Interaction of calmodulin with adrenal chromaffin granule membranes

Michael J. Geisow, Robert D. Burgoyne and Alan Harris

Division of Biophysics and Laboratory of Biological Ultrastructure, National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, England

1. INTRODUCTION

We have reported that calmodulin bound specifically to catecholamine-containing secretory granules of adrenal medullary chromaffin cells [1]. Binding was dependent upon μM levels of Ca^{2+} and saturated at a low level (3.3. pmol calmodulin/mg granule membrane protein). Additionally, we observed that the phosphorylation of membrane polypeptides of M_r 59 000, 58 000 and 53 000 was stimulated by calmodulin.

Recent studies of calmodulin localization on plasma membrane and secretion granule preparations from sea urchin oocytes have drawn attention to a direct role of calmodulin in exocytosis [2]. We have re-examined the association of calmodulin with chromaffin cell membranes, to confirm the identity of the recipient membranes and to characterise further this interaction.

Our results affirm that both granule and plasma membranes bind calmodulin in a calcium-dependent manner. Using a technique in which ^{125}I-labelled calmodulin binds to membrane proteins after separation on SDS—PAGE, calmodulin was found to bind predominantly to a polypeptide co-migrating with cytochrome $b561$, the major cytoplasmically-oriented protein of the granule membrane.

2. MATERIALS AND METHODS

Chromaffin granules were purified from bovine adrenal medullae on discontinuous gradients of Percoll® [3] and granule membranes obtained by hypotonic lysis and washing in 10 mM Tris—maleate buffer (pH 6.5). Microsomal and plasma membranes were further purified from the supernatant remaining after centrifugation at 25 000 × g_{av} for 20 min. The plasma membrane was purified on two discontinuous density gradients of Percoll® and sucrose as in [3] except that the plasma membrane was recovered at highest specific activity from the 20%/32% (w/w) sucrose interface. Calmodulin was iodinated with ^{125}I as in [1] to spec. act. 48 Ci/mmol.

The concentration-dependence of ^{125}I-calmodulin binding to granule and plasma membrane was determined by microfuge assay as in [1]. These assays used ^{125}I-calmodulin in the absence of unlabelled protein. Membranes pre-incubated with ^{125}I-calmodulin for 5 min at 20°C in 0.3 M sucrose 10 mM Hepes (pH 7.0) at 10^{-7} M Ca^{2+} or 10^{-4} M Ca^{2+} (adjusted with calcium/EGTA [1]) were layered onto linear 12 ml 20% (w/w)—40% (w/w) sucrose density gradients and centrifuged 150 000 × g_{av} for 90 min. Fractions were collected from the bottom of the gradients.

Dopamine-β-hydroxylase activity was assayed essentially as in [5] and acetylcholinesterase as in [6]. The binding of ^{125}I-calmodulin to granule membrane proteins separated by SDS—PAGE was performed as in [7] and visualized by means of autoradiography using an intensifying screen and pre-flashed X-ray film [8].

3. RESULTS

3.1. *Sucrose density gradient analysis of ^{125}I-calmodulin-labelled chromaffin granule membranes*

Chromaffin granule membranes isolated by discontinuous Percoll® density gradients [3] were low

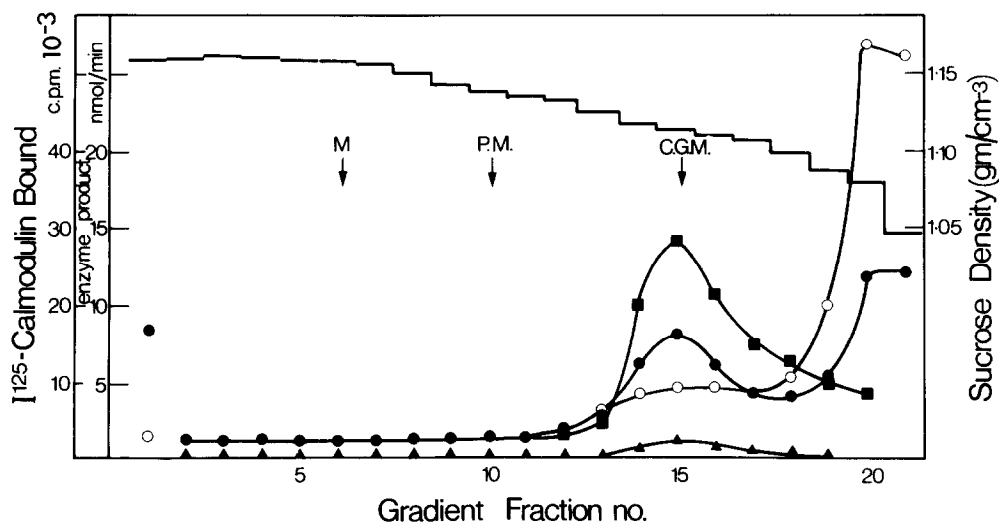Fig.1. Sucrose density gradient analysis of the interaction of ^{125}I-calmodulin with purified chromaffin granule membranes. The linear gradient is defined by the top plot. The distribution of radioactivity from 1 μg calmodulin preincubated 5 min at 20°C with granule membrane (460 μg protein) at 10^{-7} M Ca^{2+} (o——o) and 10^{-4} M Ca^{2+} (●——●) is shown. The membrane was superficially washed by pelleting in the appropriate buffer before layering on the calcium-containing gradients. The low calcium concentration was achieved by buffering with EGTA as in [1]. The distribution of the granule membrane enzyme marker dopamine β-hydroxylase is shown (■——■) as well as the plasma and granule membrane enzyme, acetylcholinesterase (▲——▲). Arrows CGM, PM and M mark the gradient positions of chromaffin granule membrane, plasma membrane and heavy microsomes. The latter two were determined in separate runs.

in mitochondrial contamination (0.7%) as assessed by electron microscopy. Plasma membranes isolated as in section 2 had high acetylcholinesterase (AchE) specific activity (enriched 7–11-fold over homogenate) and negligible dopamine β-hydroxylase activity. Heavier microsomal fractions obtained as in [4] were pooled and termed 'microsomes'. Chromaffin granule membrane pre-incubated with ^{125}I-calmodulin at high (10^{-4} M) and low (10^{-7} M) Ca^{2+} migrated within 1.08–1.13 g sucrose/cm^3 with a peak of dopamine β-hydroxylase activity at 1.11 g sucrose/cm^3. ^{125}I-labelled calmodulin co-migrated with this peak enzyme activity and there was no radioactivity corresponding to the positions of microsomes or plasma membrane in the gradient. The magnitude of the co-migrating ^{125}I-calmodulin in peak was decreased by 50% when the Ca^{2+} was reduced from 10^{-4}–10^{-7} M and more radioactivity appeared in a non-sedimentable form (fig.1).

Some AchE activity was found in the position of granule membrane which contains this enzyme [4]. No AchE activity was measured at the position

corresponding to plasma membrane in fig.1, even though the specific activity of the enzyme is much higher in the plasma membrane.

Both plasma membrane and microsomes were separately shown to bind ^{125}I-calmodulin on similar calcium-containing gradients (not shown), but the calcium-dependence of the binding to microsomes was not investigated.

3.2. *Relative binding of ^{125}I-calmodulin to granule and plasma membranes*

The microfuge assay in [1] was used to compare the concentration-dependence of binding of calmodulin to plasma and granule membranes at 100 μM Ca^{2+} and 1 mM Mg^{2+}. Binding to granule membrane was similar to [1] and showed saturable binding to a class of high affinity sites below 100 nM (K_d = 70 nM from Scatchard analysis). At higher concentrations, occupancy of lower affinity sites was significant (fig.2). Binding to plasma membrane was also specific and saturable with a higher B_{max} (B_{max} = 11.4 pmol/mg protein; K_d = 47 nM).
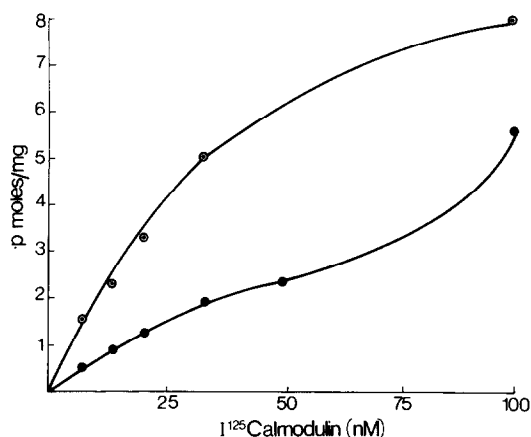
Fig.2. Concentration-dependence of specific binding of ^{125}I-calmodulin to chromaffin granule (●) and plasma membrane (○). The specific calcium-dependent binding of carrier-free ^{125}I-calmodulin to membrane (100 μg protein) was determined by microfuge assay [1]. Specific binding was calculated by subtracting counts bound at 10^{-7} M Ca^{2+} from those bound at 10^{-4} M Ca^{2+}.

3.3. Nature of the calmodulin binding sites on granule membranes

Freshly prepared granule membrane proteins were separated by SDS—PAGE and the Coomassie-blue stained gels processed as in [7]. The gels were exposed to ^{125}I-calmodulin and the polypeptides which bound calmodulin were revealed by autoradiography. The major dye-stained bands were a group of polypeptides with M_r 70 000—79 000 corresponding to subunits of dopamine β-hydroxylase and residual chromogranin A, a soluble content protein. A major band also appeared at M_r 22 000 which corresponds to cytochrome b-561 [9,10]. Autoradiography of the ^{125}I-calmodulin-stained gel revealed minor labelling of the dopamine β-hydroxylase region and major labelling of the M_r 22 000 component (fig.3). A polypeptide of M_r 25 000 was also strongly labelled and there was trace labelling of polypeptides in the region M_r 31 000—38 000.

4. DISCUSSION

The reported low stoichiometry of binding of ^{125}I-calmodulin to granule membranes led to our re-investigation of the specificity of binding, since at such low levels, the chance that calmodulin was

binding to a contaminating membrane, for example, plasma membrane, could not be ruled out. Our observation that ^{125}I-calmodulin co-migrates with the characteristic granule membrane enzyme, dopamine β-hydroxylase, on analytical sucrose density gradients supports our earlier contention that calmodulin does indeed bind to granule membrane. The amount of calmodulin co-migrating with granule membrane was sensitive to $[Ca^{2+}]$. At high $[Ca^{2+}]$ (100 μM) no calmodulin activity was present in the gradient of granule membrane fractions in positions separately established to correspond with plasma membrane and heavy microsomes. Similar gradients were used to establish that calmodulin
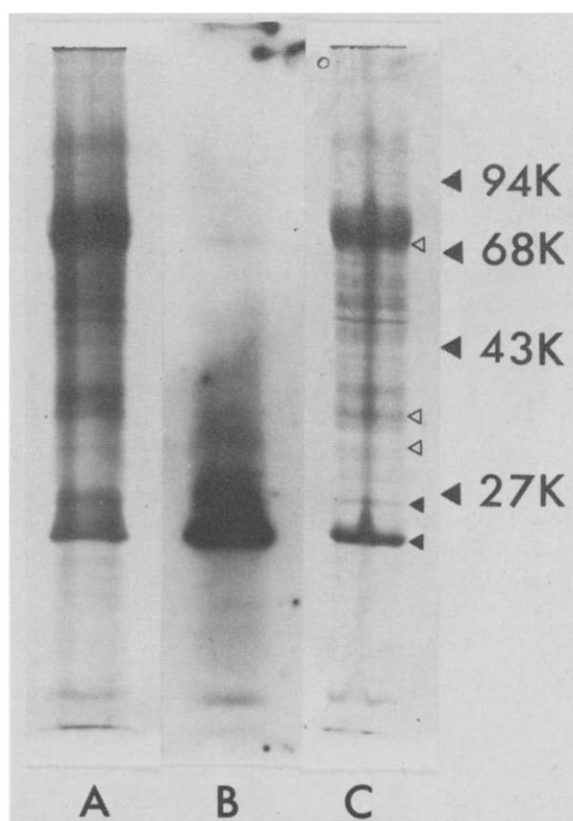

Fig.3. Coomassie-blue staining profile of chromaffin granule membrane polypeptides (A,C) and autoradiography of bound ^{125}I-calmodulin (B). 100 μg granule membrane protein was run on linear 10% (w/v) SDS—PAGE. Polypeptides which bound calmodulin are labelled (◄) and (<) for strong and weak binding, respectively.

does bind to both of these other membranes in the presence of calcium, and at relatively higher levels in terms of membrane protein. The low B_{max} for the interaction of calmodulin with granule membrane could represent a limited number of binding sites on each granule or perhaps the presence of endogenous calmodulin.

Plasma membrane and granule membrane are both clearly capable of binding calmodulin under conditions where the chromaffin cell is stimulated and the level of cytosolic calcium rises above the K_d of calmodulin for the ion [11]. Under such conditions the calcium-complexed calmodulin appears to bind to polypeptides of M_r 25 000 and M_r 22 000 present in the granule membrane. It is possible that the latter polypeptide corresponds to the membrane protein, cytochrome b-561 [9]. All the polypeptides in this M_r-region of granule membrane protein on SDS—PAGE are susceptible to labelling or partial enzymic hydrolysis in the intact granules [10]. Thus our observation of binding of a cytosolic protein is logical in terms of the intact cell, in that binding seems to occur principally to cytoplasmically-oriented polypeptides. The consequences of calmodulin binding to the granules, which may reflect a late stage in exocytosis of their content [2], remain to be determined.

NOTE ADDED IN PROOF

The gel overlay technique was repeated using [125]I Bolton—Hunter reagent-labelled ox-brain calmodulin [12] in the presence of 1 mM CaCl$_2$ and with controls in the presence of 1 mM EGTA and 1 mM fluphenazine—1 mM CaCl$_2$, respectively. The same major calmodulin-binding polypeptides were identified. However, binding to the 25 000 M_r polypeptides was Ca^{2+}-independent whereas binding to the 69 000 and 50 000 M_r components was abolished in the presence of EGTA or fluphenazine—Ca^{2+}. It is likely that these two groups of granule-membrane proteins respectively constitute the calcium-insensitive and calcium-dependent binding sites originally described [1].

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