SOLUBLE ACIDIC LIPOPROTEIN COMPONENTS OF ADRENOMEDULLARY CHROMAFFIN GRANULES. RELATION TO CHROMOGRANINS

Robert MYLROIE and Harold KOENIG

Neurology Service, VA Research Hospital; and Department of Neurology, Northwestern University Medical School, Chicago, Illinois, USA

Received 16 November 1970

1. Introduction

The adrenomedullary catecholamines are stored within special cytoplasmic particles, chromaffin granules (CG), together with ATP, phospholipids and soluble acidic protein [1]. The soluble protein [2] and its main component, chromogranin A [3, 4] are unusually rich in acidic amino acids, notably glutamic acid, and proline, but poor in cystine. CG share a number of structural and cytochemical features with lysosomes, including a selective staining by basic dyes *in vivo* and *supra vitam* [5–8]. Since the vital basophilia of lysosomal particles is due to the presence of an acidic lipoprotein (LP) [9], we have searched for a similar constituent in CG. Helle in a brief abstract [10] reported that protein in crude lysates of CG is associated with a small amount of phospholipid and exhibits a buoyant density of about 1.34 g/ml, and suggested that chromogranin may be a high density LP. We now confirm Helle's findings. We show further that the soluble fraction of CG contains a number of acidic LPs which differ in buoyant densities, lipid composition, and electrophoretic mobilities on polyacrylamide gels. These LPs seem to comprise the bulk of the soluble proteins or "chromogranins" [11].

2. Materials and methods

CG were prepared from fresh bovine adrenal medulla by differential and density gradient centrifugation [12]. The pellet, approximately 120–130 mg protein per experiment, was suspended in 10 ml of 0.2% Triton X-100 with 1 mM EDTA pH 7.2 and sonicated in a Branson Model W185D Sonifier for 10 min (in one min burst alternating with one min rest periods). In two experiments Triton X-100 was omitted from the extraction medium. The insoluble residue was sedimented by ultracentrifugation for 30 min in a no. 50 Spinco Rotor. Lipoproteins were fractionated by ultracentrifugal flotation in KBr solutions as described earlier [9]. The soluble fraction was adjusted to a specific gravity of 1.21 g/ml by adding solid KBr and centrifuged for 16 hr at 37,000 rpm in a no. 50 Spinco Rotor. An opaque yellowish surface layer, approximately 1 ml, designated the low density LP fraction (LDLP) was removed. The specific gravity of the subnatant was raised to 1.35 g/ml by further addition of KBr, recentrifuged for 16 hr at 37,000 rpm, and a second floating layer of one ml, the high density LP fraction (HDLP), separated from the subnatant, the very high density LP fraction (VHDLP). The three LP fractions were dialyzed against 1mM EDTA pH 7.2 to remove KBr. All procedures were carried out at 4°. The several fractions were analyzed for protein [13], lipid phosphorus [14], and cholesterol [15]. The phospholipid composition of lipid extracts [16] was determined by thin layer chromatography as described earlier [9]. The amino acid composition of protein hydrolysates (6 N HCl, 110°, 48 hr) was determined in an automatic amino acid analyzer. Disc gel electrophoresis [17] was carried out in 7.5% acrylamide gels at pH 8.8. Protein bands in gels were stained with Amido schwarz (1% in 7% acetic acid), phospholipid with Sudan black B or acridine orange (0.05% in 0.01 M glycine buffer pH 7.5 with 2% HCHO).

Table 1
Composition of lipoprotein fraction from bovine chromaffin granules*.

	Protein	Phospholipids		Cholesterol		moles Cholesterol
	% of total	% of total	mg/mg protein	% of total	mg/mg protein	moles Phospholipid
Soluble Fraction	67.56 ± 2.77	82.47 ± 4.85	0.42 ± 0.05	79.93	–	–
LDLP (d<1.21)	14.08 ± 0.46	43.80 ± 2.42	1.27 ± 0.20	54.10	0.50	0.81
HDLP (d=1.21–1.35)	29.52 ± 2.80	31.15 ± 0.63	0.45 ± 0.11	33.50	0.16	0.68
VHDLP (d>1.35)	56.40 ± 3.2	25.05 ± 2.48	0.18 ± 0.04	12.4	0.03	0.33
% Recovery	59.20 ± 5.67	55.19 ± 4.25	–	–	–	–
Residue	32.34 ± 2.75	17.55 ± 4.86	0.19 ± 0.01	20.07	0.04	0.35

* Mean ± standard error. Protein and phospholid values are based on three separate experiments. Cholesterol values are the mean of two experiments.

Table 2
Phospholipid composition of lipoprotein fractions from bovine chromaffin granules.

	% of Total Phospholipid		
	LDLP	HDLP	VHDLP
Phosphatidyl serine	8.2	11.0	8.9
Lysolecithin	9.2	8.8	15.1
Sphingomyelin	16.3	13.0	18.3
Lecithin	30.4	30.6	10.7
Phosphatidyl ethanolamine	23.7	21.9	24.2
Cardiolipin	12.2	14.7	22.8

The values for LDLP and HDLP are means of two experiments; the VHDLP is based on one experiment.

3. Results and discussion

On electron microscopic examination the pellets consisted almost exclusively of typical chromaffin granules with rare contaminating mitochondria, lysosomes or other structures. About 68% of the protein and 82% of the phospholipid of the granular fraction was solubilized by sonication in 0.2% Triton X-100 (table 1). The soluble fraction was subdivided by ultracentrifugal flotation into three LP fractions: a low density LP (LDLP, d<1.21 g/ml), 14%, a high density LP (HDLP, d = 1.21–1.35 g/ml), 30%, and a very high density LP (VHDLP, d>1.35 g/ml), 56% of the total soluble protein. The phospholipid and cholesterol content of these LPs varied inversely with their buoyant density, ranging from 1.27 mg phospholipid and 0.5 mg cholesterol per mg protein for the LDLP to 0.18 mg phospholipid and 0.03 mg cholesterol per mg protein for the VHDLP. The molar ratio of cholesterol to phospholipid decreased from 0.81 for the LDLP to 0.33 for the VHDLP. Blaschko et al. [18] reported a molar ratio of cholesterol to phospholipid of 0.7 for whole chromaffin granules. The residue, 32% of the total granule protein, contained considerable phospholipid, 0.19 mg, and cholesterol, 0.04 mg, per mg protein, and may include the insoluble LP which seems to originate from the membranous envelope of the CG (see below).

The phospholipid composition of the three LP fractions was similar (table 2). The relatively high lysolecithin content of the CG (18–20) was reflected in similar high values for this phospholipid in the LP fractions. The acidic phospholipids, phosphatidyl serine and cardiolipin, comprised a relatively large portion, 15–31%, of the total phospholipids. The amino acid composition of the TCA- and lipid-extracted fractions (table 3) differed only in minor details from one another and closely resembled that of the total soluble protein of CG [2] and of chromogranin A [3, 4] in its high glutamic acid and proline and low cystine content.

The soluble extract and the three LP fractions were analyzed by disc electrophoresis (fig. 1). Approximately 9 protein bands were resolved in the soluble extract. The same 9 protein components were observed in the three LP fractions with but minor quantitative variations. However a slowly moving protein, band 8, present in trace amounts in the LDLP and VHDLP, was a major component of the HDLP. All major protein bands and most of the minor components, as nearly as could be ascertained, were stained by Sudan black B, thereby

Table 3
Amino acid composition of lipoprotein fractions from bovine chromaffin granules. Comparison with soluble protein and chromogranin A

	LDLP	HDLP	VHDLP	Sol. protein	Chromogranin A
Lysine	93.7	67.8	69.6	72.6	82.6
Histidine	23.2	24.2	28.3	21.4	18.8
Arginine	50.6	58.8	71.8	68.4	60.9
Aspartic acid	81.2	107.1	92.3	81.2	81.2
Threonine	31.9	34.6	36.6	30.0	27.5
Serine	59.6	58.0	63.4	76.9	79.7
Glutamic acid	185.8	275.3	200.6	222.2	226.0
Proline	65.0	48.4	80.7	89.7	98.7
Glycine	73.0	64.4	80.3	85.5	75.4
Alanine	79.4	67.2	80.9	85.5	79.8
Half cystine	14.2	0	7.1	0	4.4
Valine	51.0	51.2	46.6	38.5	36.2
Methionine	7.0	3.1	1.5	12.8	18.8
Isoleucine	29.9	27.6	24.0	12.8	10.1
Leucine	104.0	79.8	76.2	72.6	72.5
Tyrosine	21.1	10.9	16.5	12.8	11.6
Phenylalanine	29.4	21.6	23.6	17.1	15.9

Lipoprotein fractions were precipitated with 10% trichloroacetic acid lipids extracted with chloroform–methanol (2:1, v/v), and the residual protein hydrolyzed in 6 N HCl at 110° for 72 hr, and analyzed in an automatic amino acid analyzer. Results are given as amino acid residues per 1000. The values for LDLP and HDLP are the mean of two experiments, and for VHDLP, one experiment. Soluble protein values calculated from Helle [2]. Chromogranin A values calculated from Smith and Winkler [3].

demonstrating that the phospholipids in the LP fractions were indeed associated with proteins. The various LP bands also stained metachromatically with the basic fluorochrome acridine orange, indicating the presence of anionic groups.

In two experiments the CG were lysed by suspending in 1 mM EDTA pH 7.2 at 4° without Triton X-100 or sonication, and the insoluble residue removed by ultracentrifugation. Electron microscopic examination showed that the majority of CG were disrupted, membrane profiles with little or no granular contents making up the bulk of the pellet. Chemical analysis revealed that 40–65% of the protein and 30–61.8% of the phospholipid of the CG was present in the soluble fraction, the remainder occurring in the residue. On disc electrophoresis, the same LP components were found in the water lysate as were demonstrated in Triton X-100 extracts. These findings indicate that the soluble protein originates primarily from the internal matrix of CG, the insoluble residue representing mainly membranous envelopes of CG. Moreover, sonication in 0.2% Triton X-100 seems to cause no qualitative changes in the soluble LPs, but increases their yield in the soluble fraction.

It is clear from these experiments that the bulk of the soluble protein from bovine CG consists of acidic LPs. Our results are at variance with a recent report by Winkler et al. [21] in which most of the phospholipid and cholesterol of bovine CG was present in the insoluble residue. The method of lysis used by these workers, repeated freeze-thawing of CG in a tris-sodium succinate buffer, pH 5.9 and I 0.015, may have caused a dissociation of lipid and protein moieties of the soluble LPs. In this regard it is pertinent to note that DL-lysine (10 mM) caused a dissociation of the phospholipid from the protein in lysates of CG [10].

It has been suggested that the acidic proteins or chromogranins may serve to provide binding sites for storage of the catecholamines [1–4]. The lipoprotein nature of the chromogranins may increase their binding and storage capacity for the catecholamines. During perfusion of adrenal glands *in vitro*, granule proteins are released into the perfusate in response to stimulation by secretagogues, together with ATP and catecholamines, without a concomitant efflux of phospholipid or cholesterol [22, 23]. Instead, these lipids are retained in the adrenomedullary cells in

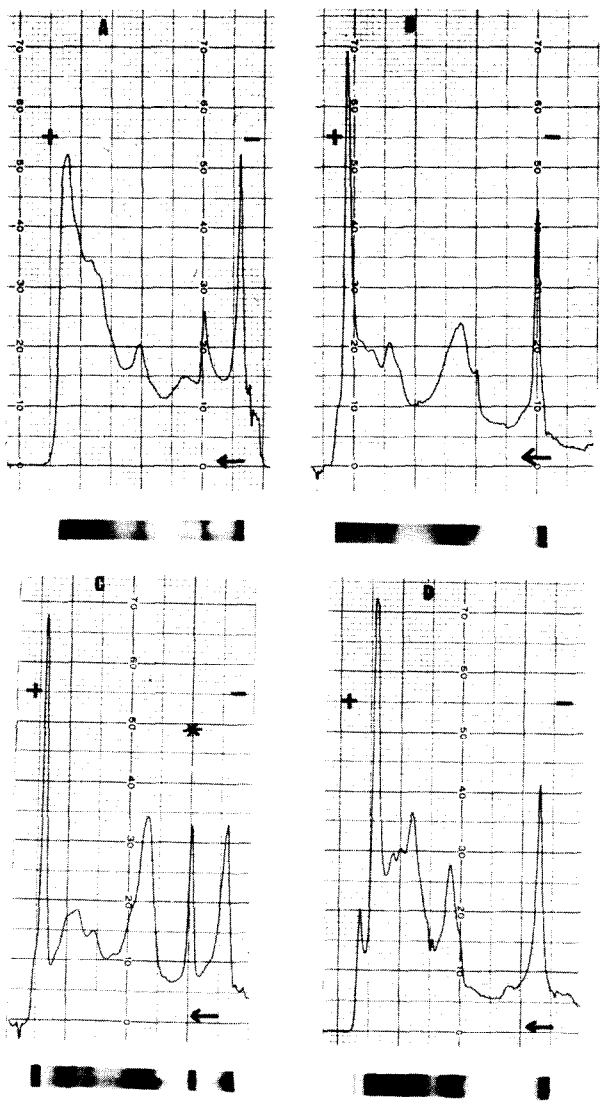

Fig. 1. Densitometer scans and disc gel electrophoretograms of soluble lipoprotein fractions from bovine chromaffin granules. A) Total soluble fraction; B) LDLP; C) HDLP; D) VHDLP. Asterisk in C) indicates band 8.

granules with a reduced specific gravity [24]. These findings suggest that the LPs may undergo delipidation during the sectory process. It is interesting to note in this context that a number of lysosomal hydrolases are secreted from the adrenal gland coincident with the secretion of catecholamines, ATP, and chromogranins [25].

Acknowledgements

This investigation was supported by the Veterans Administration, and by grants from NIH (NSO5509, NSO1456 and NSO6838), from the Natl. Mult. Scler. Soc. (MS–372 and MS–512), and AEC (Contract No. AT(11–1)–1180). We thank Mr. E. Washington for technical assistance, and Mr. C. Hughes for electron microscopic examination of pellets.

References

[1] N.-Å. Hillarp, Acta Physiol. Scand. 47 (1959) 271.
[2] K.B. Helle, Mol. Pharmacol. 2 (1966) 298.
[3] A.D. Smith and H. Winkler, Biochem. J. 103 (1967) 483.
[4] W.J. Smith and N. Kirschner, Mol. Pharmacol. 3 (1967) 52.
[5] H. Koenig, J. Histochem. Cytochem. 11 (1963) 120.
[6] H. Koenig, in: Lysosomes in Biology and Pathology, Vol. 2, eds. J.T. Dingle and H. Fell (North-Holland, Amsterdam, 1969) p. 111.
[7] H. Koenig, R. Mylroie, D. Gaines and T. McDonald, Neurology 15 (1965) 289.
[8] H. Koenig, J. Histochem. Cytochem. 3 (1965) 706.
[9] A. Goldstone, E. Szabo and H. Koenig, Life Sci. 9 (1970) 607.
[10] K.B. Helle, Biochem. J. 109 (1968) 43p.
[11] H. Blaschko, R.S. Comline, F.H. Schneider, M. Silver and A.D. Smith, Nature 215 (1967) 58.
[12] A.D. Smith and H. Winkler, Biochem. J. 103 (1967) 480.
[13] O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, J. Biol. Chem. 193 (1951) 265.
[14] G.R. Barlett, J. Biol. Chem. 234 (1959) 466.
[15] A. Zlatkis, B. Zak and A.J. Boyle, J. Lab. Clin. Med. 41 (1953) 486.
[16] J. Folch, M. Lees and G.H. Sloane-Stanley, J. Biol. Chem. 226 (1957) 497.
[17] L. Ornstein and B.J. Davis, Disc Electrophoresis, Parts I and II, Distillers Products Industries Preprint, Rochester, New York (1961).
[18] H. Blaschko, H. Firemark, A.D. Smith and H. Winkler, Biochem. J. 98 (1966) 24p.
[19] H. Blaschko, H. Firemark, A.D. Smith and H. Winkler, Biochem. J. 104 (1967) 545.
[20] W.W. Douglas, A.M. Poisner and J.M. Trifaró, Life Sci. 5 (1966) 809.
[21] H. Winkler, H. Hortnägl, H. Hortnägl and A.D. Smith, Biochem. J. 118 (1970) 303.
[22] F.H. Schneider, A.D. Smith and H. Winkler, Brit. J. Pharmacol. 31 (1967) 94.
[23] J.M. Trifaró, A.M. Poisner and W.W. Singer, Biochem. Pharmacol. 16 (1967) 2095.
[24] A.M. Poisner, J.M. Trifaró and W.W. Douglas, Biochem. Pharmacol. 16 (1967) 2101.
[25] F.H. Schneider, Biochem. Pharmacol. 19 (1970) 833.