IDENTIFICATION OF Ca^{2+}- INDEPENDENT CALMODULIN BINDING PROTEINS IN BRAIN MICROSOMES AND SYNAPTIC PLASMA MEMBRANES

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ABSTRACT

The effect of Ca^{2+} and Mg^{2+} on the interaction of calmodulin with brain microsomes and synaptic plasma membranes was investigated. Mg^{2+} increases the binding of calmodulin to both types of membranes, but Ca^{2+} has a more potent stimulatory effect. When both cations are added together to the reaction medium, a superimposed effect is observed, but it is not additive. Cation-dependent binding of calmodulin is specific and saturable with a maximal capacity which is slightly higher in synaptic membranes than in microsomes. (Ca^{2+}, Mg^{2+}) - dependent calmodulin receptors were identified by photoaffinity cross-linking of ^{125}I-calmodulin to the membranes. Furthermore, we also observed that Mg^{2+} alone permits interaction of calmodulin with some proteins of the membranes, although the extent of the process is significantly reduced as compared with that observed in the presence of Ca^{2+}. On the other hand, we found that synaptic plasma membranes, but not microsomes, contain a component (P-57) which does not require either Ca^{2+} or Mg^{2+} to bind calmodulin. The relationship between the calmodulin-receptor systems and the phosphoinositide lipids of the membranes is discussed in terms of the signal transduction at the cellular level.

INTRODUCTION

Calmodulin is the Ca^{2+} receptor which mediates many Ca^{2+} - dependent processes

Key-Words: Calmodulin-binding proteins; synaptic plasma membranes; brain microsomes; signal transduction.

in the cells (1-4). In the presence of this cation, calmodulin interacts with target proteins which are stimulated to produce a physiological response (1,2). Calmodulin has been described as a modulator of various nerve terminal functions , such as neurotransmitter synthesis (5),neurotransmitter release (6-8) and glycogen metabolism (9-10). Thus, calmodulin receptors have been investigated in brain membranes (12-16),but no comparative studies in microsomes and synaptic plasma membranes have been reported.

In this work, we studied the effect of Ca^{2+} and Mg^{2+} on the interaction of calmodulin with both types of membranes. It is reported that similar(Ca^{2+}, Mg^{2+})- dependent calmodulin-binding proteins exist either in synaptic plasma membranes or microsomes, but we observed that these membranes contain different calmodulin receptors which do not require Ca^{2+} to bind calmodulin with high affinity.

MATERIAL AND METHODS

Preparation of calmodulin-depleted synaptic plasma membranes and microsomal membranes.

The synaptic plasma membranes were isolated from sheep brain cortex by a modification[1] of the method previously described (17). Sheep brain cortex was homogenized in 9 volumes of a solution containing 0.32 M sucrose and 10 mM Hepes-Tris (pH 7.4). Then, it was centrifuged for 10 min. at 900 g and the supernatant obtained was centrifuged again at 10,000 g for 20 min.The supernatant was collected and used to prepare microsomal membranes according to the method of Hajós (18),whereas the pellet was utilised to prepare synaptic plasma membranes. Thus, the pellet was lysed in 25 volumes of 5 mM Hepes-K (pH 8.0) and the suspension was centrifuged for 10 min. at 8,000 g. The supernatant was centrifuged again for 30 min. at 35,000 g and the pellet obtained was applied to a discontinuous density gradient solution of dextran T 110 (1.005:1.05 g/ml),which was centrifuged for 2 hr at 60,000 g. Synaptic plasma membranes were collected at the interface of the gradient and they were washed in a solution containing 10 mM Tris-maleate (pH 7.0) and 50 mM KCl.

The endogenous calmodulin was removed by washing twice the synaptic and microsomal membranes with 10 mM Tris-maleate (pH 7.0), 50 mM KCl and 1.5 mM EDTA. Then , they were washed twice with the same solution without EDTA and, finally,they were resuspended in the same medium.

Protein concentration was determined by the biuret method (19).

Preparation of azido - ^{125} I-calmodulin.

Pure bovine brain calmodulin, prepared according to Alface and Pires (20) , was iodinated by the iodogen method (21), and the derivative azido-125 I-calmodulin was performed using methyl-4-azidobenzoimidate reagent, as described by Andreasen et al. (22).

Photoaffinity cross-linking of azido-125 I-calmodulin to microsomes and synaptic plasma membranes.

Membrane proteins (600 µg) were incubated with azido-125 I-calmodulin(10-200 nM) for 30 min.,at room temperature,in a medium containing 25 mM Hepes-Tris (pH 7.2) , 13,5 mM KCl, 10 mM $MgCl_2$ (if present) and 0.7 mM $CaCl_2$ (if present),in a total volu

[1] Gonçalves, P. P., M. S. Santos and A. P. Carvalho (1987). A simple method for isolating synaptic plasma membrane from brain. (Submitted).

me of 400 µl. Photo-irradiation was performed for 8 min. with an U.V.mineralight source type UVS-11, positioned 1.0 cm above the sample. Then, the sample were spun for 5 min. in an Eppendorf microcentrifuge, Model 5412, and the pellets were washed three times with 1,5 ml of a solution containing 10 mM Hepes (pH 7.4), 130 mM KCl, 500 µM $MgCl_2$ and 50 µM $CaCl_2$. Finally, the radioactivity was counted in a gamma counter. Controls were performed by adding 8 mM EDTA to the reaction medium without cations.

Separation of proteins by SDS-polyacrylamide gel electrophoresis.

SDS-polyacrylamide gel eletrophoresis was performed by the method of Laemmli (23). After electrophoresis, the gels were stained with Coomassie brilliant blue R and dried. Then, they were autoradiographed by exposing during one week, at -80°C, to a Dupont Cronex 2Dx-ray film with a Dupont Cronex Lighting - Plus intensifying screen.

RESULTS

Ca^{2+} and Mg^{2+} stimulation of ^{125}I-calmodulin binding to brain membranes.

Calmodulin interacts with brain membranes in a cation-dependent manner. Fig. 1 shows that Mg^{2+} increases the amount of ^{125}I-calmodulin bound to the membranes, but Ca^{2+} has a more potent stimulatory effect. About 1.5 pmol. mg^{-1} protein are

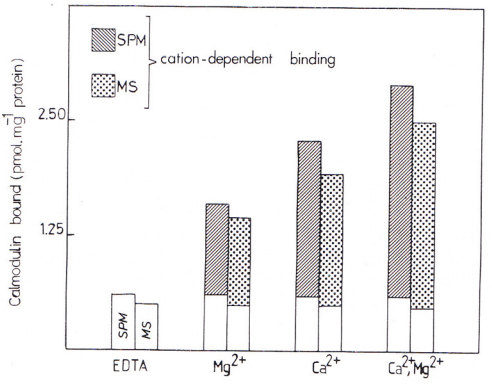

FIGURE 1

Effect of cations on the interaction of azido-125 I-calmodulin with synaptic plasma membranes and microsomal membranes. Brain membranes (600 µg) were cross-linked with azido-125 I-calmodulin (100 nM) in the presence of EDTA (8 mM) or Mg^{2+} + EGTA (10 mM + 8mM) or Ca^{2+}(0,7 mM) or Ca^{2+} + Mg^{2+} (0.7 mM + 10 mM) as described under "Material and Methods". The open bars represent cation-independent binding of calmodulin to the *membranes; the cross-hatched bars represent the (Ca^{2+} + Mg^{2+})* - dependent binding of calmodulin to synaptic plasma membranes (SPM), and the stippled bars represent (Ca^{2+} + Mg^{2+}) - dependent binding of calmodulin to microsomal membranes (MS).

bound by synaptic membranes in presence of Mg^{2+}, whereas in the presence of Ca^{2+}, the binding increases to about 2.3 pmol. mg^{-1} protein. Moreover, when both cations are added together to the reaction medium, the binding of calmodulin is further increased to about 2.75 pmol. mg^{-1} protein. It appears that both cations have synergistic but not additive effects, which suggests that they act by a common mechanism.

Although synaptic plasma membranes and microsomes have a similar behaviour with respect to cation-dependent calmodulin binding, synaptic membranes have a slightly higher calmodulin binding capacity as compared to that of microsomes (Fig. 1).

In the absence of cations, the membranes bind about 0.63 pmol. calmodulin.mg^{-1} protein. However, most of this fraction of calmodulin binding appears to be non specific. It is neither saturable (Fig.2 A and Fig. 3 A) nor displaced by unlabelled calmodulin (data not shown).

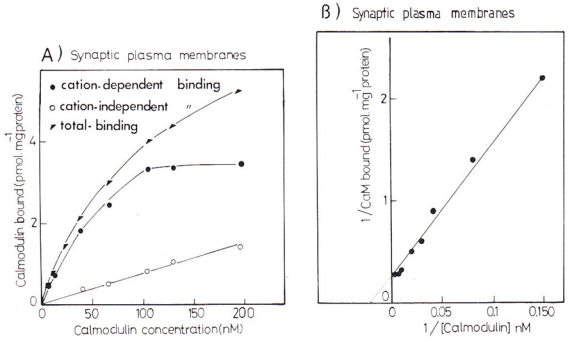

FIGURE 2

Calmodulin binding to synaptic plasma membranes as a function of calmodulin concentration. Increasing amounts of azido-125 I-calmodulin (10--200 nM) were incubated with membrane protein (600 µg) for 1 h at room temperature. After photolysis the extent of calmodulin binding to the membranes was determined as described under "Material and Methods". Cation--dependent binding of calmodulin was obtained by subtracting the binding obtained in the absence of cations from the total binding obtained in the presence of Ca^{2+} and Mg^{2+}. A - calmodulin binding curve; B - Lineweaver-Burk analysis.

In contrast, (Ca^{2+}+ Mg^{2+}) - dependent binding appears to be specific for calmodulin. It is displaced by unlabelled calmodulin and it is saturable at about 100nM of calmodulin concentration (Fig. 2 A and 3 A). Lineweaver-Burk analysis (Fig.2 B) shows that synaptic plasma membranes contain one class of cation-dependent calmodulin receptors which bind calmodulin with high affinity ($K_D \simeq 47$ nM) and a maximal capacity (B_{max}) of about 4 pmol. mg^{-1}. protein. The microsomal membranes (Fig. 3 B) bind calmodulin with similar affinity ($K_D \simeq 48$ nM), although the capacity appears to be slightly lower (3.5 pmol. mg^{-1}. protein) than that of synaptic membranes, which is in agreement with the results depicted in Fig. 1.

Calmodulin binding measurements gave information about the presence of cation-

-dependent calmodulin receptors in brain membranes. However, this type of analysis is not sensitive enough to detect high affinity cation-independent binding, since this fraction is masked by relative high non- specific calmodulin binding.

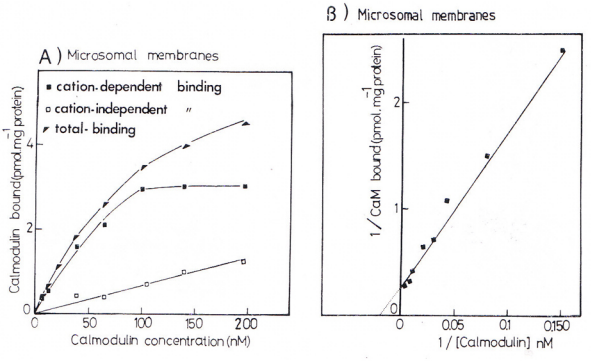

FIGURE 3

Calmodulin binding to microsomal membranes as a function of calmodulin concentration. The experiments were carried out as described in Fig. 2. A - calmodulin binding curve; B - Lineweaver-Burk analysis.

Detection of calmodulin-binding proteins in brain membranes by photoaffinity cross-linking.

By using a photoaffinity cross-linking method (Fig.4) , we could distinguish between three types of calmodulin receptors: Ca^{2+} - dependent, Mg^{2+} - dependent and cation-independent receptors.

In absence of Ca^{2+} and Mg^{2+}, an ^{125}I-containing product is observed in synaptic membranes (Fig.4, lane 2) which corresponds to the formation of a complex between calmodulin (17000 Da)and a protein of about 57000 Da. This component has been found in citosol and in membrane preparations of brain, under conditions of absence of Ca^{2+} and presence of Mg^{2+} (24,25). Although we observed P-57 under these conditions (Fig. 4,lane 4), our results also indicate that P-57 does not require either Ca^{2+} or Mg^{2+} to bind calmodulin (Fig. 4, lane 2).Furthermore , it is detectable only in synaptic membranes (Fig 4 , lanes 2 and 4) and not in microsomes (Fig. 4, lanes 6 and 8).

Most of the calmodulin-binding proteins require Ca^{2+} for the binding (Fig. 4 , lanes 3 and 7), but the effect is increased wether Mg^{2+} co-exists in the reaction medium (Fig 4, lanes 1 and 5).

Concerning the Mg^{2+}-dependent calmodulin binding, we observed that it is particularly evident in microsomes (Fig 4, lane 8), which contain two Mg^{2+} - dependent ^{125}I-containig products (45 kDa and 90 kDa).

It is concluded that similar (Ca^{2+} + Mg^{2+}) - dependent calmodulin receptors exist either in brain microsomes or in synaptic plasma membranes. However, these membranes differ in their content of Mg^{2+} - dependent and cation-independent calmodulin receptors. Mg^{2+} is essentially required for calmodulin binding in microsomes, whereas synaptic plasma membranes contain a calmodulin binding protein which does not require divalent cations.

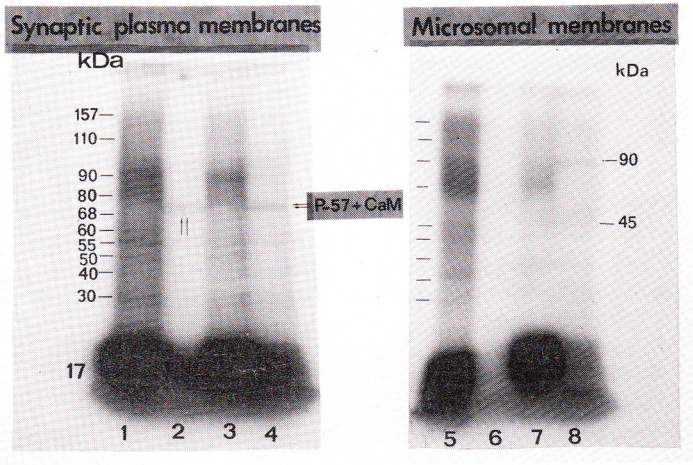

FIGURE 4

Autoradiograms showing (Ca^{2+}, Mg^{2+}) *- dependent and* Ca^{2+} *-independent calmodulin binding proteins in microsomes and synaptic plasma membranes .* The membranes were cross-linked with azido-125 I-calmodulin (CaM) as described under "Material and Methods". Electrophoresis was performed in polyacrylamide gradient (7%-15%) and autoradiography was carried out as described in "Methods". Lanes 1 and 5 ; presence of Ca^{2+} + Mg^{2+}. Lanes 2 and 6 ; absence of cations (8 mM EDTA). Lanes 3 and 7 ; presence of Ca^{2+}. Lanes 4 and 8 ; presence of Mg^{2+}.

DISCUSSION

Synaptic plasma membranes and microsomes bind exogenous calmodulin in a cation--dependent manner. Mg^{2+} stimulates calmodulin binding but Ca^{2+} has a more pronounced stimulatory effect. In presence of both cations, these effects are synergistic but they are not additive (Fig. 1), which indicates that they act by a similar mechanism. It is well established that Ca^{2+} interacts successively with four binding sites of the calmodulin molecule causing various conformers which are recognized by different target proteins (26). Since Mg^{2+} has been described as a low affinity competitive agent for the same binding sites (26,27), it appears reasonable to think that Mg^{2+} stimulates calmodulin binding to its effectors by inducing molecular conformations similar, but not identical, to those of Ca^{2+}. Indeed, the presence of Ca^{2+} seems to be essential for the interaction of calmodulin with most of the target proteins, although a perfect calmodulin activation is observed in the presence of both cations. These results are in agreement with those previously reported by Vandermers et al (28) in brain membranes, Louis et al (29) in lens plasma membranes and Kristensen (30) in erythrocyte membranes.

Under optimal conditions (presence of Ca^{2+} and Mg^{2+}), both types of membranes show one class of high affinity sites which bind calmodulin (Fig. 2 and 3).The fraction of total 125 I-calmodulin binding, which is cation-dependent is saturable and

specific for calmodulin, whereas the cation-independent binding is non specific and not saturable. However, a minor protein of about 57 kDa, detectable in synaptic membranes by photoaffinity cross-linking, appears to be a high affinity calmodulin binding protein which does not require either Ca^{2+} or Mg^{2+} for the binding event (Fig. 4, lane 2). Probaly, this protein corresponds to that previously described as a neurospecific calmodulin-binding protein (24,25,31,32). In contrast with others calmodulin receptors, the affinity of P-57 for calmodulin is lowered by Ca^{2+}. Therefore, it has been proposed that this protein may function to localize and concentrate calmodulin at the membranes surface and release it in response to increases of intracellular Ca^{2+} (24). The process appears to be induced due to phosphorylation of P-57 by protein kinase C (32). It was suggested that this regulatory mechanism serves to decrease the response time for Ca^{2+}-calmodulin-regulated processes (32).

The P-57 has been found in both, membrane and soluble fractions of the bovine cerebral cortex. It has been isolated from heterogeneous membrane preparations (24), but the results reported here indicate that it is essentially localized in synaptic plasma membranes (Fig. 4, lanes 2 and 4).

Synaptic plasma membranes and particularly microsomal membranes contain other calmodulin-binding proteins which bind calmodulin in the absence of Ca^{2+}. However, these proteins require Mg^{2+} for the binding.

Ca^{2+}-independent calmodulin binding proteins appears to be differentially localized in plasmatic and microsomal membranes, whereas Ca^{2+}-dependent calmodulin receptors show a similar pattern in both types of membranes (Fig. 4, lanes 1 and 5).

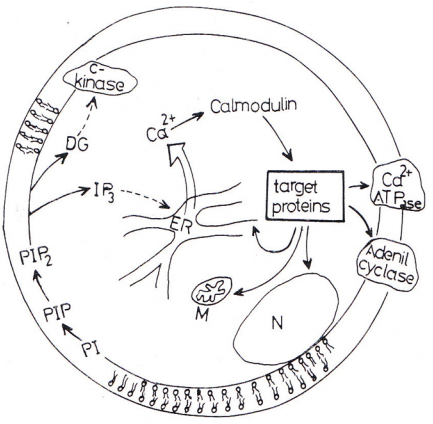

FIGURE 5

Relationship between calmodulin binding proteins and membrane phosphoinositide lipids at the level of cellular transducing signals. Receptor-mediated metabolism of the phosphoinositide lipids produces inositol-trisphosphate (IP_3) which promotes Ca^{2+} release from the endoplasmic reticulum. This cation is bound to calmodulin in the cytoplasm and forms a complexe which is recognized by various calmodulin-receptors whose activity triggers a physiological response.

It is suggested that either Ca^{2+}-dependent or Ca^{2+} - independent calmodulin receptors may account for the equilibrium of the regulatory system of the cells. The first kind of receptors has a physiological role in transmiting the message of Ca^{2+}, whereas the second one has a structural role in facilitating contact between calmodulin and target proteins.

Calmodulin binding proteins are the final step of a transducing signal system which is related with the metabolism of the phosphoinositide lipids of the cellular membrane (Fig. 5).

The signal carried by hormones, neurotransmitters, growth factors, etc, enter into the cell through interaction with specific receptors which promote hydrolysis of phosphatidylinositol 4, 5 - bifosfate (PIP_2) by activation of phospholipase C (33 - 35). The resulting products, diacylglicerol (DG) and inositol 1,4,5 - triphosphate (IP_3), induce stimulation of protein-kinase C and liberation of Ca^{2+} from endoplasmic reticulum, respectively (36, 37). The Ca^{2+} is complexed by calmodulin, changing its conformation, so that, it is recognized by various Ca^{2+} - dependent calmodulin binding proteins which promote a physiological response (1 - 3). On the other hand, protein-kinase C regulates the activity of many enzymes of the cell machinery (38), as well as the binding of calmodulin by the Ca^{2+} - independent calmodulin receptors (P-57) (32) as discussed above.

It is concluded that calmodulin binding proteins and phosphoinositide lipids are the extremes of a long sequence of events leading to a cellular response induced by an exogenous signal.

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