SPECIFIC BINDING OF ^{125}I-CALMODULIN TO AND PROTEIN PHOSPHORYLATION IN ADRENAL CHROMAFFIN GRANULE MEMBRANES

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Received 18 June 1981; revised version received 29 June 1981

1. Introduction

In the adrenal medulla cholinergic stimulation of the chromaffin cells leads to the release of catecholamines from the granules within which they are stored [1,2]. Release is brought about by fusion of granule and plasma membranes and subsequent exocytotic release of the granule contents by a Ca^{2+}-dependent mechanism [3–5]. The details of this mechanism are unknown though a requirement for Mg-ATP has been demonstrated [3].

A role for protein phosphorylation in stimulus–secretion coupling has been suggested from work on a number of systems. An increase in cAMP-dependent protein phosphorylation has been shown to accompany amylase secretion in the rat parotid [6] and Ca^{2+}-dependent phosphorylation has been suggested to be involved in neurotransmitter release from synaptosomes [7], insulin secretion from pancreatic β cells [8] and insulinoma cells [9] and in the secretion from mast cells [10] and platelets [11]. Furthermore a possible role for calmodulin in stimulating Ca^{2+}-dependent phosphorylation in some of these systems has been demonstrated [7–9,11]. In the case of the chromaffin cells cholinergic stimulation resulted in a Ca^{2+}-dependent increase in the level of phosphorylation of two polypeptides just prior to secretion itself [12,13]. The subcellular localisation and calmodulin dependency of the phosphorylation of these polypeptides are unknown.

In view of the possible involvement of calmodulin and protein phosphorylation in stimulus–secretion coupling in the chromaffin cell we have examined the interaction of calmodulin with chromaffin granule membranes and its effect on membrane protein phosphorylation. These results show that ^{125}I-labelled calmodulin binds to chromaffin granule membranes with high affinity in a Ca^{2+}-dependent fashion. Furthermore, the granule membranes possess calmodulin-dependent, cAMP-dependent, and -independent protein kinases.

2. Materials and methods

Chromaffin granules were prepared from bovine adrenal medullae homogenised in 0.3 M sucrose, 1 mM EGTA [14]. The washed large granule fraction was purified through a density step of 1.7 M sucrose, 1 mM $MgSO_4$, 0.5 mM EGTA. The granules were lysed by homogenisation in 20 mM Hepes (pH 6.5) 1 mM $MgSO_4$ and extensively washed in the same buffer. Before use granule membranes were washed and allowed to re-seal in isotonic buffer (137 mM K^+-glutamate, 20 mM MES (pH 6.5), 1 mM $MgSO_4$).

Bovine brain calmodulin [15] (40 μg) was iodinated to spec. act. 2.5 μCi/μg protein in 100 μl PBS containing 250 μCi carrier-free K^{125}I by exposure at 4°C for 10 min to 300 μg Iodogen (Pierce and Wariner (UK) Ltd, Chester) plated onto a plastic microfuge tube. Unincorporated counts were removed by Sephadex G-25 chromatography.

For the assay of ^{125}I-calmodulin binding to granule membranes 50–75 μg membrane protein was incubated in 1 ml binding buffer [137 mM K^+-glutamate, 20 mM MES (pH 6.5), 1 mM $MgSO_4$, 5 mM EGTA and varying $CaCl_2$] containing 5–50 nM ^{125}I-calmodulin (diluted with unlabelled calmodulin to spec. act. 2 Ci/mmol). Three samples were incubated with and 3 without 4 μM unlabelled calmodulin. After incubation at 20°C for 15 min the samples were either filtered under vacuum through 2.5 cm Whatman GF/B glass-

Published by Elsevier/North-Holland Biomedical Press
00145793/81/0000–0000/$02.50 © 1981 Federation of European Biochemical Societies

fibre filter discs and washed 3 times (filtration assay) or centrifuged at 14 000 × *g* for 5 min and the pellets twice washed with binding buffer (centrifugation assay). Filters and pellets were counted in a Packard 3002 auto-gamma counter. Specific binding was defined as that in the absence minus that in the presence of excess unlabelled calmodulin.

For the incorporation of phosphate from [γ-^{32}P]-ATP, membranes (0.75 mg/ml) were incubated in binding buffer with 10 μM ATP, 10 μCi [γ-^{32}P]ATP (spec. act. 3000 Ci/mmol; Radiochemical Centre, Amersham) and, where stated, 5 μM cAMP or 1.2 μM calmodulin. Incubations were at 37°C for varying times, the reaction stopped by the addition of an equal volume of dissociation buffer [125 mM Tris–HCl (pH 6.8), 1.25% SDS, 1% 2-mercaptoethanol, 10% sucrose, 2 mM EDTA], proteins solubilised by heating to 100°C for 1 min and 100 μl aliquots analysed by sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis. Autoradiography was carried out on dried gels. Molecular masses (M_r) were determined by comparison to known M_r standards; β-galactosidase (130 000), bovine serum albumin (67 000), ovalbumin (43 000), chymotrypsin (25 000), myoglobin (17 800) and cytochrome *c* (12 400). Protein determination was done as in [16].

Varying concentrations of free calcium in the binding buffer were set up by varying the amount of added $CaCl_2$ based on calculations from the known dissociation constants of EGTA–Ca^{2+} and EGTA–Mg^{2+}. Final free [Ca^{2+}] was determined by comparison to standard solutions of Ca^{2+} (BDH) using a calcium electrode [17].

3. Results

3.1. *Binding of ^{125}I-calmodulin to chromaffin granule membranes*

Binding of ^{125}I-calmodulin to chromaffin granule membranes was carried out using either a 'filtration' or a 'centrifugation' assay. Essentially identical results were obtained with either method. Examination of specific ^{125}I-calmodulin binding over a range of calmodulin concentrations indicated that binding was saturable with a linear Scatchard plot (fig.1). An apparent K_d of 3.1 × 10^{-8} M and a maximal binding capacity (B_{max}) of 3.3 pmol/mg protein were found. At higher concentrations of ^{125}I-calmodulin evidence for a low affinity binding site(s) was obtained (not shown).

The level of specific binding of ^{125}I-calmodulin

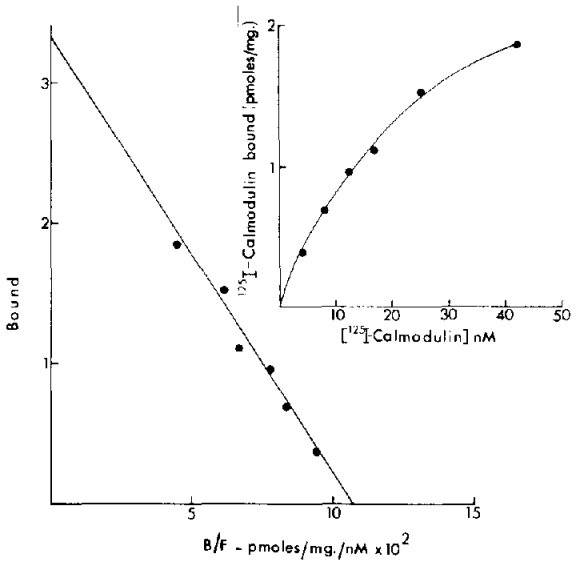

Fig.1. Dependence of ^{125}I-calmodulin binding to chromaffin granule membranes on concentration of calmodulin. The binding of ^{125}I-calmodulin to granule membranes was assayed over a range of concentrations of ^{125}I-calmodulin at 10^{-4} M Ca^{2+}. Data are shown as a Scatchard plot and saturation curve (inset) of specific binding expressed as pmol/mg membrane protein. From the Scatchard plot r = 0.96, K_d = 3.1 × 10^{-8} M and B_{max} = 3.3 pmol/mg.

over a range of free [Ca^{2+}] was determined (fig.2). A small Ca^{2+}-independent component was found in all experiments. Such a component had been seen with ^{125}I-calmodulin binding to brain membranes [18,19]. Stimulation of binding occurred with increasing Ca^{2+} with half-maximal binding occurring at 5 × 10^{-6} M free Ca^{2+}.

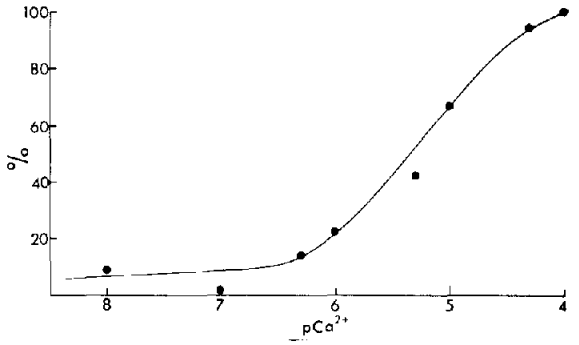

Fig.2. Dependence of ^{125}I-calmodulin binding to chromaffin granule membranes on free [Ca^{2+}]. The binding of ^{125}I-calmodulin to granule membranes was assayed at 50 nM ^{125}I-calmodulin over a free [Ca^{2+}] range. Data were calculated as ^{125}I-calmodulin specifically bound (pmol/mg protein) and expressed as a percentage of the maximum binding found.

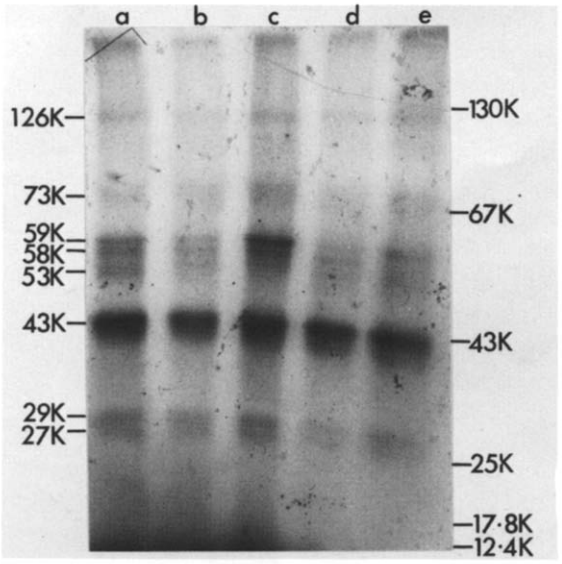

Fig.3. Sodium dodecyl sulphate–polyacrylamide gel electrophoretic analysis of phosphate incorporation from [γ-^{32}P]ATP into chromaffin granule membranes. Membranes were incubated with [γ-^{32}P]ATP at 37°C for 30 s with: (a) calmodulin, (b) no additions, (c) cAMP, (d) inhibitor of cAMP-dependent protein kinase, (e) cAMP and inhibitor of cAMP-dependent protein kinase and then analysed by SDS–polyacrylamide gel electrophoresis and autoradiography. The M_r-values of detected phosphorylated polypeptides are shown on the left and those of standards on the right. This experiment was carried out with 10^{-4} M Ca^{2+} in all incubations. In a separate experiment we compared the pattern of phosphorylation in controls with either 10^{-4} M Ca^{2+} or EGTA only present. No difference was seen in the two gel patterns indicating that the control pattern in the presence of Ca^{2+} does not include a Ca^{2+}-dependent component due to endogenous calmodulin.

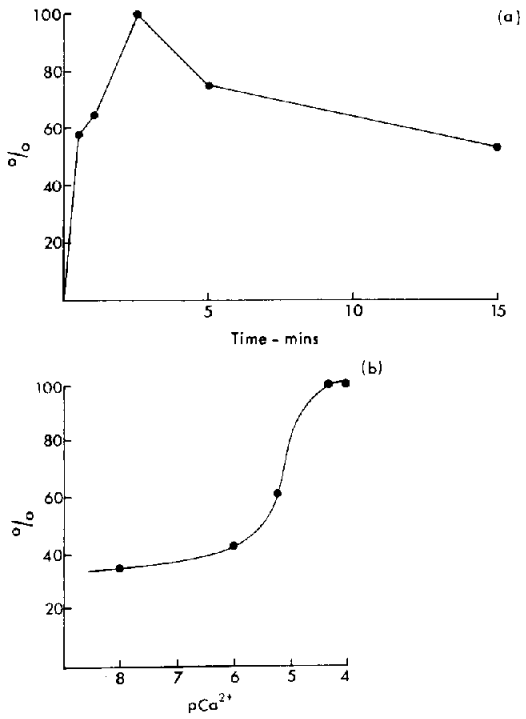

Fig.4. Dependence of the degree of phosphorylation of the major 43 000 M_r polypeptide on (a) time of incubation and (b) free [Ca^{2+}]. Chromaffin granule membranes were incubated with [γ-^{32}P]ATP and calmodulin at 37°C at 10^{-4} M Ca^{2+} for varying times or for 30 s at varying free [Ca^{2+}]. The degree of phosphorylation of the 43 000 M_r polypeptide was quantitated by cutting out dried gel pieces and counting radioactivity by liquid scintillation spectrometry. Each data point was expressed as a percentage of the maximum value in that experiment.

3.2. *Phosphorylation of chromaffin granule membrane proteins*

A number of polypeptides incorporated phosphate from [γ-^{32}P]ATP under those conditions used for examination of ^{125}I-calmodulin binding (fig.3). Examination of the time course of phosphorylation indicated a peak in the level of phosphorylation after incubation at 37°C for 2.5 min with a subsequent decline after incubation for 5 or 15 min presumably due to the action of endogenous phosphatase or protease activity (fig.4a). All polypeptides showed similar time courses of phosphorylation. In the presence of 10^{-4} M Ca^{2+}, calmodulin stimulated the phosphorylation of the major phosphorylated polypeptide of M_r 43 000 and a group of polypeptides of M_r 59 000, 58 000 and 53 000. A calmodulin/Ca^{2+} stimulation of the phosphorylation of these polypeptides was seen in 4 separate experiments. Stimulation of their phosphorylation was not brought about by Ca^{2+} or calmodulin alone. The degree of stimulation of phosphorylation by calmodulin increased with increasing [Ca^{2+}] with a half-maximal stimulation of the phosphorylation of the 43 000 M_r polypeptide at 7×10^{-6} M Ca^{2+} (fig.4b). Calmodulin-stimulated phosphorylation of the M_r 59 000, 58 000 and 53 000 polypeptides showed similar Ca^{2+}-dependency (not shown).

The phosphorylation of the 43 000 M_r protein was also stimulated by 5 μM cAMP (fig.1). A more pronounced stimulation by cAMP was seen in the case of phosphorylation of the 59 000 M_r protein. In the presence of the specific protein inhibitor of cAMP-dependent protein kinase [20] the pattern of phosphoryla-

tion was virtually indistinguishable from that seen in the controls (fig.1). Thus the basal level of phosphorylation seen in control incubations (even in the absence of Ca^{2+}) must be due to the presence of a cAMP- and calmodulin-independent protein kinase.

4. Discussion

Chromaffin granule membranes bound ^{125}I-calmodulin with high affinity at 10^{-4} M free Ca^{2+} giving a value for K_d (3.1×10^{-8} M) similar to that found for the binding of ^{125}I-calmodulin to brain membranes [18,19]. The intracellular calmodulin concentration in the adrenal medulla had been estimated to be around 10^{-6} M [21]. Thus in the intact tissue it could be expected that all of the high-affinity calmodulin binding sites on the chromaffin granules would be occupied at high $[Ca^{2+}]$. Since the internal free $[Ca^{2+}]$ in resting cells is around 10^{-7} M rising to 10^{-5} M following stimulation [5] occupancy of the calmodulin binding sites on the granule membranes would only occur following stimulation. It should be noted that our results on Ca^{2+}-dependency of ^{125}I-calmodulin binding are compatible with the known affinity constants for Ca^{2+} binding to calmodulin [22,23].

We have shown that interaction of calmodulin with chromaffin granule membranes stimulates a calmodulin-dependent protein kinase. Furthermore, we have demonstrated the presence of both cAMP-dependent and -independent protein kinase activity in the membranes. Protein phosphorylation had been demonstrated in chromaffin granule membranes [24,25]. However, the effects of calmodulin and cAMP were not examined. Cholinergic stimulation of isolated chromaffin cells resulted in a Ca^{2+}-dependent increase in the phosphorylation of two polypeptides of M_r 56 000 and 99 000 in [12] or M_r 60 000 and 95 000 in [13]. The subcellular localisation of these polypeptides showing increased phosphorylation has not been determined. The 56 000/60 000 M_r polypeptides may be related to the 53 000/58 000/59 000 M_r group of polypeptides which we have shown to demonstrate calmodulin-stimulated phosphorylation in isolated granule membranes. The M_r-values of the phosphorylated species all correspond with known polypeptides in the chromaffin granule membrane ([26], M. J. G., unpublished). The function of these polypeptides is unknown, but include a 53 000 M_r subunit of a membrane ATPase [26].

In interpreting the results of phosphorylation in granule membranes it is important to know whether the phosphorylated polypeptides are located on the external or internal facing surface of the membrane. Here we have examined both calmodulin binding and protein phosphorylation in granule membranes under isotonic conditions. Under these conditions the granule ghosts appear to reseal in their original configuration based on their ability to transport catecholamines [27]. Thus the phosphorylated polypeptides seen here are most probably on the external (cytoplasmic)-facing surface of the granule membrane.

These results are consistent with a model for Ca^{2+}-dependent exocytosis involving sequential Ca^{2+}-dependent binding of calmodulin to and calmodulin-stimulated phosphorylation in chromaffin granules preceding exocytosis. The exact functions of calmodulin- or indeed the cAMP-dependent and -independent phosphorylations in the granule membrane are unknown.

Acknowledgement

The authors wish to thank Philip Cohen for the gift of the cyclic AMP-dependent protein kinase inhibitor.

References

[1] Pollard, H. B., Pazoles, C. J., Creutz, C. E. and Zinder, O. (1979) Int. Rev. Cytol. 58, 159–197.
[2] Winkler, H. (1977) Neuroscience 2, 657–683.
[3] Baker, P. F. and Knight, D. E. (1978) Nature 276, 620–622.
[4] Douglas, W. W. (1968) Brit. J. Pharmacol. 34, 451–474.
[5] Rubin, R. P. (1975) in: Calcium and the Secretory Process, pp. 6–11, Plenum, New York.
[6] Jahn, R., Unger, C. and Soling, H.-D. (1980) Eur. J. Biochem. 112, 345–352.
[7] De Lorenzo, R. J., Freedman, S. D., Yohe, W. B. and Maurer, S. C. (1979) Proc. Natl. Acad. Sci. USA 76, 1838–1842.
[8] Gagliardino, J. J., Harrison, D. E., Christie, M. R., Gagliardino, E. E. and Ashcroft, S. J. H. (1980) Biochem. J. 192, 919–927.
[9] Schubart, U. K., Fleischer, N. and Erlichman, J. (1980) J. Biol. Chem. 255, 11063–11066.
[10] Sieghart, W., Theoharides, T. C., Alper, S. L., Douglas, W. W. and Greengard, P. (1978) Nature 275, 329–331.
[11] Nishikawa, M., Tanaka, T. and Hidaka, H. (1980) Nature 287, 863–865.

[12] Holz, R. W., Rothwell, G. E. and Ueda, T. (1980) Soc. Neurosci. abst. 6, 177.
[13] Amy, C. M. and Kirschner, N. (1981) J. Neurochem. 36, 847–854.
[14] Smith, A. D. and Winkler, H. (1967) Biochem. J. 103, 480–482.
[15] Grand, R. J. A., Perry, S. V. and Weeks, R. A. (1979) Biochem. J. 177, 521–529.
[16] Bradford, M. M. (1976) Anal. Biochem. 72, 248–254.
[17] Kim, Y. S. and Padilla, G. M. (1978) Anal. Biochem. 89, 521–528.
[18] Lau, Y.-S. and Gnegy, M. E. (1980) J. Pharmacol. Exp. Ther. 215, 28–34.
[19] Vandermeers, A., Robberecht, P., Vandermeers-Piret, M.-C., Rathe, J. and Christophe, J. (1978) Biochem. Biophys. Res. Commun. 84, 1076–1081.
[20] Walsh, D. A., Ashby, C. D., Gonzalez, C., Calkins, D., Fischer, E. H. and Krebs, E. G. (1971) J. Biol. Chem. 246, 1977–1985.
[21] Kuo, I. C. Y. and Coffee, C. J. (1976) J. Biol. Chem. 251, 1603–1609.
[22] Klee, C. B. (1977) Biochemistry 16, 1017–1024.
[23] Cox, J. A., Malnoe, A. and Stein, E. A. (1981) J. Biol. Chem. 256, 3218–3222.
[24] Trifaro, J. M. and Dworking, J. (1971) Mol. Pharmacol. 7, 52–65.
[25] Muller, T. W. and Kirshner, N. (1975) J. Neurochem. 24, 1155–1161.
[26] Abbs, M. T. and Phillips, J. H. (1980) Biochim. Biophys. Acta 595, 200–221.
[27] Phillips, J. H. (1974) Biochem. J. 144, 311–318.