Vol. 135, No. 1, 1986 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 26, 1986 Pages 323-329

THE INTERACTION OF CALMODULIN WITH RAT LIVER PLASMA MEMBRANE

Monika Gloor and Paolo Gazzotti

Eidgenössische Technische Hochschule Zürich,
Laboratorium für Biochemie, ETH-Zentrum,
Universitästrasse 16, CH-8092 Zürich, Switzerland

Received January 17, 1986

SUMMARY: Rat liver plasma membranes contain relatively high amounts of EGTA-insensitive calmodulin which seems to interact with cytoskeletal proteins. Calmodulin is particularly enriched in a subplasmamembrane fraction containing basolateral membranes. Two calmodulin-binding proteins with apparent Mr of 240 KDa and 145 KDa have been found associated with the purified plasmamembranes.

Calmodulin is an intracellular Ca-binding protein which is involved in the regulation of many Ca-dependent reactions (1). The association of calmodulin with targetting proteins is, in most cases, Ca-dependent and seems to result from a Ca-induced conformational change that expose a hydrophobic surface on the calmodulin molecule (2,3). In some cases, it has been found that calmodulin interacts with proteins in a Ca-independent manner and can not be released even after extensive washing with EGTA (4,5). This type of interaction has been also reported for calmodulin-cytoskelton protein complexes (6,7). Probably the best example of such an interaction is the calmodulin-110KDa protein complex which is involved in the anchoring of actin filaments to brush border membrane (8). We have previously

This paper is dedicated to Prof. C. Martius on the occasion of his 80th birthday.

Abbreviations:

SDS, sodium dodecylsulfate; Hepes, N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; EGTA, ethyleneglycol-bis-(amino-ethylether) N,N,-tetraacetic acid.

0006-291X/86 $1.50

reported that EGTA-insensitive calmodulin interaction is also present in a rat liver membranous fraction enriched in plasma membrane (9). In the present work we have investigated the interaction of calmodulin with purified liver plasma membrane and subplasmamembrane vesicles, with particular emphasis on its association with cytoskeletal proteins.

METHODS AND MATERIALS

Female rats fasted 20 hours were used in this study. The livers were carefully removed and quickly perfused with cold 154mM NaCl. Plasma membranes were isolated as previously described (10) in a medium containing: 1mM $NaHCO_3$, 1mM $MgCl_2$, 0.5mM K-EGTA and 0.3mM PMSF (final pH 7.8). Subplasmamembrane fractions were prepared from the purified plasma membrane using a Polytron homogenizer . The homogenization was carried out twice for period of 5 second at power setting 4. The subplasmamembrane vesicles were separated by centrifugation on a discontinous gradient. Three fraction were collected , PM-1 at first interface (34.5% w/v), PM-2 at the second interface(34.5%-39.5% w/v), PM-3 at the third interface (39.5%-44.5% w/v). The Triton X-100 insoluble fraction was obtained by incubating the plasma membrane vesicles (5mg/ml) in a medium containing 75mM KCl, 5mM Tris-Cl, 1mM K-EGTA, 0.3mM PMSF, 1mM $MgCl_2$, 1% Triton X-100, final pH 7.4. The incubation was carried out for 10 minutes on ice. An insoluble pellet was obtained by centrifuging the suspension at 105,000 g for 40 minutes. Alkaline extraction of the Triton X-100 insoluble pellet dissolved in 250mM sucrose was carried out, as previously described (10), by adjusting the pH of the suspension at 10.5 with 1M NaOH and incubating 45 minutes on ice. The isolation and identification of calmodulin in the different fractions was carried ,as previously described (9). After phenyl sepharose chromatography of a boiled extract, calmodulin was identified by studying the ability of the different fractions to activate the calmodulin-deficient phosphodiesterase. Polyacrylamide gel electrophoresis in SDS was carried using the Laemmli procedure (11). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets was carried out as previously described (12). 125-I-calmodulin overlay of the nitrocellulose sheets saturated with defatted milch powder is described in reference 13. 5'-nucleotidase was measured in the presence of 10 mM tartrate (14). Na/K-ATPase and Mg-ATPase were measured using a spectrophotomoteric procedure (15). Rabbit muscle actin was extracted and purified as previously described (16). Actin was measured using the deoxyribonuclease I-inhibition assay (17).

RESULTS AND DISCUSSION

As shown in Table 1, plasma membrane vesicles prepared in EGTA-buffer contain relatively high amount of calmodulin. No significant increase in the calmodulin content of the plasma membrane was found in vesicles prepared with an EGTA-free

TABLE 1

Calmodulin and actin content of plasma membrane and plasma membrane extracts

Fraction	Calmodulin (μg/mg prot.)	Actin (μg/mg prot.)	Protein (%)
Plasma membrane	1.86 (± 0.32)	42.2 (± 2.6)	100
Triton X-100 insoluble fraction.	8.5 (± 0.5)	82 (± 3.8)	16
Alkaline extract	14.49 (± 1.95)	n.d.*	7.5

*Actin could not be detected because it looses activity during the alkaline incubation.

isolation medium (data not shown). Such a Ca-independent interaction has often been reported with cytoskeletal proteins-calmodulin complexes (6,7). In order to verify if this was also our case, we have treated the purified plasma membrane with 1% Triton X-100, a procedure which solubilize most membrane proteins but not the cytoskeletal proteins. The Triton X-100 insoluble fraction was found to be enriched in calmodulin and actin (Table I). Most of the calmodulin in the pellet could be extracted by alkaline treatment, a procedure which is often used to solubilize cytoskeletal proteins (10). The enrichment in actin content of the different fractions, especially the alkaline extract, is also shown in the gel pattern of Fig.1. Of the other bands enriched in the extract, the 48 KDa and 56 KDa have been previously identify as tonofilaments (10). The presence of calmodulin-binding proteins in the different plasma membrane extracts was investigated using ^{125}I-calmodulin overlay of gel blots. A general behaviour of calmodulin binding proteins is that they mantain their high binding affinity for calmodulin even after SDS-polyacrylamide gel electrophoresis (13). The purified plasma membrane contains two polypeptides with Mr of ca.240 KDa

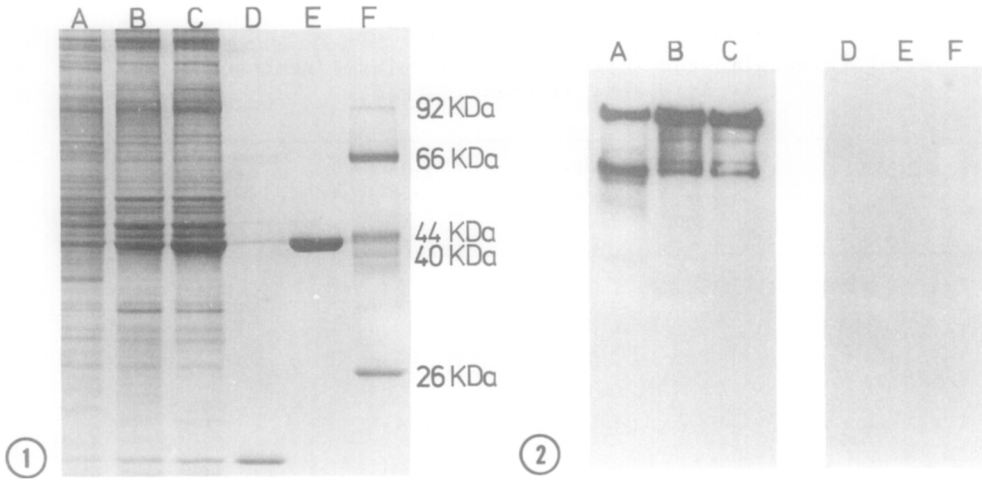

Fig.1: SDS-polyacrylamide gel electrophoresis pattern of plasma membrane extracts. A: plasma membranes; B: Triton X-100 insoluble fraction; C: alkaline extract; D: rat liver calmodulin; E: rabbit skeletal muscle actin; F: Mr standards.

Fig.2: The identification of calmodulin-binding proteins in plasma membrane extracts using ^{125}I-calmodulin overlay of gel blots. A: plasma membrane; B: Triton X-100 insoluble fraction; C: alkaline extract. D-F as A-C with 1mM EGTA in the overlay buffer.

and 145 KDa which can bind the iodinated calmodulin added in the nM range (Fig.2). The 240 KDa protein is concentrated in the fractions contaning the cytoskeletal proteins (Fig.2B and 2C). This protein has the same molecular wight as desmocalmin a calmodulin binding protein which has been recently identified in desmosomes isolated from bovine muzzle epidermis (18). It is therefore possible that our 240 KDa protein is also associated with the desmosomes which are found in liver plasma membrane preparations. Even though the calmodulin associated with the plasma membrane can not be removed by washing with EGTA, we have found that the binding of calmodulin to the blotted proteins occurs only in the presence of Ca (Fig.2). It is quite possible that calmodulin interacts with the native proteins in the plasma membrane both in a Ca-dependent (probably hydrophobic interaction) and Ca-independent (ionic or other types of interaction) manner so that the mere addition of EGTA is not

TABLE 2

Calmodulin content and marker enzymes distribution in subplasmamembrane fractions

Activity measured	Homogenate	Plasma membrane	PM-1	PM-2	PM-3
Na/K ATPase	0.22	40.85	n.d.*	44.1	39.2
Mg-ATPase	0.88	62.5	284	86.3	79.5
5'-nucleotidase	2.7	27.4	186	49.5	42.5
Actin (µg/mg)	n.m.**	48.3	39.5	59.4	32.4
Calmodulin (µg/mg)	n.m.	2.08	1.76	5.48	2.41

The data in the Table are taken from a representative experiment. Experimental variation was within 10% of the data reported. Marker enzyme activities are expressed as µmoles Pi released/hr/mg protein.
*n.d., not detectable.
**n.m, not measured.

enough to dissociate the complex. The Ca-independent interaction would then be lost in the denatured calmodulin binding protein.

We have also investigated the distribution of calmodulin and calmodulin binding proteins in subplasmamembrane fractions enriched in canalicular and basolateral domains. As shown in Table 2, the subplasmamembrane fraction PM-1 has the marker enzyme pattern typical of canalicular membranes, that is, high 5-nucleotidase and Mg-ATPase and no Na-K-ATPase. The enzyme pattern of the PM-2 and PM-3 fractions is typical of basolateral membranes. The subplasmamembrane fraction PM-3 is the most abundant, accounting for more then 70% of the proteins in the three fractions (not shown). As shown in Table 2, the PM-2 fraction is particularly enriched in both calmodulin and actin. This seems to indicate that the PM-2 fraction contains the anchoring site for some cytoskeletal proteins. The autoradiogram of Fig.3 shows that the 240 KDa calmodulin-binding proteins is

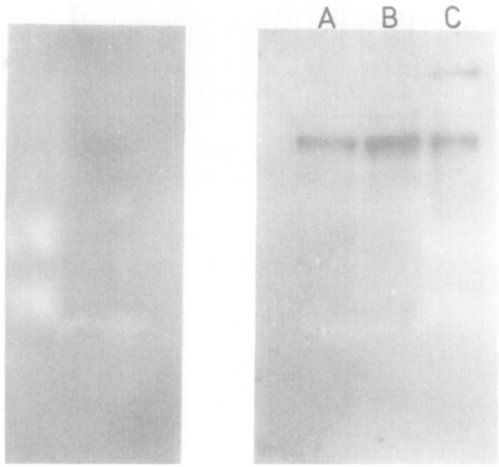

Fig.3: The identification of calmodulin-binding proteins in subplasmamembrane fractions using ^{125}I-calmodulin overlay of gel blots. A: PM-1; B: PM-2; C: PM-3. The autoradiogram of the same gel carried out in the presence of EGTA is shown on the left side of the figure.

found only in the PM-3 fraction. It has been previously reported that this fraction consists, at least in part, of membranous structures connected by junctional complexes (19). Therefore, it is possible that the 240 KDa protein interacts with junctional proteins. Indeed, the presence of calmodulin in gap junctions (20) and desmosomes (18) has been reported. In conclusion, the data reported here strongly suggest an association between calmodulin and cytoskeletal-junctional structures of liver plasma membrane.

ACKNOWLEDGMENTS

We wish to thank Prof. E. Carafoli for encouragement and helpful discussions. This work was supported by the Swiss Nationalfonds (Grant Nr. 3.658-084).

REFERENCES

1. Cheung, W.Y. (1980) Science 207, 19-27.
2. La Porte, D.C., Wierman, B.M., and Storm, D.R. (1980) Biochemistry 19, 3814-3819.
3. Tanaka, T., and Hidaka, H. (1980) J. Biol. Chem. 255, 11078-11080.
4. Cohen, P.A., Burchell, G., Foulkes, P.T.W., Coehn, T.C., Vanaman, and Nairn A.C. (1978) FEBS Lett. 92, 287-293.

5. Derry W.J., Means, A.R., and Brinkley, B.R. (1984) J. Cell Biol. 98, 904-910.
6. Andreasen, T.J, Luetje, C.W., Heideman, W., and Storm, D.R. (1983) Biochemistry 22, 4615-4618.
7. Glenney, J.R., Bretscher, A. and Weber, K. (1980) Proc. Natl. Acad. Sci. USA 77, 6458-6462.
8. Mooseker, M.S., Bonder, E.M., Conzelman, K.A. Fishkind,D.J., Howe, C.L., and Keller III, C.S. (1984) J. Cell Biol. 99, 104s-112s.
9. Gazzotti, P., Flura, M. and Gloor, M. (1985) Biochem. Biophys. Res. Commun. 127, 358-365.
10. Hubbard, A.L. and Ma, A. (1983) J. Cell Biol. 96, 230-239.
11. Laemmli, U.K. (1970) Nature 227, 680-685.
12. Towbin, H., Staehelin,T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350-4354
13. Flanagan,S.D. and Yost, B. (1984) Anal. Biochem. 140, 510-519.
14. Aronson, N.N. and Touster, O. (1974) Methods in Enzymology 31, Part A 90-102, Acad. Press N.Y.
15. Scharschmidt, B.F., Keeffe, E.F., Blankenship, N.M. and Ockner, R.K. (1979). J. Lab. Clin. Med. 93, 790-799.
16. Pardee, J.D. and Spudich, J.A. (1982) Methods in Enzymology 85, 164-181, Acad. Press, N.Y.
17. Blikstad, I., Markey, F., Carlsson, L., Persson, T. and Lindberg, U. (1978) Cell 15, 935-943.
18. Tsukita, S.and Tsukita, S.(1985) J. Cell Biol. 101, 2070-2080.
19. Meier, P.J., Sztul, E.S., Reuben, A. and Boyer, J.L. (1984) J. Cell Biol. 98, 991-1000.
20. Hertzberg, E.L. and Gilula, N.B. (1982) Cold Spring Harbor Symp. Quant. Biol. 46, 639-645.