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# Chromogranin A processing in sympathetic neurons and release of chromogranin A fragments from sheep spleen*

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Chromogranin A (CGA) has been localized to the large dense cored vesicles (LDV) of sympathetic neurons SDS-PAGE and immunoblotting of soluble LDV proteins from ox and dog adrenergic neuronal cell bodies, axons and nerve terminals, revealed an increasing number of CGA-immunoreactive forms, consistent with proteolytic processing during axonal transport. Splenic nerve electrical stimulation (10 Hz, 2 min) revealed that, apart from CGA these CGA-processing products are released from the sheep spleen. The secretion of CGA-derived fragments from sympathetic neurons might suggest a role in the regulation of synaptic transmission.

Sympathetic neuron, Chromogranin A; Vesicle, Processing; Release

## 1. INTRODUCTION

Chromogranin A (CGA), the major soluble protein of adrenal medullary chromaffin granules, and present in various other neuroendocrine secretory granules [1–3], is extensively processed by specific endogenous proteases, yielding smaller immunologically cross-reacting fragments [1,2,4–8]. CGA has also been localized to the large dense cored vesicles (LDVs) of adrenergic neurons [9–14] which are embryonically related to the adrenal medulla [15]. Yet, although it is clear that different CGA storage granules may exhibit different cleavage patterns [4,5], very few data are available on CGA breakdown in sympathetic nerve axons [2,14] and almost none in nerve terminals, presumably because of difficulties in obtaining the appropriate vesicle preparations [16]. Moreover, while the release of CGA from sympathetic neurons has already been demonstrated [17,19], no evidence has yet been provided for the release of CGA products from adrenergic nerve terminals. Since the functional significance of CGA-derived peptides is a growing field of interest we have studied CGA breakdown in sympathetic neurons by comparing CGA immunoreactivity (CGA-IR) in preparations of LDVs from ox and dog sympathetic ganglia, splenic nerve and spleen, representing adrenergic cell bodies, axons and nerve terminals, respectively. In addition, the release of CGA breakdown products from sheep spleen, following splenic nerve electrical stimulation, was investigated.

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## 2. MATERIALS AND METHODS

Chromaffin granules were prepared from bovine, dog and sheep adrenal medulla as described previously and subjected to lysis [18]. Partly or highly purified LDVs from bovine stellate ganglia, dog celiac ganglia, splenic nerve (ox and dog) and spleen (ox, dog and sheep) were prepared essentially as reported earlier [10,20–22]. After lysis of LDV-containing fractions in the presence of inhibitors of the major classes of proteases (Bio-Rad) and boiling for 5 min, soluble proteins were subjected to SDS-gel electrophoresis (SDS-PAGE) and immunoblotting.

The isolated sheep spleen was perfused essentially as previously described [23]. Electrical stimulation with bipolar platinum electrodes was performed at 10 Hz for 2 min at supramaximal voltage (200 mA, 10 V, 3 ms) in the presence of phentolamine ($10^{-5}$ M) to prevent spleen capsule contraction and the release of residual blood. Perfusates containing protease inhibitors were immediately centrifuged ($3{,}000 \times g_{max}$/15 min) to sediment the blood cells. The resulting supernatant was boiled, dialyzed, lyophilyzed and subjected to SDS-PAGE (13% gels) and Western blotting, according to established procedures [24,25]. The antiserum employed (anti-WE 1–14; 1:400–1:1,000), raised in rabbits, is directed against a CGA peptide ($CGA_{316-329}$) common to various species [8,26]. Immunodetection was performed using biotinylated alkaline phosphatase or an Immun-Lite Chemiluminescent Assay (Bio-Rad). The degree of proteolytic processing of CGA in bovine tissues was estimated by laser densitometric scanning with an XL-Ultrascan (Pharmacia-LKB) in comparison with control fractions.

## 3. RESULTS

In partly purified LDV from bovine stellate ganglia and dog celiac ganglia, the native protein (70–75 kDa) and a predominant high molecular weight IR band (94 kDa), but almost no smaller CGA fragments, could be


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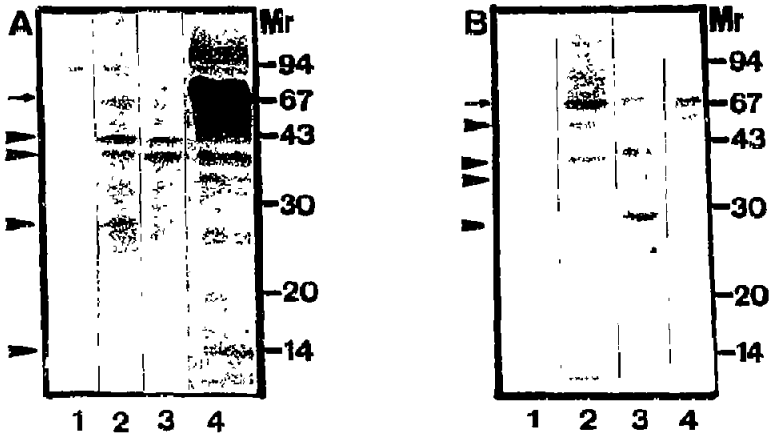


Fig. 1. Chromogranin A (CGA) immunoreactive patterns, obtained by SDS-PAGE and immunoblotting of the lysate of partly and highly purified LDV preparations and chromaffin granules from ox (A) and dog (B). Preparations of LDV from bovine stellate ganglion (A1) and dog celiac ganglion (B1), representing adrenergic cell bodies, were obtained using $D_2O$–sucrose gradients. Highly purified LDV from bovine splenic nerve (A2) as prepared with $D_2O$–sucrose gradients, and an LDV-enriched fraction (from an isopycnic sucrose gradient) of dog splenic nerve (B2) represent adrenergic axons. Semi-purified LDV preparations from bovine spleen (A3) and dog spleen (B3), containing adrenergic nerve terminals, were obtained by $D_2O$–sucrose gradient centrifugation and isopycnic gradient centrifugation, respectively. CGA immunoreactivity was also studied in bovine (A4) and dog (B4) chromaffin granule lysate. Approximately 35 μg proteins was loaded on the gel. Arrowheads indicate the most prominent fragments in splenic nerve and spleen, the arrows indicate CGA.

observed (Fig. 1, A1 and B1). The high molecular weight band accounts for the majority of IR as estimated by semi-quantitative evaluation (>75%). In highly purified axonal LDV from bovine splenic nerve, the antiserum detected, in addition to the native CGA, lower molecular size immunoreactive forms (43, 40, 34, and 28 kDa; Fig. 1, A2) consistent with 45–50% processing. In an LDV fraction from dog splenic nerve, a similar pattern was observed with, in addition to CGA, a major band at about 49 kDa and minor bands at 60, 34 and 28 kDa (Fig. 1, B2). In an LDV-enriched fraction of bovine and dog spleen, significant degradation of CGA was detected. In bovine spleen (Fig. 1, A3) degradation products similar to those in bovine splenic nerve were found (40 and 43 kDa, 34 kDa and 28 kDa), corresponding to 55–60% processing. For dog spleen (Fig. 1, B3) the degradation pattern resembled that of splenic nerve, although the ratio of IR fragments differed (60 kDa, major band at 43 kDa, 34 and 29 kDa). When the two species were compared, a difference in processing products was observed, probably due to interspecies variations. The IR pattern of LDV from both ox and dog spleen closely resembled those of the respective chromaffin granule lysates (Fig. 1, A4 and B4).

Apart from the major CGA band (70–75 kDa), CGA fragments of 43 and 30 kDa were observed in a microsomal fraction of sheep spleen (Fig. 2, lane 1). Interestingly, a similar degradation pattern was observed in an LDV-containing fraction of sheep spleen (Fig. 2, lane 3). Analysis of the perfusate of isolated perfused sheep spleen after stimulation in the presence of phentolamine ($10^{-5}$ M) at 10 Hz (2 min) showed the same CGA-IR fragments (e.g. 43 and 30 kDa) as in the semi-purified LDV of the spleen (Fig. 2, lane 2).

## 4. DISCUSSION

Subcellular fractionation of sympathetic ganglia, splenic nerve and spleen revealed the presence of CGA-IR in the LDV and not in SDV, as reflected by its co-distribution with different LDV markers [9–13]. To investigate CGA processing in sympathetic neurons, CGA-IR was studied in preparations of LDV from adrenergic cell bodies, axons and nerve terminals, in the presence of the major peptidase inhibitors. In a semi-purified fraction of perikarial LDV, CGA fragments were hardly detected. However, the high molecular weight band (94 kDa) was probably the CGA-IR pro-

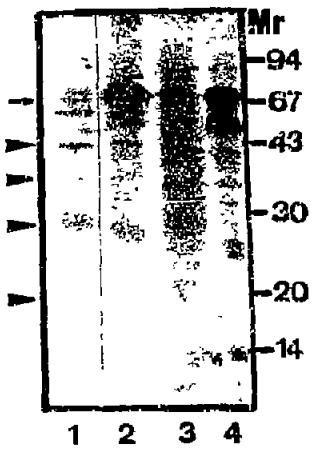


Fig. 2. CGA immunoreactive forms in (1) a microsomal fraction of sheep spleen, (2) a sheep spleen perfusate following 10 Hz stimulation (2 min, $10^{-5}$ M phentolamine), (3) an LDV-enriched fraction as obtained by $D_2O$–sucrose gradient centrifugation, and (4) a sheep chromaffin granule lysate. 35 μg proteins were loaded in each lane. Arrowheads indicate major fragments; the arrow indicates CGA.

teoglycan by analogy with the chromaffin granule lysate [4,8]. In highly purified axonal LDV, part of the native CGA appears to be degraded, while in LDV from sympathetic nerve terminals a very similar breakdown-pattern could be observed. Although it is clear that CGA is highly processed, the observed degradation pattern of CGA obtained appears to be dependent on the nature of the antiserum used. The intensity of immunostained CGA-derived bands apparently differed in chromaffin granules and splenic nerve LDV. In mature chromaffin granules, CGA appeared to be processed by endogenous proteases to a higher degree (60–70%) than in axonal (45–50%) and nerve terminal vesicles (55–60%).

It has already been shown that CGA of purified splenic nerve vesicles is processed to a more limited degree than in chromaffin granules, probably reflecting incomplete LDV-maturation [2,14].

In the presence of peptidase inhibitors, proteolytic degradation has been shown to be almost absent and the apparent processing therefore does not occur during lysis or isolation procedures [4,27,28]; thus these fragments are most likely formed during axonal transport. Moreover, using the same region-specific antibodies, the pattern of proteolysis was shown to be essentially reproducible, excluding the possibility of artifactual cleavage [8,27]. Very little is known about the nature and timing of specific proteases that are involved in the CGA processing, which seems to be a complex pH-regulated process [5,28]. Since the processing patterns of LDVs and chromaffin granules are comparable, similar proteolytic enzymes may be present in both types of organelles.

Splenic nerve stimulation of isolated perfused sheep spleen revealed a significant and $Ca^{2+}$-dependent augmentation of NA- and CGA release (to be published). In the present study, the electrical stimulation evoked release of CGA from the isolated sheep spleen revealed the presence of CGA products in the perfusate, which apparently matched those in LDV. This indicates that CGA fragments, which are formed during intracellular transport from the cell body to the nerve terminal, are released by exocytosis upon sympathetic neuronal activation. Since CGA-derived peptides have been shown to modulate CA release from chromaffin cells [29], they might also be involved in the regulation of sympathetic neurotransmission.

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