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CALMODULIN REGULATION OF THE ATP-DEPENDENT CALCIUM UPTAKE BY INVERTED VESICLES PREPARED FROM RABBIT SYNAPTOSOMAL PLASMA MEMBRANES

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Calmodulin has been shown to activate the ATP-dependent Ca^{2+} uptake in inside-out vesicles which have been prepared from rabbit synaptosomal plasma membranes by the methodology of Gill et al. (Gill, D.L., Grollman, E.F. and Kohn, L.D. (1981) J. Biol. Chem. 256, 184–192). Following extensive washings of these membranes with EGTA/EDTA solutions, the Ca^{2+} uptake activity demonstrated an affinity for calmodulin of 30 nM and an affinity for Ca^{2+} of 2 μM. The activity was completely inhibited by the anticalmodulin compound R24571 ($K_i \simeq 8$ μM). The molecular weight of the ATPase molecule, revealed by a combination of the [^{125}I]calmodulin overlay technique and [^{32}P]phosphoenzyme electrophoresis, was 145000. The overlay technique also revealed that the mechanism of activation is via a direct binding of calmodulin to the pump molecule.

Introduction

Internal calcium concentration in virtually all cells are maintained at low levels by the action of (Ca^{2+} + Mg^{2+})-ATPases which actively extrude Ca^{2+} at the expense of ATP hydrolysis. These so-called Ca^{2+} pumps have been extensively characterized in certain cases such as the erythrocyte [1] and heart sarcolemma [2]. In each example calmodulin has been shown to activate the pump molecule by directly binding to it, and increasing its V_{max} and K_m for Ca^{2+}. Other specialized cells such as rabbit neutrophils [3] and pancreatic islet-cells [4] are reported to also possess a Ca^{2+}-extruding activity and (Ca^{2+} + Mg^{2+})-ATPase, respectively, which is stimulated by calmodulin, suggesting this may be a general feature of plasma membranes.

The Ca^{2+}-uptake properties of inside-out vesicles prepared from synaptosomal plasma membranes have been extensively characterized [5]. A calmodulin-stimulated (Ca^{2+} + Mg^{2+})-ATPase activity has also been reported in a synaptosomal preparation [6]. In this study, the calmodulin dependence of the Ca^{2+}-uptake property is reported, and direct interaction of calmodulin with the pump molecule demonstrated by a combination of electrophoretic techniques.

Experimental procedures

Preparation of synaptosomal plasma membrane vesicles. Synaptosomes were isolated from rabbit forebrain by the method of Booth and Clark [7]. Synpatosomes were then lysed by osmotic shock, the plasma membranes pelleted (40000 × *g*, 10 min) and suspended into 0.32 M sucrose/1 mM $MgSO_4$/0.5 mM K-EDTA/5 mM Tris-sulfate, pH 7.4, essentially by the methodology of Gill et al.

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Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid.

[5]. This suspension, at a concentration of about 10 mg protein/ml, was frozen in liquid nitrogen and stored at −80°C. The Ca^{2+}-uptake properties of the inside-out plasma membrane vesicles produced by the methodology were essentially as described [5]. Endogenous calmodulin was depleted from the plasma membrane fraction by the following procedure. Prior to storage, the pellet was washed three times with 1 mM Tris-sulfate, pH 7.4/1 mM K-EDTA/1 mM K-EGTA, followed by two washings with the same buffer minus K-EGTA. The membranes were then resuspended at a concentration of 10 mg protein/ml in the storage buffer and frozen in 100 μl aliquots.

Ca^{2+} uptake. A thawed aliquot of plasma membrane was resuspended into 3 ml of the Ca^{2+}-uptake buffer (0.2 M sucrose/0.1 M KCl/20 mM Hepes, pH 7.5/2.5 mM $MgCl_2$/10 μM $CaCl_2$/0.1 mM dithiothreitol/0.1 mg · ml^{-1} bovine serum albumin. After equilibration at room temperature for 20 min, the suspension was centrifuged (40000 × *g*, 15 min) and the pellet resuspended into the same buffer. Aliquots of this stock solution of the plasma membrane (kept on ice) were diluted to a concentration of about 0.2 mg protein/ml in 0.5 ml of Ca^{2+}-uptake buffer and maintained with stirring in a thermostated bath (30°C). $^{45}CaCl_2$ was added such that 100 μl was equivalent to about 300000 cpm. After 5 min, a 100 μl aliquot was removed, diluted into 5 ml of ice-cold 0.15 M KCl/0.2 M sucrose/5 mM Hepes, pH 7.5 and rapidly filtered (Millipore EGWP, 0.5 μm). The filter was washed three times with the same solution (15 ml total) and removed for scintillation counting. One min after the removal of the first aliquot, ATP was added to yield a final concentration of 0.5 mM. At timed intervals, 100 μl aliquots were then removed for filtration and counting to determine the ATP-dependent Ca^{2+} uptake.

In some experiments 0.1 mM EGTA was included in the Ca^{2+}-uptake solution, as well as various additions of $CaCl_2$. The corresponding free Ca^{2+} concentrations were calculated using the EGTA stability constants [8].

[^{125}I]Calmodulin overlay. This technique was performed by a modification of that previously published [9]. Briefly, following SDS electrophoresis (5–15% polyacrylamide gradient) of 150 μg membrane protein, the gel was washed four times with 10% acetic acid/25% isopropanol (250 ml, 30 min) to remove SDS. After rinsing twice with water (5 min), the gel was incubated for 30 min in 250 ml of renaturation buffer (50 mM Tris-HCl, pH 7.5/0.15 M NaCl/20% (v/v) glycerol/1 mM $MgCl_2$/1 mM dithiothreitol/0.1 mM EDTA/0.1 mg · ml^{-1} bovine serum albumin) plus 6 M guanidinium chloride. The gel was then washed with four changes of renaturation buffer during 12 h, and then for 2 h in renaturation buffer which had been altered by adding 1 mM $CaCl_2$, increasing the bovine serum albumin to 10 mg/ml and removing glycerol. The gel was then incubated 12 h with ^{125}I-labeled calmodulin (1 μg/ml, 100000 cpm/pmol) in renaturation buffer which contained 1 mM $CaCl_2$ and from which bovine serum albumin and glycerol had been deleted. Unbound ^{125}I-labeled calmodulin was removed by washing the gel four times during 12 h with 0.15 M NaCl/50 mM Tris-HCl, pH 7.5/1 mM $MgCl_2$/1 mM $CaCl_2$/1 mM dithiothreitol. The gel was then dried under vacuum and submitted to autoradiography to visualize the calmodulin-binding bands. In some cases, the gel was fixed and stained with Commassie blue prior to autoradiography in order to observe the molecular weight marker proteins which had been electrophoresed in adjacent lanes. This procedure differs from that previously published [9] by the inclusion of the guanidinium chloride step and some components of the renaturation buffer, notably glycerol, dithiothreitol, $MgCl_2$, EDTA. Also the concentration of bovine serum albumin has been increased. These components have been shown to aid in enzyme renaturation following SDS electrophoresis [10]. Guanidinium chloride treatment also increases the efficiency of enzyme renaturation [11], presumably because the protein molecules are completely unfolded by this agent, as compared to their conformation following SDS removal.

A significant increase in bound ^{125}I-labeled calmodulin was observed following the procedure, as compared to the original methodology of Carlin et al. [9]; however, no new calmodulin-binding proteins were revealed by the altered procedure (data not shown).

Electrophoresis. SDS polyacrylamide gel electrophoresis was performed as described by Laemmli [12]. Electrophoresis of the phosphoenzyme at 4°C

was performed essentially by the method of Weber and Osborn [13] on a 7.5% acrylamide gel except that the phosphate buffer was neutralized with Tris-base and lithium dodecyl sulfate was substituted for sodium dodecyl sulfate (SDS). In this manner, precipitation of the detergent at the low temperature was avoided. Membrane samples (150 μg) were solubilized in 5 M urea/5% SDS/0.5 M dithiothreitol/50 mM Tris-HCl, pH 7.0, with brief heating (60°C, 10 s). For solubilization of the phosphoenzyme, lithium dodecyl sulfate was substituted for SDS, and Tris-phosphate, pH 7.0 for Tris-HCl.

[^{32}P]Phosphoenzyme preparation. Erythrocyte or synaptosomal plasma membranes (150 μg) were suspended in 100 μl of 0.1 M KCl/2.5 mM $MgCl_2$/0.1 mM dithiothreitol/20 mM Hepes, pH 7.5/0.1 mg · ml^{-1} bovine serum albumin/0.1 μM calmodulin and twice freeze-thawed to ensure complete accessibility of substrates. $CaCl_2$ (2 μM) or EGTA (2 mM) were also present. Following preincubation for 5 min at room temperature, the suspensions were chilled on ice for 5 min and [^{32}P]ATP (10000 cpm/pmol) added to a final concentration of 6 μM. After 15 s the reaction was quenched by the addition of 2 ml of ice-cold 5% (v/v) trichloroacetic acid. The samples were cooled on ice for 5 min and then centrifuged to pellet the proteins. The supernatant was carefully drawn off and the pellet suspended in 0.5 ml of either 0.6 M sodium acetate, pH 5.2 or 0.6 M hydroxylamine, pH 5.2. After 15 min at room temperature, 1.0 ml 15% (v/v) trichloroacetic acid was added and the protein pellet collected as described before. This pellet was dissolved as described in the 'electrophoresis' procedures.

Miscellaneous procedures and methods. Protein was determined by the method of Spector [14] using IgG (Bio-Rad) as a protein standard. Erythrocyte ghosts were prepared by the method of Blostein [15]. Calmodulin was purified from ram testis as previously reported [16] and iodinated by the iodogene method [17].

Calcium contamination in all solutions used for Ca^{2+} uptake was determined by atomic absorption spectrophotometry. ATP was neutralized with Tris base to pH 7.4 before use. [γ-^{32}P]ATP (3000 Ci/mmol) and [^{125}I]iodine (13–17 Ci/mg) were obtained from Amersham. $^{45}CaCl_2$ (50 Ci/g) was obtained from NEN. The compound R24571 was a generous gift of Dr. H. Van Belle, Janssen Pharmaceutical Research Laboratories, B-2340 Beerse, Belgium. Polyacrylamide gel electrophoresis reagents were from Bio-Rad and Serva. All other chemicals were of analytical grade.

Results

Calcium uptake

In the absence of added ATP, only a slight uptake or binding of Ca^{2+} was detected, which did not increase with time following the 5 min equilibration period. Therefore an aliquot was removed for filtering 1 min before addition of ATP, to serve as a blank for nonspecific $^{45}Ca^{2+}$ uptake. Following addition of ATP to 0.5 mM, Ca^{2+} uptake was greatly stimulated, as previously demonstrated by Gill et al. [5]. This uptake was sensitive to the presence of exogenous calmodulin, provided the membranes had been washed with EGTA prior to freezing. Without the EGTA wash procedure, no stimulation of uptake by added calmodulin was observed. In some experiments, the EGTA wash was performed after frozen storage of the membrane, immediately prior to Ca^{2+} uptake measurements. A much decreased stimulation of uptake by exogenous calmodulin was detected in this case.

The dependence of Ca^{2+} uptake upon the concentration of exogenous calmodulin is shown in Fig. 1. This curve reveals that the affinity of the Ca^{2+} pump for calmodulin is about 30 nM at a free Ca^{2+} concentration of 10 μM. In Fig. 2 the dependence of the Ca uptake upon the concentration of free Ca^{2+} is shown. Both Ca^{2+} uptake activities appear to possess a similar affinity for Ca^{2+}, about 2 μM. The stimulation by calmodulin is reflected by an increase in the velocity of Ca^{2+} uptake.

Fig. 3 demonstrates the inhibition of Ca^{2+} uptake by an anticalmodulin drug, the compound R24571 [18]. Both the calmodulin-stimulated and basal Ca^{2+}-uptake activities are inhibited in parallel by this compound; the K_i is about 8 μM.

Calmodulin overlay

^{125}I-labeled calmodulin binding to synaptosomal membrane proteins following SDS-polyacrylamide gel electrophoresis is presented in Fig.

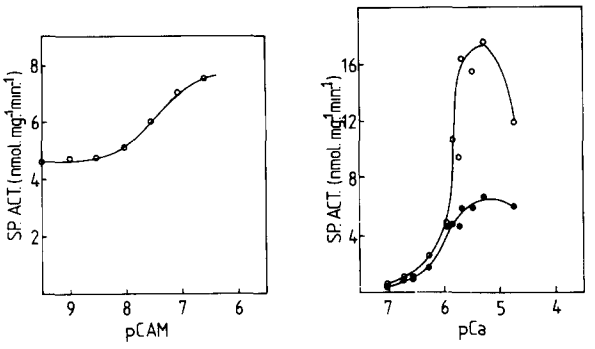

Fig. 1. The dependence of Ca^{2+} uptake upon the concentration of added calmodulin is shown. The Ca^{2+} uptake was determined as described in Experimental Procedures, with a free Ca^{2+} concentration of 10 μM. The specific activity was calculated from the initial rate of Ca^{2+} uptake.

Fig. 2. The dependence of Ca^{2+} uptake upon the concentration of Ca^{2+} is shown, in the presence (○) and absence (●) of 20 μM calmodulin. The free Ca^{2+} concentrations were calculated after the addition of 0.1 mM EGTA to the Ca^{2+}-uptake solution, as described in Experimental Procedures.

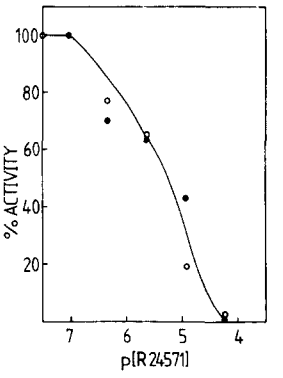

Fig. 3. The degree of inhibition of Ca^{2+} uptake as a function of the concentration of the compound R24571 is shown, in the presence (○) and absence (●) of 20 μM calmodulin.

4. For comparison, erythrocyte membranes are shown in an adjacent lane. At least four major calmodulin-binding proteins are present in the brain membranes, as well as a number of minor components. In contrast only one calmodulin-binding protein is indicated in the erythrocyte membrane. The molecular weight of this band, 145 000, is similar to that of the previously characterized calmodulin-binding erythrocyte $(Ca^{2+} + Mg^{2+})$-ATPase (140 000) [19]. A much more intense band of similar molecular weight is found in the synaptic membrane. It is important to point out that although the large difference in intensity between the two bands may reflect a different concentration of the Ca^{2+} pump in each membrane, the difference could also arise from a different efficiency of renaturation. The other intense calmodulin-binding bands correspond to proteins of molecular weight 250 000, 62 000 and 51 000.

These proteins may be tentatively identified on the basis of their molecular weight. The 250 kDa protein is probably calspectin, a spectrin-like calmodulin-dependent actin binding protein [20]. The calmodulin-binding subunit of calcineurin, recently described as a calmodulin-dependent protein phosphatase [21], is a 60 kDa protein [22]. The 51 kDa protein probably corresponds to a mjaor component of the post-synaptic density [8] which is a likely contaminant of this preparation. One or more of the other bands likely represents a calmodulin-dependent protein kinase, which has also been described in the post synaptic density [23].

Calmodulin binding to all bands was completely inhibited when 1 mM EGTA or 1 mM chlorpramazine were included in the ^{125}I-labeled calmodulin overlay solution, verifying the specific nature of this interaction.

In order to unambiguously determine the molecular weight of the $(Ca^{2+} + Mg^{2+})$-ATPase, for comparison with that indicated by the calmodulin overlay experiment, the membranes were incubated with [γ-^{32}P]ATP under conditions known to cause an accumulation of the transient phosphorylated intermediate (see Experimental Procedures). Autoradiography of the electrophoretic results allowed the molecular weight of the proteins phosphorylated under these conditions to be determined (Fig. 5). Again, erythrocyte membranes were included for comparison purposes. EGTA was included in some reaction mixtures to inhibit the Ca^{2+}-dependent phosphorylation; also, subsequent treatment with hydroxylamine allowed the more stable phosphoserine and phosphothreonine bands to be discerned. Several phosphorylated bands are found in erythrocyte and synaptosomal plasma membrane with the predicted properties of the $(Ca^{2+} + Mg^{2+})$-ATPase. That is phosphate incorporation is stimulated by Ca^{2+}, and the phospho-band is degraded by subsequent

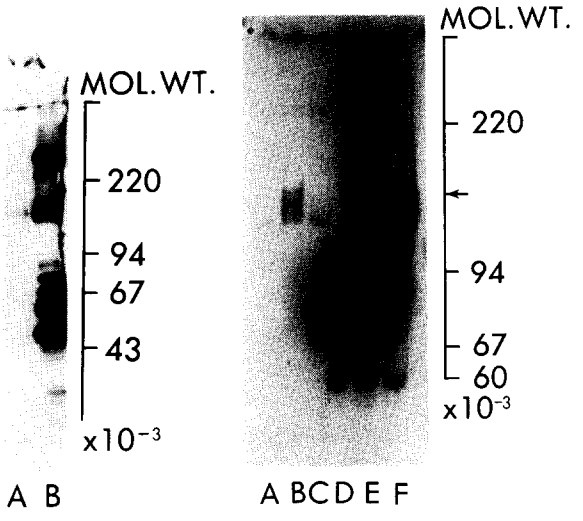

Fig. 4. ^{125}I-labeled calmodulin overlay. The autoradiogram reveals calmodulin-binding proteins in erythrocyte membranes (lane A) and synaptosomal plasma membranes (lane B). The molecular weight scale was determined with the following marker proteins (Pharmacia) as revealed by Coomassie blue staining: ferritin, 220000; phosphorylase *b*, 94000; bovine serum albumin, 67000; catalase, 60000; lactate dehydrogenase, 36000; carbonic anhydrase, 30000. For clarity several of these markers have been omitted from the figure.

Fig. 5. [^{32}P]Phosphoenzyme electrophoresis. The autoradiogram reveals phosphorylated protein bands in erythrocyte membranes (lanes A–C) and synaptosomal membranes (lanes D–F). The experimental conditions were as follows: lanes A, D: 2 mM EGTA; lanes B, E: 2 μM $CaCl_2$; lanes C, F: 2 μM $CaCl_2$, followed by treatment with 0.6 M hydroxylamine. Sample preparation is detailed in the Experimental Procedures. The molecular weight markers were the same as in Fig. 4.

hydroxylamine treatment. The highest molecular weight indicated in each preparation is 154000, similar to that determined in the calmodulin overlay experiment (145000). Bands of lower molecular weight with similar properties in the synaptosomal membranes may indicate proteolytically degraded (Ca^{2+} + Mg^{2+})-ATPase molecules, as their intensity was observed to increase at the expense of the 154 kDa band in aged preparatoins (date not shown). It is possible that the 140 kDa band, not inhibited by EGTA, represents an active Mg^{2+}-ATPase. A similar band is seen in the erythrocyte preparation. Phosphorylated bands which are not degraded by hydroxylamine treatment (90 and 60 kDa), represent proteins phosphorylated on serine or threonine residues, indicating the presence of protein kinase activity which is not Ca^{2+} dependent.

Discussion

In synaptosomal plasma membranes, Ca^{2+} uptake into inside-out vesicles is stimulated by calmodulin, provided endogenous calmodulin has been depleted by EGTA washes. A calmodulin-stimulation of 2–3-fold was obtained (Figs. 1 and 2) in contrast to results with erythrocyte membranes where the calmodulin stimulation of Ca^{2+}-uptake by inside-out resealed ghosts is about 5-fold [24]. This difference appears to arise from the elevated basal Ca^{2+} uptake (in the absence of added calmodulin) measured in the synaptosomal preparation. Studies with the erythrocyte Ca^{2+} pump have indicated that a certain basal activity is an intrinsic property of the enzyme, since it is also observed when the (Ca^{2+} + Mg^{2+})-ATPase is isolated by calmodulin-affinity chromatography and is, by definition, calmodulin-free [1]. However, the calmodulin-free erythrocyte enzyme has a decreased affinity for Ca^{2+}, as well as a decreased V_{max}. Therefore, the basal Ca^{2+}-uptake activity and small calmodulin-stimulation observed with the synaptosomal preparation likely represents a certain portion of the pump molecules which retain tightly bound calmodulin throughout the EGTA washes, since Ca^{2+} uptake in the presence and absence of added calmodulin have a similar calcium dependence (Fig. 2). In addition, the calmodulin antagonist R24571 inhibits both the basal and calmodulin-stimulated activities in parallel (Fig. 3). Although the calmodulin-free activity of the erythrocyte Ca^{2+} pump is also inhibited by R24571, the K_i is 28-times greater than that for inhibition of the calmodulin-stimulated activity [24]. Such a biphasic inhibition pattern was not observed with the synaptosomal preparation.

The affinity of the synaptosomal plasma membrane Ca^{2+} pump for calmodulin was found to be 30 nm, at a free Ca^{2+} level of 10 μM. Although the apparent affinity of calmodulin for the pump may be Ca^{2+}-dependent, as it is in the erythrocyte [25] at this concentration of free Ca^{2+}, the increase in affinity would be near maximal.

Although these first results demonstrate the

stimulation of Ca^{2+} pump activity by calmodulin, the nature of this activation remained unclear. In order to detect a direct interaction of calmodulin with the pump molecule, as opposed to an indirect activation via a calmodulin-dependent protein kinase as observed in the heart sarcoplasmic reticulum [26], the calmodulin gel overlay and [^{32}P]phosphoenzyme electrophoretic experiments were done. Taken together, these results suggest that indeed, the (Ca^{2+} + Mg^{2+})-ATPase interacts directly with calmodulin; since the molecular weight of the (Ca^{2+} + Mg^{2+})-ATPase in both erythrocyte and synaptosomal plasma membranes corresponds (given the different electrophoretic conditions) to that of a major calmodulin-binding protein in each source. In addition, this molecular weight (145 000) is similar to that previously determined for the purified erythrocyte (Ca^{2+} + Mg^{2+})-ATPase (140 000) [19]. These properties of the synaptosomal plasma membrane (Ca^{2+} + Mg^{2+})-ATPase are also similar to those of the heart sarcolemmal enzyme [2] suggesting that perhaps the calmodulin-binding, 145 kDa (Ca^{2+} + Mg^{2+})-ATPase is an intrinsic marker enzyme of most, if not all, plasma membranes. In fact, utilizing antibodies directed toward the erythrocyte (Ca^{2+} + Mg^{2+})-ATPase, Verma et al. [27] have demonstrated significant cross-reactivity with Triton X-100 extracts from rat corpus luteum plasma membranes and rat brain synaptic plasma membranes.

Robinson [28], in a Ca^{2+}-phosphorylation experiment similar to that described here, obtained a molecular weight for the (Ca^{2+} + Mg^{2+})-ATPase from rat brain microsomes of 100 000. Although this observation could correspond to one of the lower molecular weight bands observed in Fig. 5, the lack of any higher molecular weight (Ca^{2+} + Mg^{2+})-ATPase in his preparation would suggest that a major proteolysis problem existed.

While this manuscript was in preparation, Hakim et al. [29] reported the isolation of the solubilized synaptosomal plasma membrane (Ca^{2+} + Mg^{2+})-ATPase from rat brain, utilizing a calmodulin-affinity column. Besides demonstrating the direct calmodulin binding to the pump molecule reported here, the authors also determined the molecular weight of their purified enzyme to be 138 000, a value similar to our results. And finally, based on cross-reactivity between the synaptosomal molecule and antibodies directed against the erythrocyte (Ca^{2+} + Mg^{2+})-ATPase, these workers suggested that a high degree of homology exists between the Ca^{2+} pumps from the two sources.

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