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Membrane retrieval in the guinea pig neurohypophysis: biochemical characterization of a retrieval structure

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[^{3}H]Choline and [^{35}S]methionine injected into the guinea pig hypothalamus in vivo were incorporated into the lipids and proteins, respectively, of secretory vesicles transported to the neural lobe. Prolonged in vivo stimulation of hormone secretion by dehydration decreased the [^{3}H]choline content of secretory vesicles, with a concomitant increase in the [^{3}H]choline content of a membrane fraction isolated on sucrose gradients. After stimulation of neural lobes in vitro in the presence of horseradish peroxidase, this extracellular fluid marker was found in the same membrane fraction. SDS electrophoresis of membrane proteins radiolabelled by [^{35}S]methionine in vivo demonstrated that this fraction contained at least one major protein also present in the secretory vesicle membrane. These results suggest that we have isolated a membrane fraction containing the structure(s) involve in membrane retrieval in the neurohypophysis.

Introduction

Recent morphological evidence suggests that following exocytosis of secretory vesicle contents, membrane retrieval in the neurohypophysis proceeds via vacuoles [1,2], rather than by coated microvesicles [3,4]. However, a specific retrieval structure has not yet been isolated or characterized biochemically, perhaps because of the lack of suitable internal membrane markers.

[^{3}H]Choline injected into the hypothalamus in vivo is incorporated into the membranes of secretory vesicles [5] which are then rapidly transported to the neural lobe. We have recently used [^{3}H]choline incorporation as a marker for secretory vesicle membranes to provide biochemical evidence that coated microvesicles are not involved in membrane retrieval in the neurohypophysis [6,7]. However, we did find a subcellular fraction which incorporated [^{3}H]choline at the same time as secretory vesicles, and which increased its [^{3}H]choline content with dehydration, concomitantly with a decrease in the radiolabelling of secretory vesicle membranes. We have now further characterized this fraction, and in this paper present evidence that it may be involved in membrane retrieval in the guinea pig neurohypophysis.

Materials and Methods

Radiolabel incorporation in vivo. Under halothane anaesthesia, guinea pigs (Hartley, 500–700 g) were given bilateral injections (two times 20 μCi) of [^{3}H]choline (70 Ci/mmol) or [^{35}S]methionine (800 Ci/mmol, Amersham International) into the paraventricular nuclei. Some

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Abbreviations: Mes, 4-morpholineethanesulphonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid.

animals were dehydrated by replacing drinking water with 2% NaCl for 4 days before and 1 day after injection.

Subcellular fractionation. Neural lobes were removed 24 h after injection, homogenized at 0°C in 300 mM sucrose containing 1 mM Mes, 1 mM EDTA, 0.02% sodium azide (pH 6.5) and centrifuged at $2900 \times g_{av}$ for 10 min. The supernatant (400 μl) was placed on top of 250 μl 1.2 M buffered sucrose and centrifuged at $100\,000 \times g_{av}$ for 30 min to remove a pellet containing secretory vesicles, mitochondria and nerve endings. The resulting supernatant was placed on a buffered sucrose gradient (0.9–1.2 M) and centrifuged at $100\,000 \times g_{av}$ for 40 min. In other experiments, secretory vesicles were isolated on Percoll gradients as described in Ref. 6. Secretory vesicles were lysed by incubation in 100 mosM sucrose for 30 min at 0°C and centrifuged at $100\,000 \times g_{av}$ for 60 min.

In vitro incubations. Neural lobes were partially bisected, washed and incubated in oxygenated medium [8] containing 5 mg/ml horseradish peroxidase (HRP, Sigma, Grade IV, 5 mg/ml) and 56 mM K^+, for 30 min at 37°C.

Assays. Fractions from sucrose gradients were assayed for $(Na^+ + K^+)$-ATPase [9] and acid phosphatase [10]. Horseradish peroxidase activity was assayed as in Ref. 11. The ^{3}H-labelled components in secretory vesicle membranes were identified by thin-layer chromatography (TLC) as described in Ref. 12. Components were identified by comparison with the mobility of known standards, and ^{3}H radioactivity estimated by scintillation counting.

SDS electrophoresis and autoradiography were carried out according to Laemmli [13]. All membrane fractions were diluted 1 to 50 in 100 mM KCl/25 mM EGTA/10 mM Mes (pH 6.5) and centrifuged at $100\,000 \times g_{av}$ for 60 min before electrophoresis. The pellets were resuspended directly in the sample buffer [13]. The relative incorporation of [^{35}S]methionine into proteins was estimated by scanning densitometry of the autoradiograms at 500 nm.

Results

Twenty-four hours after hypothalamic injection of [^{3}H]choline secretory vesicles were the major ^{3}H-labelled structure in the neurohypophysis. Analysis of the radiolabelled secretory vesicle membranes by TLC showed that most of the ^{3}H was found in phosphatidylcholine, with smaller amounts in sphingomyelin and cholesterol (Fig. 1).

After removal of the secretory vesicles and nerve endings, at least two additional ^{3}H-labelled fractions could be distinguished on sucrose gradients (Fig. 2, upper panel). The lighter fraction sedimented in a similar position to membranes from lysed secretory vesicles (Fig. 2, middle panel), whereas the other fraction sedimented in the middle of the gradient (1.03 ± 0.02 M sucrose, mean $\pm$ S.D., $n = 3$) and was the only component whose ^{3}H-labelling was significantly increased by dehydration (control $3.6\% \pm 1.1$; dehydrated, $5.7\% \pm 1.5$, $n = 9$, $p < 0.02$).

Fig. 2 also shows the distribtion of horseradish peroxidase in membrane fractions after in vitro stimulation of neural lobes, followed by sucrose density gradient centrifugation (Fig. 2, lower panel). The fraction showing increased [^{3}H]choline labelling after dehydration (dotted line) also contained horeseradish peroxidase activity after stimulation in vitro. Note, however, that horeseradish peroxidase activity was also present in several

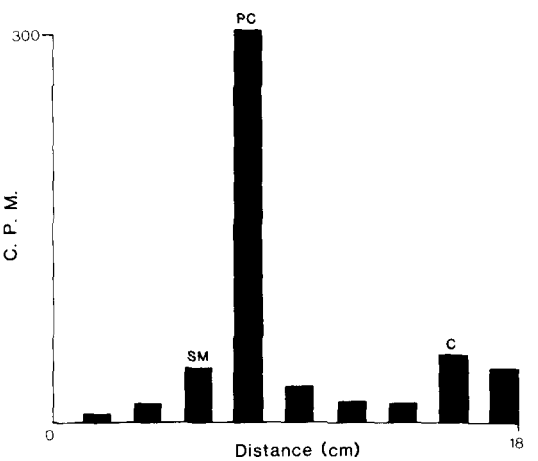

Fig. 1. Radiolabelled secretory vesicle membranes were extracted in chloroform/methanol/water (14:6:1, v/v) and analysed by TLC. Most of the radiolabel was incorporated in phosphatidylcholine (PC), with lesser amounts found in the positions of sphingomyelin (SM) and cholesterol (C). The extract contained more than 85% of the total radioactivity in the secretory vesicle preparation. Distance is given in cm from the origin.

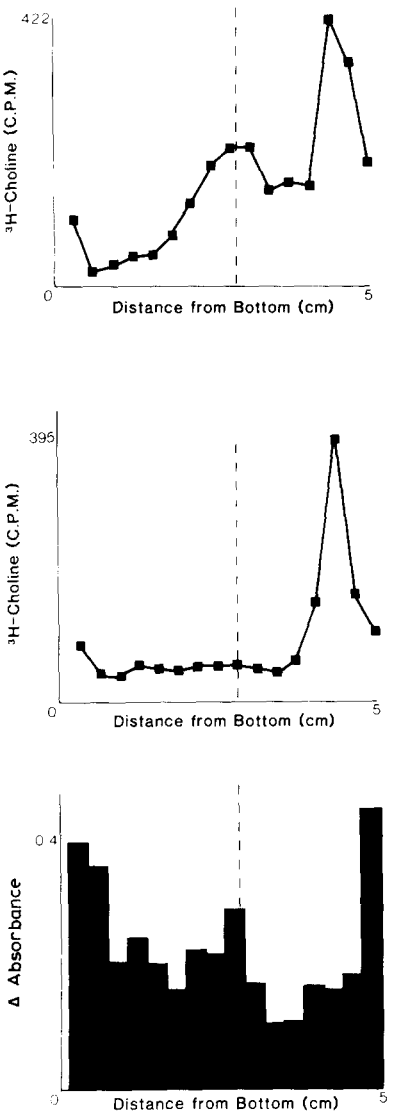

Fig. 2. A membrane fraction obtained after removal of secretory vesicles and nerve endings was centrifuged on a sucrose gradient (upper panel). Prior to removal of the neural lobes the animals were dehydrated and injected with [^{3}H]choline. The dotted line indicates the only fraction which significantly increased its [^{3}H]choline content during dehydration (see text). The middle panel shows the sedimentation position of [^{3}H]choline-labelled secretory vesicle membranes after lysis. The lower panel shows the distribution of horseradish peroxidase activity after in vitro incubation of intact neural lobes with horseradish peroxidase.

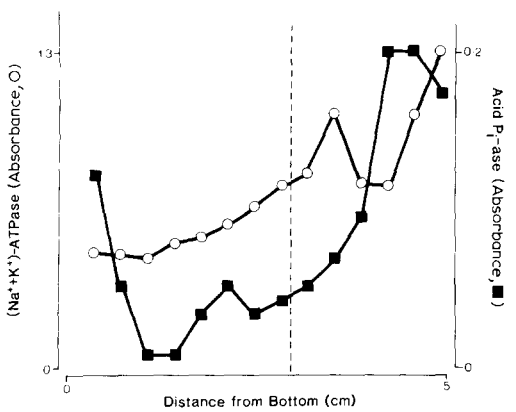

Fig. 3. Fractions prepared by gradient centrifugation as in Fig. 2 were analysed for their content of acid phosphatase and $(Na^+ + K^+)$-ATPase activities. The dotted line indicates the position of the fraction shown in Fig. 2 to incorporate both [^{3}H]choline and horseradish peroxidase. 0.1 absorbance unit corresponds to 1 nmole of phosphate, and 1.3 nmoles of ADP liberated over 30 min by acid phosphatase and $(Na^+ + K^+)$-ATPase, respectively. Each analysis was performed on material pooled from three neural lobes.

other fractions not labelled with [^{3}H]choline. These sucrose gradients separated the [^{3}H]choline-labelled fraction from the bulk of lysosomal and plasma membrane components, as shown by the distributions of $(Na^+ + K^+)$-ATPase and acid phosphatase on identical sucrose gradients (Fig. 3).

Twenty-four hours after hypothalamic injection, [^{35}S]methionine was incorporated into a number of membrane proteins which could be analysed by SDS electrophoresis and autoradiography of the gels (Fig. 4). The fraction containing the putative retrieval structure (Fig. 4, upper trace) and the secretory vesicle membrane fraction (Fig. 4, middle trace) show a similar profile of radiolabelled proteins, though their relative proportions vary. In particular, a major labelled protein (molecular weight approx. 100 000) was found in both fractions (arrow), but not in membrane material from the upper part of the sucrose gradient (Fig. 4, lower trace).

Discussion

Since [^{3}H]choline and [^{35}S]methioninė injected into the hypothalamus in vivo are incorporated into the constituents of neural lobe secretory vesicles, they offer useful internal markers with which to study the fate of secretory vesicle mem-

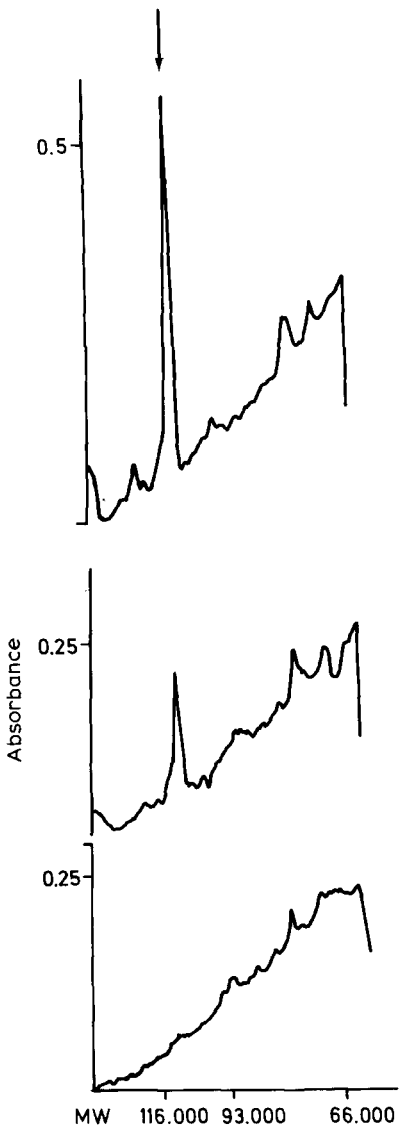

Fig. 4. Neural lobes from animals injected with [^{35}S]methionine were fractionated as described and fractions from the sucrose gradients were subjected to SDS electrophoresis. After autoradiography of the gels the films were scanned at 500 nm (absorbance). The upper trace represents the putative retrieval structure (the four fractions surrounding the dotted line in Fig. 2). The middle trace shows the labelled proteins in a preparation of secretory vesicles run in parallel on the same gel. The lower trace corresponds to the upper 4 fractions from the sucrose gradient shown in Fig. 2. The arrow shows the major ^{35}S-labelled membrane protein which is common to both secretory vesicle membranes and the fraction containing the proposed retrieval structure. MW shows the relative mobility of molecular weight markers on these gels.

branes after exocytosis. In a previous study [6] we demonstrated that prolonged dehydration, a physiological stimulus for release of neurohypophysial hormones, decreased the neural lobe content of [^{3}H]choline-labelled secretory vesicles, presumably as a result of increased exocytosis. Although we were unable to show any changes in [^{3}H]choline incorporation into coated microvesicles, which had been proposed as membrane retrieval structures in this tissue [3,4], we obtained preliminary evidence for a membrane fraction whose [^{3}H]choline content did increase during dehydration [6]. We have now further characterized this fraction and attempted to provide biochemical evidence for its role in membrane recapture in the neurohypophysis.

After removal of the denser organelles (secretory vesicles, mitochondria, nerve endings) two major [^{3}H]choline-labelled peaks could be separated on shallow sucrose gradients. One of these peaks showed a significant increase in ^{3}H content following dehydration, as would be expected of a structure involved in direct recapture of radiolabelled secretory vesicle membranes. It is unlikely that this fraction was derived from plasma membrane or lysosomes, which might also have increse their [^{3}H]choline content during stimulation, since its distribution was consistently different from that of lysosomal and plasma membrane markers. Furthermore, it is unlikely that this ^{3}H-labelled fraction derived from ruptured secretory vesicle membranes produced by the homogenization or centrifugation procedures since it was clearly separated from lysed secretory vesicle membranes on sucrose gradients.Structures involved in membrane retrieval after exocytosis should internalise markers of extracellular space, and horseradish peroxidase has often been used as such a marker to trace membrane recapture [2,14,15]. After high K^+ stimulation of neural lobes in vitro, horseradish peroxidase was detected in the [^{3}H]choline-labelled fraction, and also in several other structures. This is to be expected, since the neural lobe is not solely comprised of nerve terminals, but contains numerous blood vessels and pituicytes which may also be affected by stimulation [16]. The use of [^{3}H]choline as an internal marker for the secretory vesicle membranes enabled us to identify which horseradish peroxidase-associated structures derived from

nerve terminals and were involved in recapture of the [^{3}H]choline-labelled secretory vesicle membranes.

The major [^{35}S]methionine-labelled protein in secretory vesicle membranes also represented the major labelled protein in the putative retrieval structure. Since this protein was not found in other membrane fractions, its presence in the retrieval structure cannot simply be due to nonspecific absorption of a cytoplasmic protein, nor to it being a ubiquitous membrane protein. It is also unlikely that the presence of this major protein is a result of contamination of one fraction by the other since the relative proportions of radiolabelled components are different in each fraction.

It is not possible to identify unambiguously a membrane retrieval structure solely using subcellular fractionation methods. Nevertheless, the fraction we have isolated contains a membrane structure which incorporates [^{3}H]choline and is the only component that increases its radiolabelling when exocytosis is stimulated, concomitantly with the fall in secretory vesicle membrane labelling [6]. Furthermore, this structure internalises an extracellular fluid marker upon stimulation of exocytosis, is not derived from plasma membrane or lysosomes, and contains the major membrane protein found in secretory vesicles. Taken together, these results strongly suggest that this fraction does contain a structure involved in direct membrane recapture after exocytosis in the neural lobe. It is tempting to speculate that these structures may correspond to the vacuoles suggested by Morris and Nordmann [2] to be involved in membrane recapture in this tissue. Further studies combining electron microscopy and autoradiography are necessary to establish the precise identity of the membrane retrieval structures in the fraction characterized in our study.

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