Evidence for the Presence of a Chromogranin-like Protein in Bovine Splenic Nerve Granules

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SUMMARY

Evidence is presented that a protein having immunological properties identical with those of a chromogranin purified from chromaffin granules (obtained from bovine adrenal medullae) is present in granules isolated from bovine splenic nerve. The possibility that chromogranin plays a role in noradrenaline storage in splenic nerve granules as well as in chromaffin granules is discussed.

In the adrenal medulla adrenaline and noradrenaline are stored in chromaffin granules together with ATP (1) and soluble proteins (2), now termed chromogranins (3). In sympathetic nerves noradrenaline is stored in similar, but smaller, granules (4). The nerve granules also contain ATP and have a catecholamine to ATP molar ratio which corresponds closely to that of chromaffin granules (5). In view of the similarity between these two types of storage granules, an attempt has been made to detect a chromogranin-like protein in splenic nerve granules by using an antiserum prepared against the major chromogranin fraction from bovine adrenal chromaffin granules.

The reference sample of chromogranin used in the present experiments was the main peak of protein (peak II) obtained by molecular sieving of a crude chromaffin granule lysate on Sephadex G-200 (6). A small portion of protein appeared in the void volume, together with phospholipids (peak I). The amino acid compositions of both fractions are given in Table 1, from which it may be seen that the amino acid compositions of these two protein components are identical and are very similar to those reported for other highly purified preparations of the major lysate protein.

The antichromogranin serum used in the present work was prepared against the total protein peak (peaks I + II) without separation of the phospholipid-rich component (9). The specificity of the antiserum was demonstrated by the fact that the peak of immunologically active protein obtained by fractionation of the granule lysate on Sephadex G-200 closely paralleled that of the major protein peak (peak II). The phospholipid-rich component (peak I) was also found to react with the antiserum after concentration, and a pattern of complete identity was obtained between peaks I and II; this finding is in agreement with their similarity in amino acid composition. In adsorption studies, 0.290 mg of protein could be collected as a precipitate after 0.150 mg of chromogranin had been allowed to react with an optimal amount of antiserum. This demonstrates that the antiserum can quantitatively remove purified chromogranin from solution.

Dopamine β-oxidase activity, found to account for less than 1% of the lysate pro-

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TABLE 1

Amino acid composition of chromogranin antigen

Amino acid analyses of chromogranin peaks I and II were carried out on hydrolysates obtained after 24, 48, and 72 hr of hydrolysis. The values presented were obtained by extrapolation to zero time.

Amino acid	Chromogranin			Chromogranin A[b]
	Peak I	Peak II	S_1[a]	
	g amino acid/100 g protein			
Aspartic acid	7.8	8.0	10.2	8.35
Threonine	2.6	2.6	2.5	2.45
Serine	5.5	5.9	6.1	6.20
Glutamic acid	27.2	26.4	27.3	26.01
Proline	10.3	9.7	8.0	8.56
Glycine	3.9	4.1	3.5	3.86
Alanine	5.0	5.2	5.3	5.03
Valine	3.6	3.3	3.5	3.25
Half-cystine	0.5	0.5	0.6	0.347
Methionine	1.9	1.6	2.3	2.24
Isoleucine	1.7	1.4	1.4	1.08
Leucine	7.11	6.5	6.5	7.31
Tyrosine	2.4	1.6	1.2	1.67
Phenylalanine	2.2	2.1	1.8	2.08
NH_3	1.4	1.2		1.44
Lysine	8.9	10.1	10.2	9.43
Histidine	2.0	2.0	2.0	2.35
Arginine	7.2	7.9	7.0	8.49

[a] The major protein fraction S_1 described by Sage, Smith, and Kirshner (7).

[b] The major protein fraction, chromogranin A, described by Smith and Winkler (8).

tein (7), could not be detected in our preparations of chromogranin.

The second, slowly emerging precipitin line previously reported to be obtained with concentrated preparations of the main protein peak without removal of phospholipids (2) reflects the presence of the high molecular weight, phospholipid-rich form of chromogranin (i.e., peak I protein). This second, slowly emerging line cannot be construed as evidence for the presence of an antibody to dopamine β-oxidase in the antiserum.

Noradrenaline was measured by the method of Häggendal (10), and ATP was determined by a modification of the firefly luminescence method (11). Protein was estimated by the method of Lowry *et al.* (12).

A preparation of nerve granules was obtained by squeezing lengths of bovine splenic nerve with a scalpel on a glazed tile and collecting the press juice from 1 g of tissue in 4 ml of ice-cold NaCl (170 mM) containing 0.02% sodium azide. The debris was removed by centrifugation at $600 \times g$ for 10 min, and the resultant supernatant was recentrifuged at $20,000 \times g$ for 20 min to yield a sediment which contained approximately 6% of the protein present in the $600 \times g$ supernatant fraction. This sediment was used as a source of nerve granules for the experiments. Electron microscopic examination of the $20,000 \times g$ pellet, after fixation with OsO_4 and staining the ultrathin sections with lead citrate, showed that it contained numerous vesicles, approximately 80–90 mμ in diameter, and several damaged mitochondria. Some of the vesicles contained electron-dense cores typical of noradrenaline storage granules found in electron micrographs of adrenergic axons. However, the majority of the vesicles did not have electron-dense cores. The noradrenaline content of the pellet was 5 mμmoles/mg of protein, and its noradrenaline to ATP molar ratio was 5.2.

Samples of granule preparations were resuspended in 155 mM NaCl containing 0.5% sodium deoxycholate and left in the cold overnight in order to lyse the granules. The lysate was tested against rabbit antiserum to bovine chromogranin by the Ouchterlony double-diffusion technique (13) on agarose plates. Samples of purified bovine chromogranin were used as controls (6). In all these experiments the lysate could clearly be seen to react with the chromogranin antiserum to form a precipitin line identical with that given by the control. Only a trace of chromogranin-like material could be detected in the $20,000 \times g$ supernatant fraction in one out of three experiments. The double-diffusion technique on agarose plates permits the detection of chromogranin at a concentration of 0.03–0.05 mg/ml. By dilution analysis, the concentration of chromogranin-like material in the nerve granule lysate was estimated to be 6% of the total lysate protein, or 0.4% of the total protein present in the

600 × *g* supernatant fraction of the press juice.

Absorption studies, in which a mixture of nerve granule lysate and antiserum against purified chromogranin was placed in one well of a double-diffusion plate and allowed to diffuse against purified chromogranin placed in an adjacent well, showed that the chromogranin antibody was completely removed by the presence of the nerve granule lysate. The concentration of chromogranin-like material in this preparation was 0.04 mg/ml. The purified chromogranin used as the control in these experiments also completely removed the chromogranin antibody from the anti-serum used. Moreover, immunoelectrophoresis of the nerve granule lysate showed it to contain a component which has a mobility identical with that of pure chromogranin A.

These studies show that granules from bovine splenic nerve contain material that has immunological properties identical with those of a purified fraction of the chromogranins present in the chromaffin granules of bovine adrenal medulla. Our observation is in agreement with the finding that a material reacting with antiserum to chromaffin granules is present in splenic nerve (14, 15).

The detection of the chromogranin-like protein in the granules of sympathetic nerve fibers tempts us to assume an involvement of this protein in the storage of noradrenaline by these particles in a manner similar to that suggested for the adrenal medullary granules. A concentration of chromogranin-like material of approximately 6% of the total lysate protein of the splenic nerve granules would thus be responsible for the accommodation of about 80 mμmoles of noradrenaline per milligram of chromogranin-like protein, a value at least 50-fold less than that found in the chromaffin granules of the medulla (16). The nerve granules are known to lose their content of noradrenaline at 37° without loss of ATP (5). Thus some of the agranular vesicles present in the 20,000 × *g* sediment may be chromogranin-containing vesicles depleted of noradrenaline during the isolation procedure, or granules at a stage of development prior to being filled with noradrenaline and ATP. In view of the molar ratio of noradrenaline to ATP of 5.2 found for the 20,000 × *g* sediment, the latter seems to be the more likely possibility. On the other hand, the amount of noradrenaline bound per milligram of chromogranin may normally be less in sympathetic nerves than in the adrenal medulla.

Furthermore, since chromogranin is known to be secreted from the adrenal medulla together with the low molecular weight contents of the chromaffin granules (3, 17, 18), the possibility now arises that the release of transmitter at sympathetic nerve endings may also be attended by the simultaneous release of protein.

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