Calmodulin binding and protein phosphorylation in adrenal medulla coated vesicles

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Coated vesicles from bovine adrenal medulla contained clathrin and major detergent-insoluble polypeptides of 120–100, 51 and 49 kDa. Intact coated vesicles and vesicles lacking clathrin light chains were bound by immobilized calmodulin in the presence of Ca^{2+}. Clathrin in the form of 700 Å cages was not bound. The calmodulin binding components in intact coated vesicles are therefore contributed by the enclosed vesicle or by the 120–100, 50 or 49 kDa polypeptides. The 51 kDa component incorporated $^{32}P_i$ from labelled ATP by an endogenous kinase activity; no other coat or vesicle membrane protein was phosphorylated in vitro, either by intrinsic or exogenous kinases.

Ca^{2+}calmodulin *Chromaffin cell* *Phosphorylation* *Adrenal medulla* *Clathrin* *Coated vesicle*

1. INTRODUCTION

While coated vesicles are relatively stable after isolation, they are believed to be transient structures in the cell [1]. It is not understood how the necessary polymerisation and dispersal of the coat protein is regulated, but there are indications that calmodulin-requiring and ATP-dependent steps are involved. Thus calmodulin co-caps with lymphocyte cell surface receptors during endocytosis via coated pits [2] and calmodulin antagonists inhibit receptor uptake [3,4]. Isolated brain coated vesicles bind calmodulin with high affinity in the presence of Ca^{2+} [5].

It has been suggested that ATP is a co-factor in a process which removes coat from coated vesicles [6]. Brain coated vesicles also exhibit protein kinase activity [7,8], although the identities of the enzymes and their physiological substrates remain to be determined. We report here on the protein composition of homogeneous adrenal medulla coated vesicles and describe the sites of interaction with calmodulin and protein kinase.

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2. MATERIALS AND METHODS

2.1. Materials

Bovine adrenal glands were supplied by British Beef Co., Watford. Bovine brain clathrin triskelions were the kind gift of Dr E. Ungewickell. Calmodulin–Sepharose was produced by coupling 40 mg bovine calmodulin to 10 ml Affigel 15 (Bio-rad) in 0.1 M Hepes–HCl (pH 7.5). Fluphenazine–Sepharose was prepared as in [9].

^{125}I-labelled Bolton and Hunter reagent and [^{32}P]ATP were from Amersham. TLCK-treated α-chymotrypsin and the catalytic subunit of protein kinase (beef heart) were from Sigma (London).

2.2. Isolation of medullary coated vesicles

Bovine adrenal glands were collected on ice and perfused briefly with Ca^{2+}- and Mg^{2+}-free saline. After dissection of the medullae, small pieces of the tissue were washed well with isolation buffer A (0.1 M 2-(*N*-morpholino)ethanesulphonic acid (Mes), 1 mM EGTA, 1 mM $MgSO_4$, 1 mM NaN_3 and 1 mM phenylmethanesulphonyl fluoride (PMSF) at pH 6.5 (with NaOH). The tissue was homogenised in an equal volume of buffer A (Waring Blendor, 3 × 5 s) and filtered through

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muslin. Centrifugation at $1000 \times g_{av}$ for 10 min and at $30000 \times g_{av}$ for 20 min gave a supernatant from which microsomes were pelleted by centrifugation at $80000 \times g_{av}$ for 60 min. The pellet was resuspended in 50 ml buffer A, incubated for 30 min at 20°C with pancreatic ribonuclease A (10 units/ml) and recentrifuged at $100000 \times g_{av}$ for 60 min. The pellet was resuspended in 7.0 ml isolation buffer and clarified at $9000 \times g_{av}$ for 10 min in an SW55 Ti rotor. The supernatant was layered onto a step gradient containing the salts of buffer A and 8% (w/v) sucrose in D_2O adjusted to a pD of 6.5 [10] and centrifuged ($100000 \times g_{av}$, 2 h) in an SW40 Ti rotor at 10°C. The pellet was resuspended in buffer A, clarified at $10000 \times g_{av}$ for 10 min and applied to a 4 ml continuous gradient formed from 9% D_2O, 10% (w/v) Ficoll 400 in buffer A and 98% D_2O, 20% Ficoll and the salts of buffer A [11]. This was centrifuged at $100000 \times g_{av}$ for 16 h at 10°C. The coated vesicles were purified to morphological homogeneity (negative stain) by passage over Sepahacryl S-1000 (50 × 1.5 cm) in buffer A. Coated vesicles eluted in a sharp peak following smooth membrane contaminants in the void volume.

2.3. Isolation of adrenal triskelions and light chains

The purification of clathrin was based on the method in [12]. Coated vesicles (1 mg/ml) were suspended with a Dounce-type homogeniser in a 1:1 mixture of 1 M Tris–HCl (pH 7.0) and buffer A. After 15 min at 20°C, vesicle membranes were sedimented at $100000 \times g_{av}$ for 60 min at 4°C. The supernatant was chromatographed in Tris–buffer A on a 1.5 × 100 cm column of Sepharose 4B. Triskelions eluted after the vesicular material in the void volume; the subsequent peak contained 100 and 50 kDa proteins. Clathrin light chains were prepared by heating the triskelion fraction to 95–100°C for 5 min followed by centrifugation at $20000 \times g_{av}$ for 20 min. After dialysis against PBS light chains were labelled by reaction with 100 μCi ^{125}I-labelled Bolton and Hunter reagent using the procedure recommended by the manufacturers. Binding of ^{125}I-labelled light chains to light chain-depleted brain clathrin was carried out using the centrifugation assay developed in [13,14].

2.4. Phosphorylation of coated vesicle protein

This was performed in 50 μl of 40 mM NaF, 5 mM $MgCl_2$ and 100 mM Na–Mes (pH 6.5). Each incubation was started by the addition of Na_2 ATP to a final concentration of 10 μM containing 10 μCi [^{32}P]ATP. After 5 min at 37°C, the reaction was terminated by heating at 95–100°C for 5 min. Labelled proteins were separated on SDS–PAGE and autoradiographed.

2.5. Electron microscopy

Coated vesicle fractions were examined after attachment to carbon-coated electron-microscope grids. Grids were washed with distilled H_2O and negatively stained with 1% (w/v) uranyl acetate. Affigel conjugates were washed 3 times by sedimentation at 1 × g in the incubation buffer, fixed and processed for electron microscopy as in [15].

3. RESULTS

3.1. Coat proteins from adrenal medulla

Three distinct size populations were present in coated vecicles from this tissue (fig.1). Vesicles of

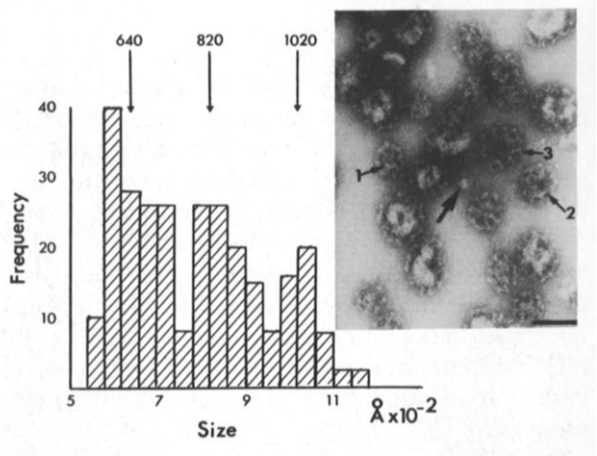

Fig.1. Frequency distribution of coated vesicle diameters. Three negatively stained fields of vesicles, from two different preparations were analysed for vesicle diameters, measured across the extremities of the cage. The measurements were recorded and analysed by an Apple II microcomputer equipped with a standard graphics tablet. Diameters were the average of two orthogonal directions to compensate partially for the distortion introduced during binding to the carbon-coated grid. The means of the size distributions (arrows) were 640 ± 46, 820 ± 91 and 1020 ± 100 Å. Inset: coated vesicles from the first sucrose/D_2O gradient. Structures with 3 distinct average diameters are present (numbered arrows). Bar, 0.1 μm.

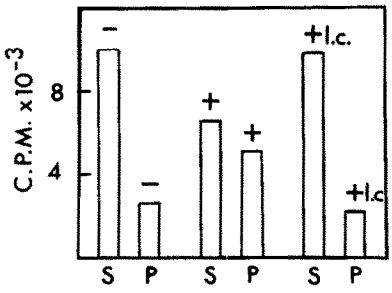

Fig.2. Formation of hybrid clathrin–adrenal light chain cages. ^{125}I-labelled adrenal light chains were incubated either alone (−), with light-chain free brain clathrin cages (+) or in the presence of excess brain light chain (+l.c.) using the centrifugation assay described in [14]. S and P, counts in the supernatant and in the pelleted cages, respectively.

similar diameters had previously been observed in electron microscopic observations of cultured chromaffin cells (unpublished). Coat proteins solubilized by 0.5 M Tris re-assembled into 700 Å baskets after dialysis (20–50 μg protein/ml) against isolation buffer. SDS–PAGE of 9 preparations gave a consistent pattern of major polypeptides with molecular masses of 180, 120, 110, 100, 51, 49, 33 and 31 kDa (fig.4, lane I). All resisted detergent extraction, sedimenting repeatedly from 1% (w/v) Triton X-100.

After separation of coat proteins by Tris extraction and Sepharose 4B chromatography, the 33 and 31 kDa polypeptides partitioned with clathrin. Both polypeptides remained soluble at 98°C, while clathrin was denatured and aggregated. After centrifugation to remove denatured clathrin, the 33 and 31 kDa polypeptides were radioiodinated by the Bolton and Hunter method. Both peptides bound to light chain-free brain clathrin as determined by the centrifugation assay of [13,14]. The specific nature of this heterologous binding was confirmed by the effective competition of authen-

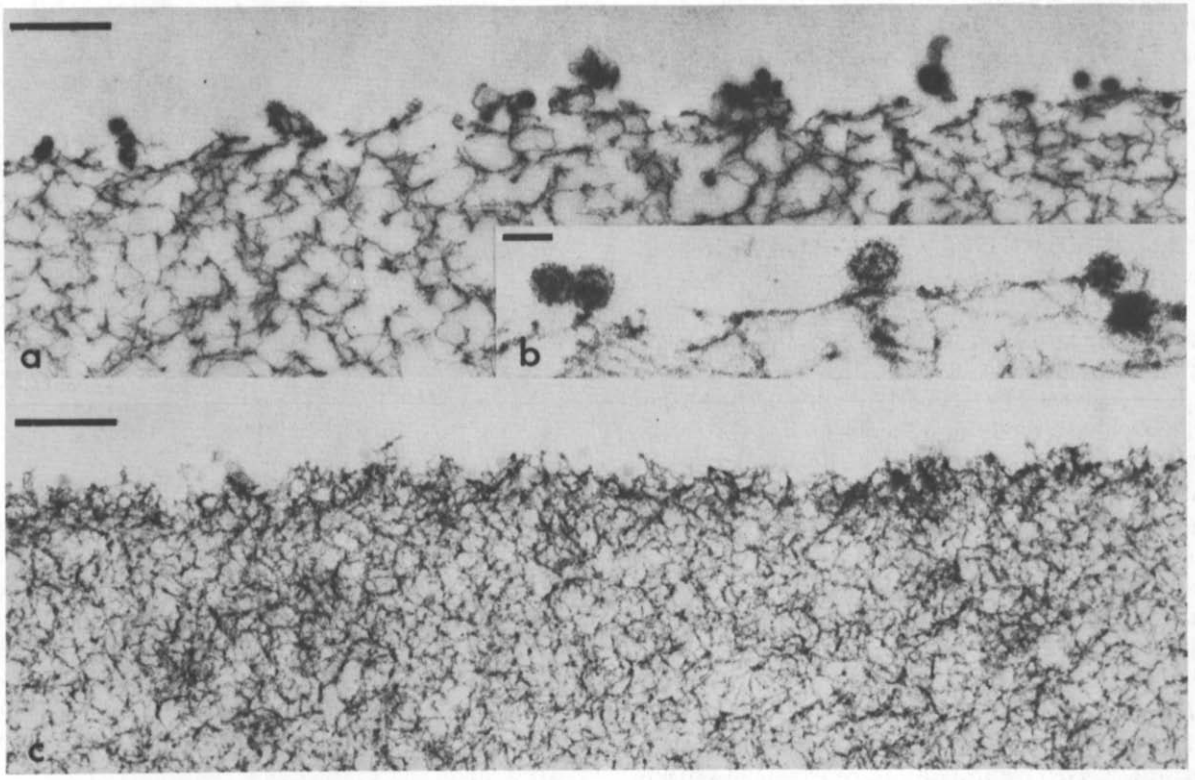

Fig.3. Thin section micrographs showing binding of intact adrenal coated vesicles to calmodulin-derivatized Affigel 15. (a,b) Binding of coated vesicles to calmodulin–Affigel in buffer A containing 100 μM Ca^{2+}. (c) Surface of Affigel–calmodulin incubated with coated vesicles in buffer A (1 mM EGTA). No coated vesicles were seen adherent to the beads in (c). Bars: (a,c) 0.5 μm; (b) 0.1 μm.

tic brain light chains (fig.2). These observations unambiguously identify 33 and 31 kDa polypeptides as adrenal medulla clathrin light chains, functionally equivalent to brain 36 and 33 kDa polypeptides.

3.2. *Calmodulin binding by adrenal medulla coated vesicles and coat proteins*

To examine the binding of calmodulin to adrenal medulla coated vesicles, an electron microscope assay was developed using calmodulin immobilized on Affigel beads. Coated vesicles bound to derivatized beads when Ca^{2+} was present, but did not bind under similar ionic conditions in the absence of Ca^{2+} (fig.3). In control experiments, coated vesicles did not bind to calmodulin–Affigel in the presence of 1 mM Mg^{2+}, nor to ethanolamine-substituted Affigel in the presence of Ca^{2+}. Some small membrane vesicles and empty coats were present in the incubation, but only intact coated vesicles with an inner membrane were observed to bind to the beads. Reassembled clathrin cages were not retained by calmodulin–Affigel in the presence of 0.1 mM Ca^{2+}.

It was possible that the coated vesicle proteins themselves responded to Ca^{2+} by forming hydrophobic sites in a manner analogous to calmodulin. Since a number of calcium-binding proteins interact with the phenothiazine trifluoperazine [16,17], electron microscopic observation was made of coated vesicles incubated with Sepharose–trifluoperazine. Coated vesicles did not bind to these beads either at high or low Ca^{2+}.

It has been reported that brain clathrin light chains bind calmodulin when isolated [18] or when present in intact coated vesicles [5,19]. However, more than 70% of the ^{125}I-labelled adrenal 33 and 31 kDa polypeptides was eluted from calmodulin–Affigel at 0.2 mM Ca^{2+} and the original ratio of 33 and 31 kDa polypeptides was not affected by passage through the column. To examine whether light chains present on intact coated vesicles could bind calmodulin, coated vesicles were treated with TLCK-treated chymotrypsin under conditions where 33 and 31 kDa polypeptides were quantitatively cleaved by proteolysis. Most of the 110 and 100 kDa polypeptides was also removed, but clathrin, 120, 51 and 49 kDa polypeptides were not detectably different from untreated controls (fig.4). There was essentially no difference in the extent of binding of protease treated and untreated coated vesicles to calmodulin–Affigel.

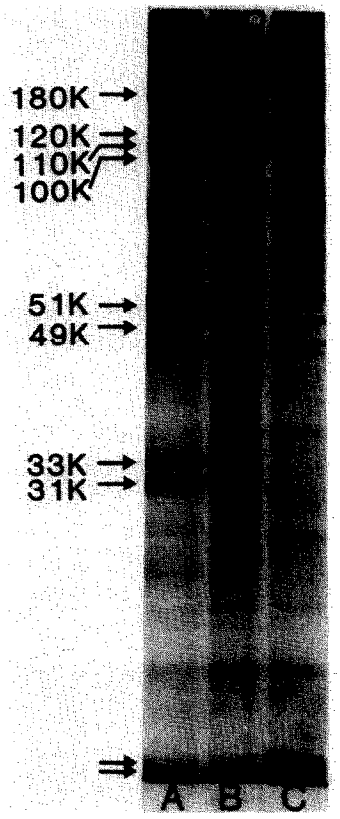

Fig.4. Proteolysis of clathrin light chains. (A) Untreated coated vesicles. (B) Coated vesicles incubated in buffer A with no PMSF for 30 min at 25°C. (C) As for B except that α-chymotrypsin was present at a ratio of 2 μg/mg coated vesicle protein. The digestion was terminated by 2 mM PMSF. Two unlabelled arrows indicate light chain proteolysis products. K, kDa.

3.3. *Phosphorylation of adrenal medulla coated vesicles*

Coated vesicles were phosphorylated on the 51 kDa component after addition of [γ-^{32}P]ATP (fig.5). No other coated vesicle component was a substrate when Ca^{2+} alone, Ca^{2+} and brain calmodulin or cAMP was added to the incubation mixture. Nor was there any new phosphorylation after addition of medulla cytosol (100 000 × g_{av} supernatant); the extent of phosphorylation of the 51 kDa component was slightly reduced (fig.5). The presence of kinase activity in this cytosol was evident from the cAMP-dependent appearance of

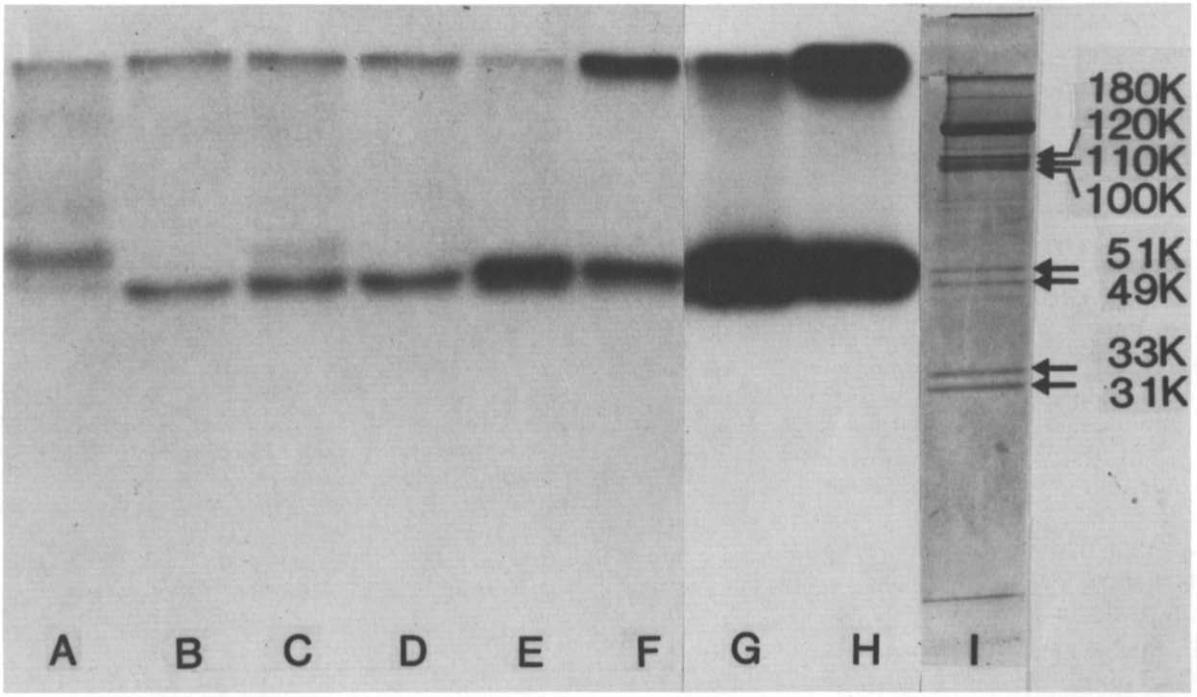

Fig.5. Autoradiograph showing phosphorylation of adrenal coated vesicle proteins. Adrenal coated vesicles (c.v.) (20 μg protein) were incubated alone, with 2 μg of cAMP-dependent protein kinase or with 50 μg medullary cytosol. Lanes: A, cytosol + 5 μM cAMP; B, c.v. + cytosol + 1 mM Ca^{2+}; C, c.v. + cytosol + 5 μM cAMP; D, c.v. + cytosol + 1 mM EGTA; E, c.v. + protein kinase catalytic subunit; F, c.v. (no additions); G–H, 10× longer autoradiographic exposure of lanes E and F; I, protein staining of lane F. K, kDa.

a 58 kDa cytosol phospoprotein (fig.5, lanes A,C). The 51 kDa component was, however, a substrate for cAMP-dependent bovine cardiac muscle protein kinase. Addition of the pure catalytic subunit significantly increased the extent of phosphorylation of 51 kDa polypeptide (fig.5, lanes E,G). It is not excluded, of course, that the phosphate is added to the 51 kDa polypeptide at different sites from those phosphorylated by the endogenous kinase.

4. DISCUSSION

Adrenal coated vesicles were first described and partially characterised in [20]. The major coat proteins are analogous to those from brain, but differ in detail. Despite the lower relative molecular mass of the adrenal light chains, these proteins are able to hybridise with brain clathrin. Light chains from placenta [14] and liver [21] have also been shown to form heterologous complexes with brain clathrin.

The other adrenal coat proteins described were assigned to the coated vesicles using as criteria, purity of the preparation, co-purification with clathrin in several different separation protocols and resistance to detergent extraction. These criteria would not exclude the possibility that one or more proteins was tightly bound, but external to the clathrin lattice.

Adrenal coated vesicles bound to calmodulin–Affigel in the presence of Ca^{2+}. Control experiments indicated that this association was calmodulin-specific, rather than unspecific ionic or hydrophobic binding. This also receives support from the inability of reassembled adrenal clathrin cages to bind to the beads.

Brain clathrin light chains have been reported to bind calmodulin [5,18,19] but light chains isolated from adrenal were not bound by calmodulin-columns even though they retained their ability to bind brain clathrin after labelling. Furthermore complete proteolysis of these molecules did not im-

pair the binding of otherwise intact coated vesicles to calmodulin beads. The most probable sites of calmodulin binding are therefore the 100 or 50 kDa protein groups. This is assuming that the immobilized calmodulin would have difficulty in gaining access to the surface of the enclosed vesicle itself.

The 51 kDa protein was the sole site of protein phosphorylation in adrenal coated vesicles. This may also be the situation in the intact cell, since neither protein kinases present in the gland homogenate, nor the purified catalytic subunit of beef heart cAMP-dependent protein kinase introduced phosphate into any other coated vesicle protein. Phosphorylation of a 50 kDa component of brain coated vesicles has also been reported [7,8]. Since no function has been assigned to a protein of this molecular mass, there is no evidence to exclude the possibility that the 51 kDa polypeptide is a component of the endogenous kinase which undergoes autophosphorylation. It may be significant that calmodulin binds to polypeptides which are substrates for cAMP dependent protein kinases [22]. Thus the 51 kDa component could be the site of calmodulin binding in intact coated vesicles.

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