The EMBO Journal Vol.1 No.10 pp.1167–1170, 1982

Calelectrin self-aggregates and promotes membrane aggregation in the presence of calcium

Th.Chr.Südhof*, J.H.Walker, and J.Obrocki

Abteilung Neurochemie, Max-Planck-Institut für biophysikalische Chemie, Postfach 2841, D-3400 Göttingen, FRG

Communicated by V.P.Whittaker
Received on 20 July 1982

Calelectrin is a protein that can be purified to homogeneity from the cholinergically innervated electric organ of *Torpedo marmorata* where it is present in large amounts. It has been shown to bind to the membranes of the electric organ in a Ca^{2+}-dependent and specific manner. Using the purified protein we now report that it is specifically self-aggregated by Ca^{2+} in micromolar concentrations but not by Mg^{2+} at much higher concentrations. Sr^{2+} is also completely inactive, while Ba^{2+} and the trivalent lanthanides Tb^{3+}, Eu^{3+}, and La^{3+} can substitute for Ca^{2+}. Calelectrin also greatly enhances the Ca^{2+}-induced aggregation of isolated synaptic vesicle membranes from the cholinergic nerve terminals of *T. marmorata* and of chromaffin granule membranes from the bovine adrenal medulla. The potentiation of membrane aggregation is mainly due to the appearance of a fast aggregatory phase in the presence of calelectrin. It is saturable with respect to calelectrin and can be demonstrated at very low calelectrin concentrations, suggesting a specific calelectrin membrane-binding component. This component seems to be of lipid nature since the aggregation of total extracted lipids from *Torpedo* electric organ and from chromaffin granules could also be enhanced by calelectrin. The Ca^{2+}-induced self-association of calelectrin and its aggregation enhancing effect may be of great importance to the structural organization of neural and secretory cells and the mechanism of exocytosis.

Key words: calelectrin/calcium-binding protein/membrane aggregation/exocytosis/cytoskeleton

Introduction

Exocytosis is a widely accepted mechanism of secretion for many neurotransmitters and hormones (Smith and Winkler, 1972; Whittaker, 1980). It requires that the molecules to be secreted are stored in high concentrations in specialized cytoplasmic membrane-bound vesicles and that calcium triggers fusion of these vesicles with the plasma membrane after depolarisation, leading to the externalisation of the contents of the vesicles. It is unknown, however, how Ca^{2+} triggers the rapid fusion of the vesicle and plasma membranes.

The extensive study of Ca^{2+}-induced membrane aggregation and fusion in recent years has shown that the Ca^{2+}-induced interactions of biological membranes alone are poor models for the exocytosis process because of high Ca^{2+}-requirements, slow speeds, and low amplitudes under physiological ionic strength conditions, although important conclusions have been drawn about the physical mechanisms of membrane aggregation and fusion (Haynes *et al.*, 1979a, 1979b; Morris *et al.*, 1979a, 1982b; Portis *et al.*, 1979). Attention has focused on aggregation and fusion enhancing factors, notably the Ca^{2+}-binding protein synexin, which has been isolated from the adrenal medulla and shown to self-aggregate in the presence of Ca^{2+} and to enhance the Ca^{2+}-induced aggregation and fusion of all membranes tested so far (Creutz *et al.*, 1978, 1979; Morris and Hughes, 1979; Morris *et al.*, 1982a; Hong *et al.*, 1982; Creutz and Pollard, 1982). However, although synexin can be isolated from tissues other than the adrenal medulla, attempts to show its presence in the central nervous system have failed, and its tissue concentrations in the adrenal medulla appear to be very low. Therefore it seems important to look for other, more widely distributed proteins with similar properties.

One of us recently reported the isolation of a protein from the cholinergically innervated electric organ of *Torpedo marmorata* that is selectively bound to membranes by very low Ca^{2+} concentrations (Walker, 1982). This protein, here referred to as calelectrin, can be demonstrated by immunofluorescence to be present in several secretory tissues, including the adrenal medulla of rats and rat brain, in a distinct distribution compatible with that of synaptic and secretory vesicle membranes (Walker, Zimmermann, Südhof, and Obrocki, unpublished data).

Here we demonstrate that calelectrin is selectively self-aggregated by Ca^{2+} and greatly enhances the Ca^{2+}-induced membrane aggregation of isolated secretory vesicles with characteristics which imply the existence of a specific calelectrin-binding membrane component. In view of the wide distribution and high concentration of calelectrin these findings may have important implications for both the mechanism of exocytosis and the cytoskeleton of secretory tissues.

Results

Self-association of calelectrin

Calelectrin avidly self-associates in the presence of Ca^{2+}. As shown in a typical experiment in Figure 1a, the self-association is apparent at micromolar concentrations of Ca^{2+} with a final amplitude of several hundred times that of the original scattering of the calelectrin solution in 0.15 M buffered KCl. Lineweaver-Burke plots (Figure 1b) reveal two different apparent binding sites for Ca^{2+}: a high affinity site with a K_m of $\sim 150-200$ μM and a low affinity site in the millimolar range. The K_ms of the Ca^{2+}-induced self-aggregation varied slightly between preparations and were very sensitive to storage times and conditions. Mg^{2+} and Sr^{2+} had no aggregating effect on calelectrin, while Ba^{2+} could completely substitute for Ca^{2+} and the trivalent lanthanides Tb^{3+}, La^{3+}, and Eu^{3+} were even more effective. Like Ca^{2+}, Tb^{3+} also exhibits a two affinity mechanism of self-aggregation, although with much lower K_m values. Mg^{2+}, although itself ineffective, partially inhibits the Ca^{2+}- and Tb^{3+}-induced aggregation of calelectrin at high concentrations in a concentration-dependent manner.

Ca^{2+}-aggregation of calelectrin could be reversed by EDTA but not by EGTA; the latter effected partial reversal at low EGTA:Ca^{2+} ratios but strongly self-aggregated calelectrin at high EGTA:Ca^{2+} ratios. EGTA alone had no calelectrin aggregating effect at concentrations up to 50 mM. At

*To whom reprint requests should be sent.

 0261-4189/82/0110-1167$2.00/0.

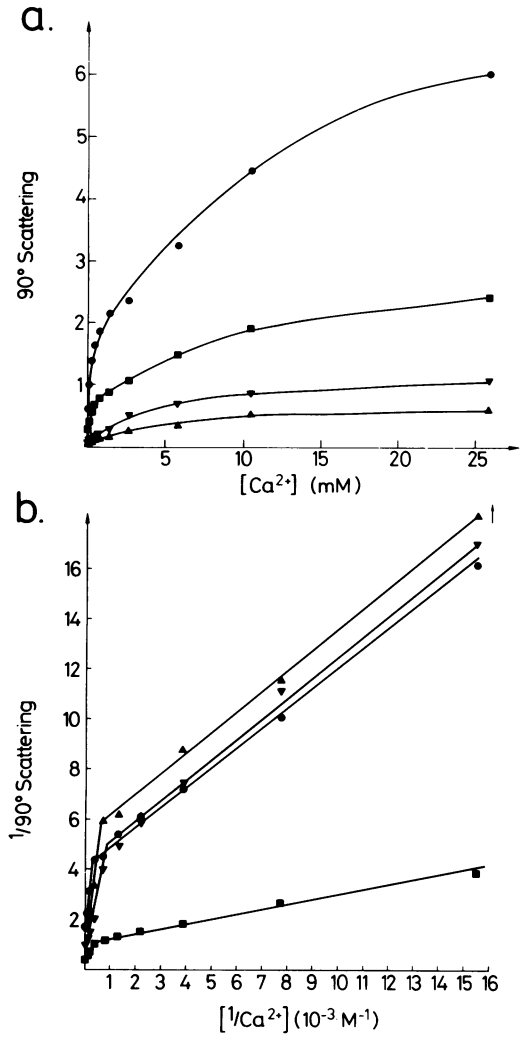

Fig. 1. Self-association of calelectrin as a function of Ca^{2+} concentration measured by 90° light scattering at four different calelectrin concentrations (14.0, 28.0, 55.0, and 105.0 μg/ml calelectrin). In **a**, the increase in light scattering is plotted against Ca^{2+} concentration. **b** Presents Lineweaver-Burke plots of these data and reveals two Ca^{2+}-binding processes with different affinities (K_{m_1} 150–200 μM, K_{m_2} 3–5 mM, respectively) independent of protein concentration (data for highest protein concentration have been multiplied by 10). For conditions, see Materials and methods.

any given EGTA concentration, the self-aggregation of calelectrin by Ca-EGTA solutions with a surplus of EGTA was much higher than the self-aggregation induced either by EGTA-Ca solutions with a surplus of Ca^{2+} or by Ca^{2+} alone without EGTA. These observations suggest that the Ca_1-EGTA complex but not the Ca_2-EGTA complex aggregates calelectrin. EGTA binding is often observed with Ca^{2+}-binding proteins (Haiech *et al.*, 1979).

Promotion of membrane aggregation by calelectrin

Calelectrin greatly increases the Ca^{2+}-induced aggregation of both isolated cholinergic synaptic vesicle membranes from *T. marmorata* and of isolated chromaffin granule membranes from the bovine adrenal medulla (Figures 2a and 2b). Without calelectrin the Ca^{2+}-induced membrane aggregation in buffers of physiological ionic strength is almost negligible, while calelectrin self-association alone cannot account for the turbidity increase observed. The aggregation reactions of chromaffin granule membranes with and without calelectrin are shown in Figure 3 and demonstrate that the calelectrin

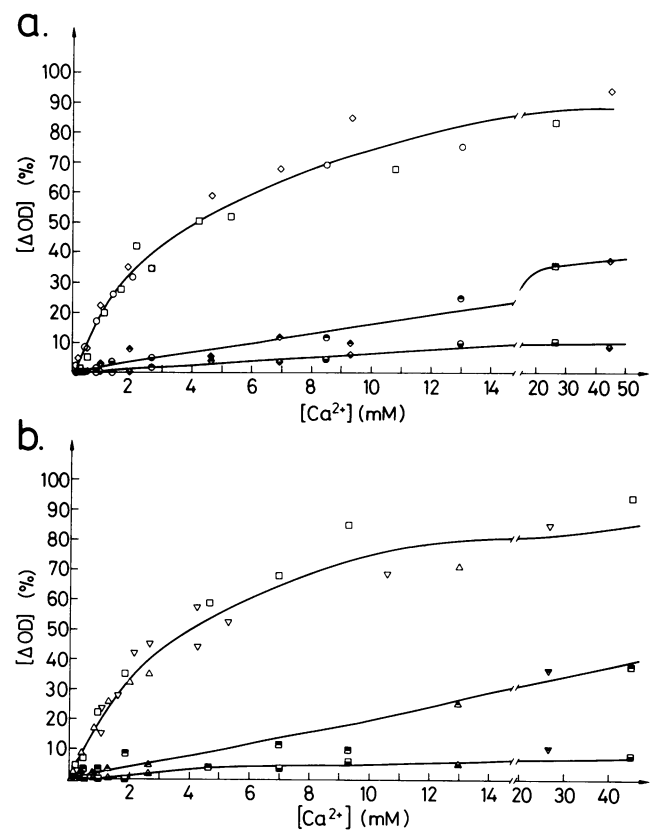

Fig. 2. Potentiation of Ca^{2+}-induced aggregation of cholinergic synaptic vesicle and of chromaffin granule membranes by calelectrin. **a** Displays the potentiation of cholinergic synaptic vesicle membrane aggregation and **b** the potentiation of chromaffin granule membrane aggregation. Membrane aggregation was followed by turbidity measurements at 320 nm and is plotted as the OD after 15 min in % of the membrane turbidity as a function of total calcium concentration. In both graphs, void symbols (upper traces) represent the turbidity increases in the presence of both calelectrin and membranes, symbols with the upper halves filled (middle traces) the turbidity increases due to calelectrin only, and symbols with the lower halves filled show the membrane turbidity increases in the absence of calelectrin (lower traces). Synaptic vesicle membrane aggregation was measured at 25°C in 0.4 M buffered KCl, and chromaffin granule membrane aggregation at 37°C in 0.15 M buffered KCl.

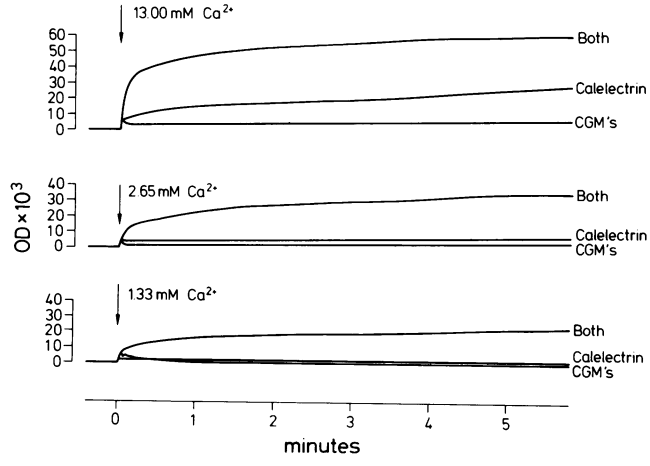

Fig. 3. Time course of the Ca^{2+}-induced aggregation with and without calelectrin of chromaffin granule membranes at three different Ca^{2+} concentrations under physiologic ionic strength conditions (0.15 M buffered KCl). Conditions were as in Figure 2b (Traces labelled CGMs = only chromaffin granule membranes were present; calelectrin = only calelectrin present; both = calelectrin and granule membranes were present).

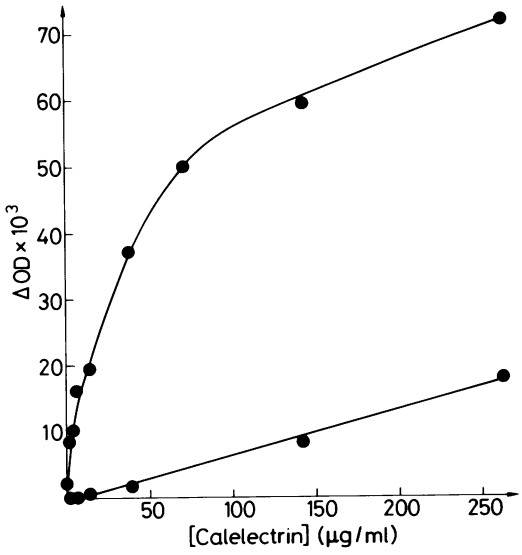

Fig. 4. Titration of cholinergic vesicle membrane aggregation at a fixed Ca^{2+} concentration (4.55 mM) against calelectrin concentration (upper curve) with a control of calelectrin only (lower curve).

potentiation of membrane aggregation is mainly due to a new fast phase, which is quite diffrent from the aggregation enhancement seen with synexin (Morris and Hughes, 1982). Identical traces were obtained for synaptic vesicle membranes.

Titrations of the membrane aggregation against calelectrin concentration at a given Ca^{2+} concentration demonstrated that the aggregation potentiation was already marked at very low calelectrin concentrations and was saturated at high concentrations for both chromaffin granule and cholinergic synaptic vesicle membranes (shown in Figure 4 for synaptic vesicle membranes). This behaviour indicates the existence of a specific calelectrin-binding membrane component.

We investigated further the ability of calelectrin to potentiate the aggregation of lipids. Calelectrin potentiates both the aggregation of total extracted lipids from the electric organ of *T. marmorata* and from bovine chromaffin granules in a concentration-dependent, saturable manner (shown with *Torpedo* electric organ lipids in Figure 5a). When these were further fractionated (Richardson *et al.*, 1982), the aggregation enhancement was most pronounced with the 'neutral lipid' fraction. This demonstrates the lipid nature of the calelectrin membrane binding component; unlike synexin, however, whose aggregation enhancing effect is also lipid mediated (Morris *et al.*, 1982a), calelectrin does not enhance the aggregation of phosphatidylserine liposomes (Figure 5b), but slightly inhibits it. Phosphatidylcholine liposome aggregation, on the other hand, requires much higher calcium concentrations and was not at all affected by the presence of calelectrin (6 μg/ml) up to concentrations of 10 mM Ca^{2+} (data not shown).

Discussion

In this report, we identify and characterize two properties of a protein isolated in pure form from the electric organ of *T. marmorata* which may be of great importance to the cytoplasmic organization of neural and secretory tissues and to the mechanism of exocytosis: these are, Ca^{2+}-dependent and Ca^{2+}-specific self-association at low Ca^{2+} concentrations, and potentiation of Ca^{2+}-induced membrane interactions at

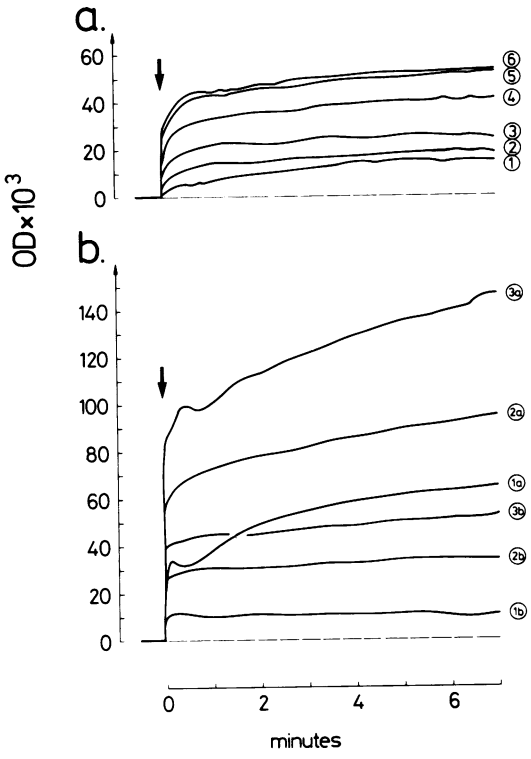

Fig. 5. Effect of calelectrin on Ca^{2+}-induced lipid aggregation. **a** Shows the concentration dependent and saturable potentiating effect of calelectrin on the aggregation of total lipids from *Torpedo* electric organ (trace 1: 0.0 μg/ml, 2: 12 μg/ml, 3: 24 μg/ml, 4: 60 μg/ml, 5: 120 μg/ml, 6: 240 μg/ml calelectrin, arrow indicates addition of 40 mM Ca^{2+}). In **b** the inhibitory effect of calelectrin on phosphatidylserine liposome aggregation is displayed at three different calcium concentrations (traces 1a/b: 0.180, 2a/b: 0.360, 3a/b: 0.541 mM Ca^{2+}; suffix a) denotes absence, suffix b) presence of 6 μg/ml calelectrin). For conditions, see Materials and methods.

higher Ca^{2+} concentrations. In view of the wide distribution of calelectrin, its high tissue concentration, and its apparent evolutionary conservation these data suggest a major role of calelectrin in secretory tissues.

Two different self-aggregation mechanisms for calelectrin were observed which differed in their affinity for Ca^{2+}. Although the preparations used were >99% pure as judged by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), the two different affinity mechanisms might not necessarily reflect binding sites on the same molecule but could also correspond to two functionally different classes of calelectrin (e.g., by post-translational modification) or be due to the interaction with a minor contaminant, a question which cannot be resolved with the techniques presented here. The interaction of calelectrin with Ba^{2+} and lanthanide ions might be of relevance to the unexplained pharmacological actions of these ions in excitatory tissues.

The Ca^{2+} requirements for the membrane aggregation seem to be rather high to be of physiological significance except in the zone of cytoplasm close to the plasma membrane during depolarisation (Abraham-Shrauner, 1975). The rapidity of exocytosis and the fact that only well below 50% of the transmitter store of a cell can be released by extensive stimulation, suggest that only secretory vesicles close to the plasma membrane may be exocytosed. Furthermore, in isolating the protein many associated factors have been removed which could influence the interactions of the protein

with itself and with the cell membrane. In order to appreciate the *in vivo* importance of calelectrin the whole system of proteins will have to be reconstituted step by step and its association with other cytoskeletal proteins characterized.

Calelectrin behaves in many ways like synexin (Creutz *et al.*, 1978, 1979; Morris *et al.*, 1982a), which is also self-aggregated by Ca^{2+} and promotes membrane aggregation, possibly with a higher activity than calelectrin. However, the two proteins are difficult to compare because synexin has never been purified to homogeneity and its activity has been almost exclusively characterized in low ionic strength buffers. Synexin differs from calelectrin in mol. wt. and isoelectric point. Calelectrin is self-aggregated by Ba^{2+} and trivalent lanthanides as well as Ca^{2+}; these ions are known to have prominent effects on many Ca^{2+}-mediated processes *in vivo*, but they are completely ineffective in promoting membrane aggregation in the presence of synexin (Morris and Hughes, 1979). Furthermore, there is a difference between calelectrin and synexin in the kinetics of the potentiated chromaffin granule aggregation and in the mechanism of action, since synexin enhances Ca^{2+}-induced phosphatidylserine liposome aggregation while calelectrin is inhibitory. In this respect calelectrin is similar to calmodulin and prothrombin, two other Ca^{2+}-binding proteins with different physical characteristics, which, however, also inhibit Ca^{2+}-induced phosphatidylserine liposome aggregation (Hong *et al.*, 1982).

Materials and methods

Materials

All chemicals used were reagent grade and used without further purification. All solutions for the isolation of cholinergic synaptic vesicle membranes and for the purification of calelectrin were buffered with 10 mM Tris-HCl, pH 7.4 (at 20°C). All other solutions used were buffered with 10 mM HEPES-KOH, pH 7.4 (at 20°C). All isolation procedures were carried out at 2–4°C.

Isolation of calelectrin

Electric organ tissue (200 g) stored in liquid nitrogen was crushed with a pestle to form a coarse powder to which 200 ml 0.4 M buffered NaCl containing 1 mM $CaCl_2$ were added. The mixture was homogenized in a Waring-type blender for 30 s on the low speed setting and the homogenate centrifuged for 30 min at 39 000 g_{max} to yield a pellet P_1 and supernatant S_1. P_1 was processed as described (Walker, 1982) except that in the last step, instead of using DEAE-cellulose chromatography, it was found possible to freeze-dry the partially purified calelectrin, resuspend the powder in deionized water and remove contaminants by centrifuging at 20 000 g_{max} for 15 min. The supernatant obtained by this treatment was >99% pure as judged by SDS-PAGE.

Isolation of cholinergic synaptic vesicles

Supernatant S_1 from above was centrifuged at 100 000 g_{max} for 2 h and the resulting pellet containing the synaptic vesicles resuspended in 0.8 M buffered sucrose containing 1 mM EGTA. The vesicles were then isolated by flotation gradient centrifugation as described (Carlson *et al.*, 1978). The vesicles formed a light band with a high ATP/protein ratio and with a protein composition very similar to that described (Tashiro and Stadler, 1978). The vesicles were lysed in 10 mM HEPES-10 mM KCl, centrifuged for 2 h at 100 000 g_{max}, and the resulting membrane pellet washed twice before resuspension in 0.4 M buffered KCl.

Isolation of chromaffin granule membranes

Chromaffin granules were isolated as described (Südhof, 1982) and further purified by centrifugation through a cushion of 1.6 M buffered sucrose (30 000 g_{max}) for 20 min in the presence of 1 mM EGTA (Smith and Winkler, 1972). The pure chromaffin granules were lysed in 10 mM HEPES in a large volume, and the membranes pelleted by centrifugation (30 000 g_{max} for 20 min). The particles were washed once by repeating this procedure and the final pellet resuspended in 0.15 M buffered KCl.

Lipid extraction from the electric organ of T. marmorata

Total lipid extraction by chloroform-methanol and further lipid subfractionation of *Torpedo* electric organ lipids were performed as described (Richardson *et al.*, 1982).

Small unilamellar liposome vesicles

These were prepared from bovine brain phosphatidylserine and dimyristoyl phosphatidylcholine as described by Morris *et al.* (1982a) except that they were prepared in 0.15 M buffered KCl.

Membrane aggregation measurements

These were performed by following the turbidities of the suspension as a function of time at 320 nm in a ZEISS PMQ 3 spectrophotometer (Morris *et al.*, 1979b). For cholinergic synaptic vesicle membranes (starting ODs adjusted 0.080) and extracted lipids from *Torpedo* electric organ (starting ODs 0.200), measurements were performed at 25°C in 0.4 M buffered KCl, for chromaffin granule membranes (starting ODs 0.100), chromaffin granule extracted lipids (starting ODs 0.080) and liposomes (starting ODs of phosphatidylserine vesicles 0.080, of phosphatidylcholine vesicles 0.300) at 37°C in 0.15 M buffered KCl. All measurements were performed in 0.5 ml volume, are dilution corrected, and have been repeated at least twice with independent preparations.

Calelectrin self-association

This was measured by 90° light scattering at 400 nm in an MPF4 Perkin Elmer spectrofluorometer. Determinations were made in solutions of the pure protein at different concentrations in 0.15 M buffered KCl at 37°C as a function of metal ion concentration.

Protein determinations

These were carried out according to Bradford (1976).

Acknowledgements

We would like to thank Dr.V.P.Whittaker for many helpful discussions and Dr.P.Feretti for help in extracting the lipids.

References

Abraham-Shrauner,B. (1975) *J. Math. Biol.*, **72**, 333-339.
Bradford,M.M. (1978) *Anal. Biochem.*, **72**, 1188-1199.
Carlson,S.J., Wagner,J.A., and Kelly,R.B. (1978) *Biochemistry (Wash.)*, **17**, 1188-1199.
Creutz,C.E., Pazoles,C.J., and Pollard,H.B. (1978) *J. Biol. Chem.*, **253**, 2858-2866.
Creutz,C.E., Pazoles,C.J., and Pollard,H.B. (1978) *J. Biol. Chem.*, **253**, 2858-2866.
Creutz,C.E., Pazoles,C.J., and Pollard,H.B. (1979) *J. Biol. Chem.*, **254**, 553-558.
Creutz,C.E., and Pollard,H.B. (1982) *Biophys. J.*, **37**, 119-120.
Haiech,J., Devancourt,J., Pecheche,J.-F., and Demaille,J.G. (1979) *Biochemistry (Wash.)*, **18**, 2753-2758.
Haynes,D.H., Kolber,M.A., and Morris,S.J. (1979a) *J. Theor. Biol.*, **81**, 813-843.
Haynes,D.H., Lansman,J., Cahill,A.L., and Morris,S.J. (1979b) *Biochim. Biophys. Acta*, **557**, 340-353.
Hong,K., Düzgünes,N., and Papahadjopoulos,D. (1982) *Biophys. J.*, **37**, 297-305.
Morris,S.J., Chio,V.W.K., and Haynes,D.H. (1979a) *Membr. Biochem.*, **2**, 163-201.
Morris,S.J., Hellweg,M.A., and Haynes,D.H. (1979b) *Biochim. Biophys. Acta*, **553**, 342-350.
Morris,S.J., and Hughes,J.M.X. (1979) *Biochem. Biophys. Res. Commun.*, **91**, 345-350.
Morris,S.J., Hughes,J.M.X., and Whittaker,V.P. (1982a) *J. Neurochem.*, **39**, 529-536.
Morris,S.J., Südhof,T.C., and Haynes,D.H. (1982b) *Biophys. J.*, **37**, 117-118.
Portis,A., Newton,C., Pangborn,W., and Papahadjopoulos,D. (1979) *Biochemistry (Wash.)*, **18**, 780-790.
Richardson,P.J., Walker,J.H., Jones,R.T., and Whittaker,V.P. (1982) *J. Neurochem.*, **38**, 1605-1614.
Smith,A.D., and Winkler,H. (1972) in Blaschko,H., and Muscholl,E. (eds.), *Catecholamines, Handbook of Experimental Pharmacology*, Volume **33**, Springer-Verlag Berlin, FRG, pp. 538-617.
Südhof,T.C. (1982) *Biochim. Biophys. Acta*, **684**, 27-39.
Tashiro,T., and Stadler,H. (1978) *Eur. J. Biochem.*, **90**, 479-487.
Walker,J.H. (1982) *J. Neurochem.*, **39**, 815-823.
Whittaker,V.P. (1980) in Nover,L., Lynen,F., and Mothers,K. (eds.), *Cell Compartmentation and Metabolic Channeling*, Fischer Verlag, Jena, GDR, pp. 509-523.