum of Ca^{2+}-dependent enzymes and Ca^{2+}-dependent cellular processes [1–3].

It has been reported that calmodulin binds to chromaffin granule membranes at micromolar Ca^{2+}-concentrations [4]. Kenigsberg and co-workers [5,6] demonstrated that calmodulin antibodies as well as trifluoperazine, a calmodulin inhibitor, blocked secretion from chromaffin cells in culture. In studies on the interaction between islet cell secretory granules and plasma membranes, it has been shown [7] that calmodulin, in the presence of Ca^{2+}, increased the binding of secretory granules to the cytoplasmic surface of the plasma membranes. These findings suggested that a Ca^{2+}-calmodulin complex may bind to specific secretory granule or plasma membrane proteins and that this may lead to fusion. As expected, the presence of calmodulin-binding proteins could be demonstrated on chromaffin granules [8–10], as well as on chromaffin cell plasma membranes [10]. Two research groups have already investigated the calmodulin-binding proteins on the chromaffin granule membranes [4,8–10] but their results were not identical. Recently, calmodulin-binding proteins in chromaffin cell plasma membranes were described by Fournier and Trifaró [11] but, due to their low yield of plasma membranes, only a few calmodulin-binding proteins could be demonstrated. The aim of the present study was to compare the calmodulin-binding proteins from chro-

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maffin cell membranes with those from chromaffin granule membranes. For this purpose use was made of a membrane purification method which combines a considerable yield with a high purification.

Material and Methods

Preparation of chromaffin granule membranes

Chromaffin granules were prepared as described by Smith and Winkler [12]. Briefly, bovine adrenal glands were obtained from a local slaughterhouse and kept at 0 °C in an isotonic buffer containing 0.3 M sucrose, 10 mM Tris-HCl and 1 mM EGTA, pH 7.4 (buffer A). The medullae were homogenized gently in 3 volumes of buffer A using an Ultra-Turrax homogenizer at half-maximum speed for 20 s followed by two up- and down-strokes in a Potter-Elvehjem homogenizer at 500 rpm. The homogenate was centrifuged at 600 × *g* for 10 min in a MSE 6 × 300 fixed angle rotor. The supernatant was then centrifuged at 20000 × *g* for 20 min in a MSE 8 × 50 fixed angle rotor.

The pellet thus obtained was resuspended in buffer A and layered on 1.7 M sucrose in the same buffer. After centrifugation at 100000 × *g* for 75 min in a Beckman 60 Ti fixed angle rotor, a pellet of highly purified chromaffin granules was obtained. Granules were lysed by resuspension in 10 mM Tris (pH 7.4), freeze-thawed three times and then centrifuged at 100000 × *g* for 30 min in a Beckman 60 Ti rotor to separate granule lysate from granule membranes. This whole procedure was performed at 4 °C.

Preparation of chromaffin cell plasma membranes

Plasma membranes were prepared in EGTA-free buffer A (buffer B) essentially as described by Kidroni and co-workers [13] for the purification of plasma membranes from pancreas.

Medullae were dissected free of cortex, cut into small pieces and 5 volumes of buffer B were added. The tissue was homogenized gently using an Ultra-Turrax homogenizer at half-maximum speed for 90 s followed by ten strokes with a Potter-Elvehjem homogenizer at 500 rpm.

The homogenate was centrifuged in a MSE 6 × 300 rotor at 300 × *g* for 10 min yielding a fraction of nuclei and unbroken cells (N-fraction) and a supernatant SN_1.

SN_1 was centrifuged at 15600 × *g* for 15 min in a MSE 8 × 50 rotor to obtain a mitochondrial pellet (M) and a supernatant SN_2.

SN_2 was centrifuged in a Beckman 60 Ti rotor at 40000 × *g* for 40 min yielding a lysosomal fraction (L) and a supernatant SN_3 which was centrifuged at 100000 × *g* for 2 h in a Beckman 60 Ti rotor to obtain a microsomal pellet (P) and a final supernatant SN_4.

The combined L and P pellets were gently resuspended by rotation for 3 h on a rotatorque in 30 ml of buffer B. This avoided fragmentation of plasma membranes into smaller structures. The suspension was brought to a 1.58 M sucrose concentration by addition of 27 ml of a buffer containing 3.0 M sucrose, 10 mM Tris-HCl (pH 7.4). A final purification of the plasma membranes was achieved by a discontinuous sucrose density gradient centrifugation of the L + P-fractions in a Kontron TST 28/38 swing-out rotor for 15 h at 100000 × *g*. For this purpose, 9.5 ml of the L + P-fraction was applied to the bottom of each centrifuge tube and overlaid with four layers of, respectively, 1.25 M, 1.10 M, 0.91 M and 0.25 M sucrose in buffer B; each layer had a volume of 6.5 ml. After centrifugation, the gradients were collected in six distinct fractions as illustrated on Fig. 1. Highly enriched plasma membranes could be obtained from reaction P_2.

This membrane fraction was washed twice with buffer B by centrifugation at 100000 × *g* for 30 min. This whole procedure was performed at 4 °C.

Biochemical markers

Protein concentrations were measured with Coomassie blue G-250 according to Bradford [14]. DNA was quantified according to the method reported by Burton [15]. Endoplasmic reticulum was detected by NADPH–cytochrome-*c* reductase (NCCR) and monitored at 550 nm as described by Masters and co-workers [16]. Lysosomes were determined by acid phenylphosphatase activity (AcP) according to the method of Kind and King [17] and by β-glucosidase (βGl) activity according to Beck and Poppel [18].

Mitochondria were detected by monoamine oxidase (MAO) activity [19] and succinate dehydrogenase (SDH) activity [20]. Plasma membranes were localized by 5′-nucleotidase (5′N) activity according to the procedure of Morré [21]. Lactate dehydrogenase (LDH) was used as a cytosolic marker and assayed as described by Schwert and Winner [22]. The distribution of chromaffin granules and granular membranes was determined by an enzyme linked immunosorbent assay (ELISA) for dopamine-β-monooxygenase (DβM) [23] or by the enzymatic assay as described by Dillen et al. [24].

Calmodulin affinity chromatography

Chromaffin granule membranes or plasma membranes were solubilized overnight at 4 °C in a buffer containing 50 mM Tris, 250 mM NaCl and 1% (v/v) Triton X-100 (pH 7.5). After centrifugation at 100000 × *g* for 30 min in a Beckman 60 Ti rotor, $CaCl_2$ was added to the supernatant to a final concentration of 2 mM. This was loaded onto a 10 ml Calmodulin-Sepharose 4B (Pharmacia, Sweden) column equilibrated with 20 column volumes of a buffer containing 50 mM Tris, 200 mM NaCl, 1 mM dithiothreitol, 2 mM $CaCl_2$ and 0.1% (v/v) Triton X-100 (pH 7.5).

Subsequently, the column was eluted with the same buffer until electrophoretically no proteins could be

detected in the eluate (at least 200 ml or 20 column volumes). Next, calmodulin-binding proteins were eluted with the same buffer containing 2 mM EGTA instead of 2 mM $CaCl_2$. The protein-containing fractions were pooled and dialyzed against H_2O, dried with a speed-vac concentrator (Savant, New York) and dissolved in lysis buffer for two-dimensional gel electrophoresis [25].

Two-dimensional gel electrophoresis

Total and calmodulin-binding proteins (50 μg) were subjected to isoelectric focusing followed by SDS-polyacrylamide gel electrophoresis as described by O'Farrell [25]. After electrophoresis, proteins were stained as described by Wray and co-workers [26].

Immunoblotting procedure

After two-dimensional gel electrophoresis, proteins were electrophoretically transferred to nitrocellulose membranes with a Multiphor II Novablot System (LKB, Sweden). Immunodetection of dopamine-β-monooxygenase was performed with a Bio-Rad (CA, U.S.A.) Immun-Blot™ Goat Anti-Rabbit Horseradish Peroxidase Conjugate Kit according to the manufacturer's instructions. Polyclonal anti-dopamine-β-monooxygenase was used as antiserum.

Results

Preparation of plasma membranes

The results of the differential centrifugation showed that almost 55% of the initial 5'-nucleotidase activity could be recovered in the combined L- and P-fraction (Table I). The enzyme in these fractions was enriched about 3.3-times and 2-fold, respectively (Table II). Both fractions were pooled for sucrose density gradient centrifugation (Fig. 1). From the P_2 fraction of this gradient, highly enriched plasma membranes were obtained (48 × for 5'-nucleotidase activity) which were only slightly contaminated by other subcellular organelles (Table I). In a typical preparation, starting from 20 to 25 μg of adrenal medullae 10 to 15 mg of purified plasma membranes could be recovered from fraction P_2,

TABLE II

Enrichment of specific markers in enzyme-enriched fractions

Data are calculated from data given in Table I. Enrichments are expressed as the specific activity of a marker enzyme in a certain fraction compared with the specific activity of the same marker in the total homogenate. The specific activity is the activity/mg protein.

Enzyme	Fraction	Enrichment	Organelle
5'-Nucleotidase	L	3.3	plasma
	P	2.0	membranes
	P2	48	
Acidic phenyl phosphatase	P3	4.3	lysosomes
β-Glucosidase	P3	5.4	lysosomes
NADPH–cytochrome-*c*	P0	180	endoplasmic
reductase	P1	125	reticulum
Succinate dehydrogenase	M	2.1	mitochondria
Monoamine oxydase	M	2.5	mitochondria
Dopamine-β-monooxygenase	M	2.5	chromaffin granules
DNA	N	4.9	nuclei

TABLE I

Distribution of protein and markers over subcellular fractions from bovine adrenal medulla obtained by differential and gradient centrifugation as expressed by their quantity or enzymatic activity

The values ($n = 3$) were recalculated to an initial quantity of 20 g of medullae. Relative standard errors are less than 10% for all markers. Rec.: recovery; N + M + L + P + SN_4 as compared to TH and P_0-P_5 as compared to L + P. n.d.: not detectable.

Fraction	Prot. (g)	5'N (nmol P_i/min)	AcP (mmol pNp/min)	βGl (mmol pNp/min)	NCCR (nmol NADP/min)	SDH (A_{490}/min)	MAO (A_{360}/min)	DβM (mg)	LDH (μmol NAD/min)	DNA (μg)
TH	2.17	464	4.34	1.43	86.8	108	118	12.7	21.7	406
N	0.269	61	0.39	0.115	1.5	10.1	1.9	0.68	2.40	247
M	0.481	117	1.03	0.530	39.2	50.1	66.4	7.03	0.69	100
L	0.293	205	0.96	0.520	35.1	26.7	34.9	3.37	0.86	30
P	0.115	48	0.26	0.038	11.8	5.7	3.7	0.24	0.37	10
SN_4	0.882	43	1.70	0.141	4.5	5.6	10.4	1.63	13.69	48
Rec.	94%	102%	100%	94%	106%	91%	100%	102%	83%	107%
P_0	0.00093	n.d.	n.d.	n.d.	6.70	n.d.	n.d.	n.d.	n.d.	n.d.
P_1	0.00299	1.58	n.d.	n.d.	14.9	n.d.	n.d.	0.0041	n.d.	n.d.
P_2	0.0119	122	0.0267	n.d.	6.89	n.d.	n.d.	0.071	n.d.	n.d.
P_3	0.0179	38.11	0.155	0.0639	4.28	6.97	7.03	0.098	n.d.	n.d.
P_4	0.212	68.6	0.625	0.319	8.19	22.5	20.1	1.07	0.635	n.d.
P_5	0.126	4.75	1.82	0.102	3.16	3.92	9.02	2.54	0.865	n.d.
Rec.	91%	93%	81%	87%	94%	104%	94%	105%	122%	n.d.

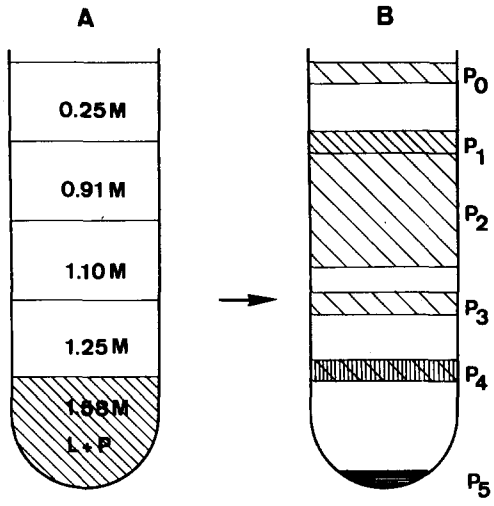


Fig. 1. Sucrose density gradient centrifugation of plasma membrane-enriched fractions from chromaffin cells. After isopycnic centrifugation on a discontinuous density gradient (see Materials and Methods) six distinct fractions can be obtained as indicated on the figure. (A) Gradient before centrifugation. (B) Gradient after centrifugation.

representing about 25% of the initial 5′-nucleotidase activity.

Calmodulin-binding proteins of chromaffin granule membranes and plasma membranes

Approximately 1% of the proteins of both the chromaffin granules and plasma membranes was bound to the calmodulin Sepharose 4B column in the presence of 2 mM Ca^{2+} and could be eluted with 2 mM EGTA.

In Fig. 2 calmodulin-binding proteins from chromaffin granule membranes and plasma membranes are compared after two-dimensional gel electrophoresis. Calmodulin-binding proteins (represented black) were given Roman numerals. This was done in order to facilitate description and comparison of the spots between the gels for both membrane preparations. Calmodulin-binding proteins which are common to both chromaffin granules and plasma membranes were numbered I–V. Due to the high plasma membrane yield and to the very sensitive silver staining at least 16 groups of calmodulin-binding proteins can be observed. In Figs. 2A and C, representing the calmodulin-binding proteins from the chromaffin granule membrane, two protein bands are heavily stained. One band (Fig. 2C; I) consists of a train of multiple spots with a p*I* varying from 4.9 to 6.0 and with an M_r of 65 000. Remarkably, the p*I* of the observed spots of these 65 kDa proteins differed from one membrane preparation to another.

These 65 kDa Ca^{2+} calmodulin-binding proteins are also present in our plasma membrane preparation (Fig. 2D, I).

The second heavily stained band (Fig. 2C; VII) has a p*I* between 5.0 and 5.7 and a M_r of about 80 000. Immunoblotting was carried out and this protein was demonstrated to be dopamine-β-monooxygenase (Fig. 3). Determination of the enzymatic dopamine-β-monooxygenase activity as well as ELISA further showed that, in comparison with the total granule membrane protein, the enzyme was enriched about 2-fold in the calmodulin-binding protein fraction. Since 25% of the protein content of these membranes consists of dopamine-β-monooxygenase while approx. 40% of the calmodulin binding proteins consists of this protein, the presence of dopamine-β-monooxygenase must be specific.

Apart from the five spots or groups of spots in common, most protein spots are restricted to one type of membrane (Figs. 2 C and D; VI–XVI) and, with the exception of dopamine-β-monooxygenase, none of these proteins have so far been characterized.

Discussion

For the identification and isolation of calmodulin-binding proteins from chromaffin cell plasma membranes, a membrane preparation method which yields considerable amounts of pure plasma membranes was chosen.

In the past plasma membranes were prepared according to Meyer and Burger [27] and to Zinder et al. [28]. In both cases considerable contamination by chromaffin granule membranes was found; contamination by endoplasmic reticulum and mitochondria was low. Since chromaffin granule membranes have almost the same density as plasma membranes, they are in contrast to intact granules difficult to remove. For that reason homogenization of the tissue has to be performed very gently in order to damage as little as possible chromaffin granules.

The results of our new plasma membrane purification were compared with those obtained in earlier studies using other methods and showed a considerable improvement using this technique [27–30]. This method combines a satisfactory yield (more than 25% of the 5′-nucleotidase activity), low contamination with other subcellular organelles and a high enrichment (more than 40-times). This approximates the maximal possible purification factor of about 52 times, which can be estimated [31,32].

Taking into account the following data:
diameter of a chromaffin granule : ± 300 nm
diameter of a chromaffin cell : ± 30 000 nm
number of granules/cell : ± 30 000

The ratio between the total area of the chromaffin granule membrane and the area of the plasma membrane for one cell can be calculated as follows:

$$\frac{S_{cg}}{S_{pm}} = \frac{(4\pi)(150)^2 \times 30000}{(4\pi)(15000)^2} = 3$$

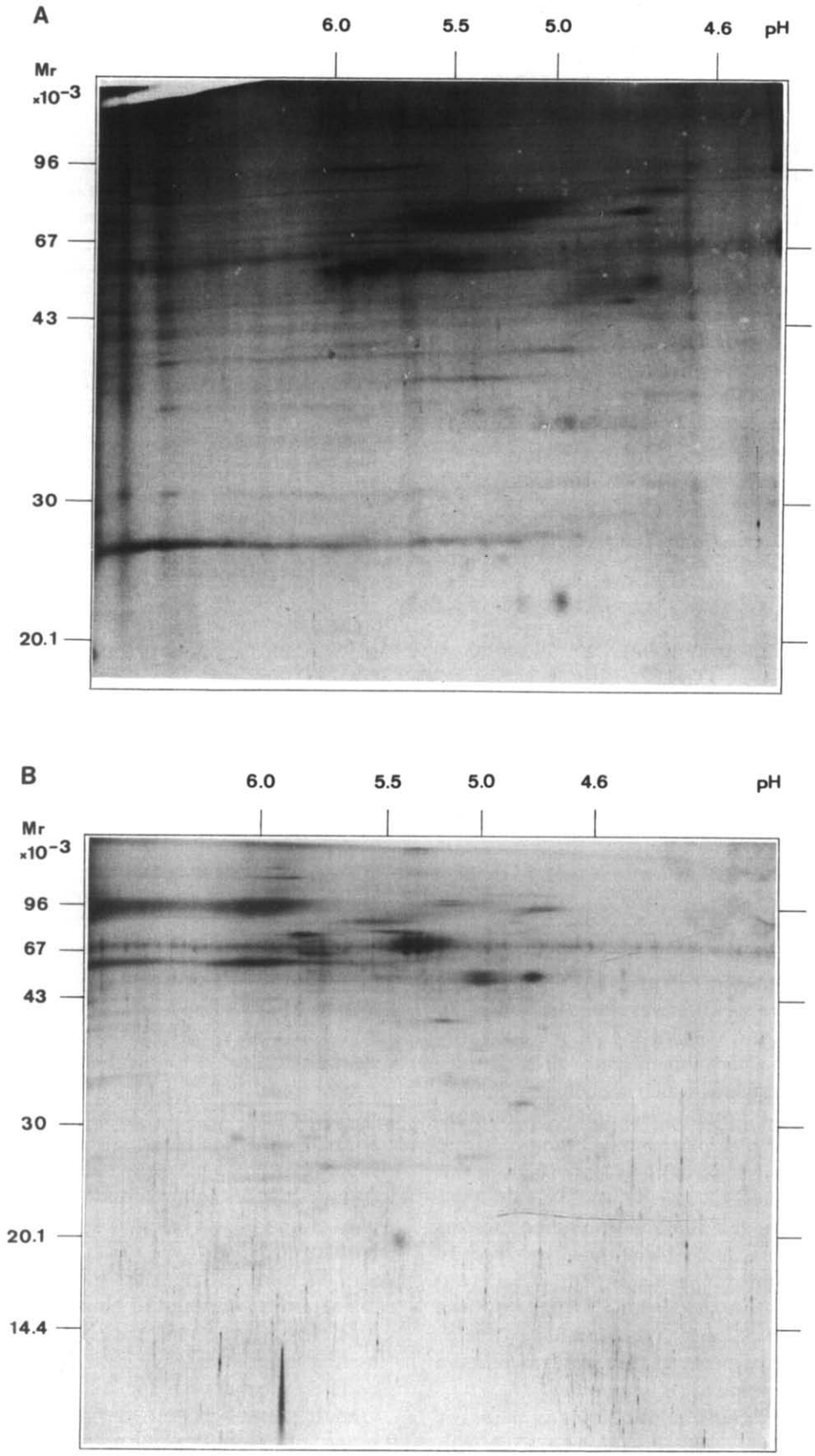
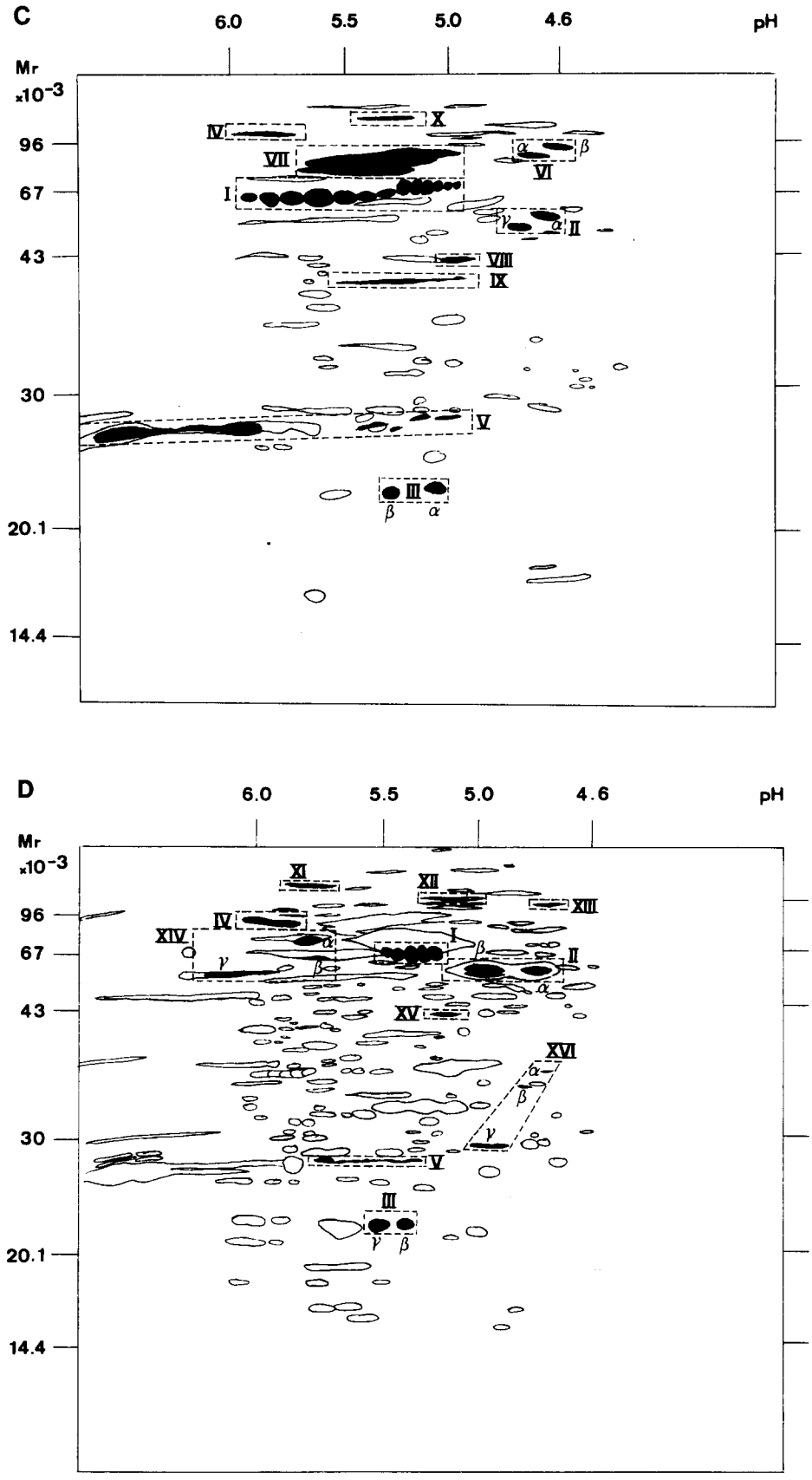


Fig. 2. Two-dimensional sodiumdodecylsulphate polyacrylamide gel electrophoresis of Ca^{2+}-calmodulin-binding proteins from granule and plasma membranes. (A) Ca^{2+}-calmodulin-binding proteins from chromaffin granule membranes (50 μg). (B) Ca^{2+}-calmodulin-binding proteins from chromaffin cell plasma membranes (50 μg). (C) Drawing of the photograph of total proteins from chromaffin granule membranes. Ca^{2+}-calmodu-



lin-binding proteins are marked black and identified with Roman numerals. (D) Drawing of the photograph of total proteins from chromaffin cell plasma membranes. Ca^{2+}-calmodulin-binding proteins are marked black and identified with Roman numerals.

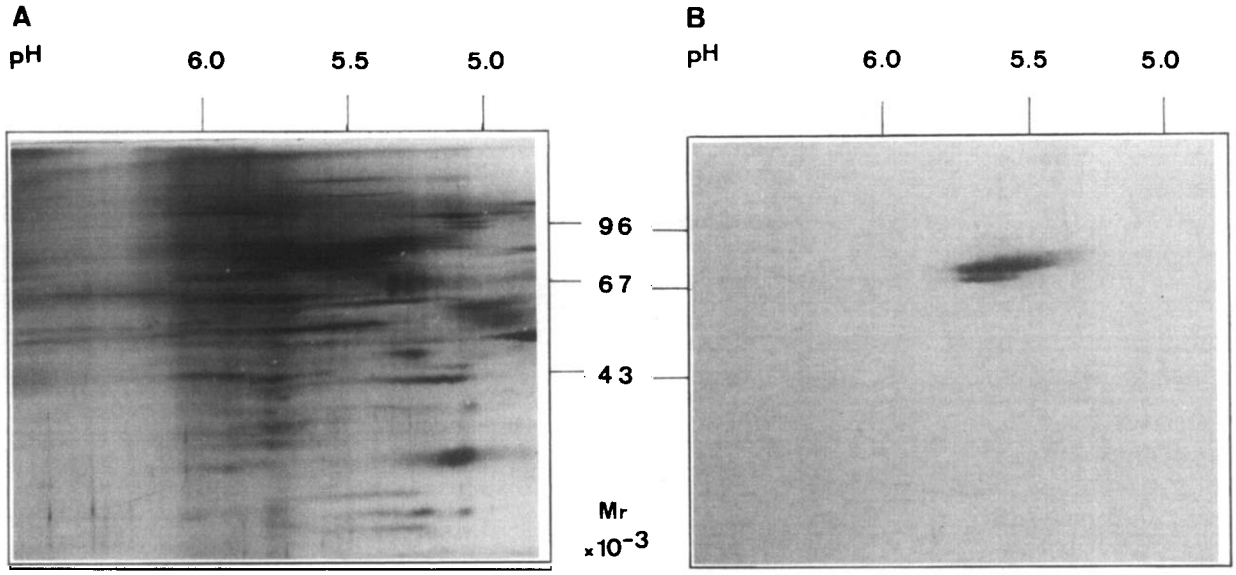


Fig. 3. Immunoblotting with anti-dopamine-β-monooxygenase. (A) Two-dimensional sodiumdodecylsulphate polyacrylamide gel electrophoresis of granule membrane proteins (fragment of the total gel). (B) Immunoblot of calmodulin-binding proteins from chromaffin granule membranes with anti-dopamine-β-monooxygenase as described in Materials and Methods.

Accepting that the ratio of proteins over phospholipids both for chromaffin granule membranes as well as plasma membranes is approximately the same and that 23% of the proteins from chromaffin granules are membrane bound [33], the total protein ratio between chromaffin granules and plasma membranes can be estimated:

$$\frac{P_{cg}}{P_{pm}} = \frac{S_{cg}}{S_{pm}} \cdot \frac{100}{23} = 13$$

Over a 1.7 M sucrose layer, chromaffin granules can be purified for almost 100%. For typical granule markers an enrichment to a factor 4 is obtained. Therefore pure plasma membranes will be enriched to a factor $4 \times 13 = 52$. This means that theoretical and experimental enrichments for plasma membranes are higher than in most other cell types.

High enrichments were found for NADPH–cytochrome-*c* reductase (endoplasmic reticulum) in fractions P_0 and P_1 and moderate enrichments in fraction P_2 (Table II). These results were reproducible ($n = 3$) and the recoveries for the enzyme ranged on each occasion between 90% and 110%, which illustrates the reliability of the enzymatic assays. An experimental enrichment factor of 125 and 180 in fraction P_0 and P_1, respectively, for the endoplasmic reticulum marker enzyme means that there are at least 2.5–4-times more plasma membranes than endoplasmic reticulum. Since 28% of the total plasma membranes and 8% of the total endoplasmic reticulum is present in fraction P_2 a maximal possible contamination by endoplasmic reticulum between 7% and 11.5% can be calculated, which is acceptable.

Essential for the method described in this paper is a well controlled and gentle homogenization procedure. It is very important to disrupt chromaffin granules as little as possible, since their membranes have almost the same density as plasma membranes and could therefore be the major cause of contamination. Equally important is that the resuspension of the membrane containing L- and P-fraction occurs very gently. This would leave the membrane structures almost intact and hence avoids the formation of very small plasma membrane fragments which are difficult to bring to equilibrium density. Fournier and Trifaró [11] isolated plasma membranes on Cytodex 1 microcarriers, a method for which one can expect relative low yields but a high purification. However, these authors obtained a rather moderate enrichment of 10-fold and 9-fold for acetylcholinesterase activity and α-[^{125}I]bungarotoxin binding, respectively, which are two plasma membrane markers. On the other hand, contamination of their plasma membranes by other subcellular organelles, such as lysosomes (β-glucosidase), endoplasmic reticulum (cytochrome-*c* reductase) and granule membranes (dopamine-β-monooxygenase) is in the same range or even lower than for our membrane preparations. We believe that this discrepancy can be explained by the fact that Fournier and Trifaró assayed their marker enzymes on membrane fractions which were still attached to the beads. This may lead to an underestimation of the enrichment of their membrane preparations as well as a possible underestimation of the contamination by other subcellular organelles. Indeed, since their plasma membranes are attached by the extracellular site on the beads this can lead to less efficient enzymatic assays through steric hindrance. It is therefore difficult to

compare the purity of our plasma membrane preparations with that obtained by Fournier and Trifaró.

For the isolation and identification of the calmodulin-binding proteins an affinity chromatography was preferred to ^{125}I-calmodulin blotting because this last technique has some disadvantages. Due to SDS denaturation calmodulin-binding sites can be lost and calmodulin-binding proteins may not be recognized after blotting. On the other hand, artefactual calmodulin-binding sites, which are actually not present in the native form, may become exposed.

The results presented here show two prominent spots of calmodulin-binding proteins from chromaffin granule membranes, one of them being dopamine-β-monooxygenase.

Also Fournier and Trifaró [8] as well as Geisow et al. [10] mentioned the presence of a Ca^{2+}-calmodulin binding protein in the molecular weight region of dopamine-β-monooxygenase. The relevance of this finding, however, is at present unclear and to our knowledge the enzyme has not yet been reported to be a Ca^{2+}-calmodulin binding protein. The enzyme is not expected to participate in the calcium-dependent molecular events after Ca^{2+} entry in the cell since it faces the granular matrix in granules and the extracellular space after exocytosis. It must also be mentioned that the possibility still exists that dopamine-β-monooxygenase itself is not a true Ca^{2+}-calmodulin-binding protein, but a protein that may be bound specifically to a calmodulin-binding protein. Whether this is the case and whether this binding would have an effect on the enzymatic activity, will however be a subject for future research.

A series of prominent spots with an M_r of 65 000 is present both in granule and plasma membranes and corresponds with the 65 kDa calmodulin-binding proteins described by Trifaró and co-workers [8,11]. According to these authors this protein was resolved into two subunits of pI 5.3 and pI 5.2. Using a sensitive silver staining we could detect at least four to five spots. Also, the 53 kDa calmodulin-binding protein described by Bader et al. [9] can be found on plasma membranes and granule membranes (Figs. 2 C and D, II).

With our approach several calmodulin-binding proteins, not yet described in the literature, could be detected. This is mainly because in the present study use has been made of a membrane preparation method which yields relatively large quantities of highly purified membranes combined with a sensitive staining method after the electrophoresis of the calmodulin-binding proteins.

It is possible that some of these calmodulin-binding proteins participate in the molecular events between cell stimulation and exocytosis. In this case they would be expected to be present in several, if not all, secretory systems. In fact this has already been demonstrated for the 65 kDa calmodulin-binding protein [12,23].

Therefore, further research on this subject will focus on the purification of these proteins so that antibodies can be raised against them. Antibodies will then be used for investigating the presence of these calmodulin-binding proteins in other secretory cells and also to study the relation between Ca^{2+}-calmodulin-dependent processes and exocytosis.

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