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Identification and primary structure of a calbindin 9K binding domain in the plasma membrane Ca^{2+} pump

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Bovine calbindin 9K has been conjugated to a bifunctional, photoactivatable cleavable and radioactive cross linker. It has been photolyzed in the presence of preparations of the purified erythrocyte Ca^{2+} pump, and shown to interact with it in the presence of Ca^{2+}. The affinity of the interaction has been studied using the fluorescence enhancement of dansylated calbindin 9K incubated with the synthetic calmodulin binding domain of the pump. Two versions of the domain have been used, one corresponding to its full length (28 residues), one to about 2/3 of it (20 residues). The affinity of the interaction was between 5 and 10 times lower than in the case of calmodulin.

Calbindin, Calcium pump, Calcium transport

1 INTRODUCTION

The Ca^{2+} pump of the plasma membrane is normally assumed to be activated by calmodulin [1,2], but a number of alternative compounds and procedures, some of them possibly physiologically significant, have also been shown to produce activation [3–11]. A recent report has claimed stimulation of the ATPase in rat duodenal basolateral membrane-enriched fractions by the vitamin D-induced Ca^{2+} binding protein calbindin 9K, which is a structural analogue of calmodulin [12]. The stimulation occurred at physiological concentrations of calbindin 9K, was not additive with that given by calmodulin, and was only observed in the absence of EGTA in the incubation medium. Whether calbindin interacted directly with the pump, or whether it bound Ca^{2+} to be delivered preferentially to it was left undecided by these experiments. It could also be mentioned that preliminary experiments on the purified erythrocyte pump have failed to show stimulation by calbindin 9K [12].

The calmodulin binding domain in the erythrocyte ATPase has been identified and sequenced using a photoactivatable, radioactive, cleavable cross-linker conjugated to calmodulin [13]. The domain has a predominance of positively-charged amino acids, and shows propensity to form an amphiphilic helix as in the putative calmodulin binding domains of calmodulin-modulated proteins. Since calbindin 9K is an EF-hand type protein analogous to calmodulin [14] it appeared possible that it activated the pump by interacting with the domain that binds calmodulin. The work described here has tested this hypothesis by conjugating calbindin 9K to the same cross-linker that was used to identify the calmodulin binding domain, and by sequencing the labelled peptide. The results have shown that calbindin 9K indeed interacts with the calmodulin-binding domain of the Ca^{2+} pump.

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Abbreviations CNBr, cyanogen bromide, DCAL, dansyl calbindin, DMF, dimethylformamide, EGTA, ethylene glycol-bis-(β aminoethyl ether) *N,N,N',N'*-tetraacetic acid, Hepes, *N*-2-hydroxyethyl piperazine-*N*-2-ethanesulfonic acid, TFA, trifluoroacetic acid TMA, dithiothreitol, SDS, sodium dodecyl sulphate

2. EXPERIMENTAL

2.1 *Materials*

All reagents used were of the highest purity grade available. All chemicals except those specifically mentioned were purchased from Fluka AG (Buchs, Switzerland). Hepes and EGTA were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The dialysis tubing was purchased from Spectrum Medical Industries (Los Angeles, CA, USA). The radioactive labelled cross-linker (the Denny Jaffe reagent [15,16]) was obtained from New England Nuclear (Newton, MA, USA). Bovine calbindin 9K was expressed in *E. coli* from a synthetic gene encoding the minor form A of calbindin 9K as previously described [17].

2.2 *Methods*

2.2.1 Preparation of dansyl-calbindin

0.45 mg of calbindin 9K were dissolved in 0.9 ml of 20 mM NH_4HCO_3 buffer, pH 7.4. The Ca^{2+}-concentration was adjusted to 5 mM. 13 μl of a stock solution of 1.06 mg/ml dansylchloride (Sigma Chemical Co., about 96%) in acetone were added to the dissolved calbindin (50 μl final dansylchloride concentration). The mixture was left at room temperature for 1.5 h and vortexed every 20 min. Prior to dialysis, the tubing (cut-off size 3500 Da) was incubated in 20 mM

buffer and kept in a 5 mg/ml solution of polyvinylpyrrolidone 40 kDa to prevent absorption artifacts. The latter was dissolved in a small volume of acetic acid to obtain a clear solution. The tubing was soaked for 20 min and rinsed exhaustively in distilled water and 20 mM buffer.

Dialysis was carried out overnight against 3 × 2 litres of buffer. Before and after dialysis the solution was centrifuged to remove dust and solids formed during the reaction. Absorption measurements at 320 nm using a molar extinction coefficient of 3400 M^{-1} cm^{-1} [18] showed about 10% incorporation of dansyl groups into calbindin. 10 µl of this solution (20 mM ammonia buffer, pH 7.5) in 3 ml Hepes buffer were used in each titration experiment. The concentration of dansyl calbindin in all experiments was 165 nM.

A 50 µM solution of dansyl calbindin was used (standardized by amino acid analysis). 10 µl of this solution (20 mM ammonia buffer, pH 7.4) in 3 ml of Hepes buffer was used for one titration experiment (167 nM calbindin).

2.2.2. Peptide synthesis

Peptides C20W (LRRGQILWIRGLNRIQTQIK) and C28W (LRRGQILWIRGLNRIQTQIKVVNISSS) corresponding to a part (C20W) or the entire (C28W) calmodulin binding domain of the plasma membrane Ca^{2+} pump [17] were synthesized on an Applied Biosystems peptide synthesizer model 431 (Foster City, CA, USA) using the Fmoc/tBU strategy with 1-methyl 2-pyrrolidone for coupling and washing according to the standard protocol for the synthesiser. Details of the synthesis, deprotection and purification are described elsewhere [19].

2.2.3. Fluorescence measurements

Fluorescence measurements were performed with a SPEX Fluorolog 1680 (Metochen, NJ, USA) double spectrofluorometer connected to a DM1B coordinator. Quartz cuvettes with a path length of 10 mm and a volume of about 3.0 ml were used. Dilution effects were <3% and the temperature of the sample was kept at 26°C.

The dansyl moiety of calbindin was excited at 340 nm and the peptides were then added. The resolution of the excitation monochromator was set at 8 nM and the samples were only stirred after the addition of the peptides dissolved in double-distilled water. Spectra were recorded from 400 to 550 nm. The titration of the fluorescence enhancement was performed by recording emission at 425 nm. One data point corresponds to fluorescence intensities integrated over a total integration time of 5 s after equilibration of the mixture. For the determination of the affinity constants the data points were calculated according to Stinson and Holbrook [20] since the method is more suitable for the determination of boundary values.

2.2.4. Labelling of calbindin

Calbindin was labelled using a modification of the manufacturers instructions. It was dissolved (1 mg/ml) in a solution containing 0.8 vols DMF and 0.2 vols of 15% TMA in water. The reaction was terminated after incubation for 2 h in the dark at room temperature by addition of a 100-fold molar excess of glycine. Unreacted label was removed on a Sephadex G-25 column in 0.1 M phosphate, pH 7.4. The fractions were stored in the dark at −20°C. The specific activity of the resultant protein (DJ-calbindin) was 10 mCi/mmol.

2.2.5. Cross-linking of the Ca^{2+} pump

The erythrocyte Ca^{2+}-ATPase was isolated essentially as described in [4] in a buffer containing 130 mM NaCl, 20 mM Hepes-NaOH, pH 7.2, 1 mM $MgCl_2$, 2 mM DTT, 0.05% (w/v) Triton X-100 (buffer A). Cross-linking to the ATPase was performed by pre-incubating the DJ-Calbindin with the ATPase for 5 min. The solution was then placed 10 cm in front of a mercury arc lamp with a saturated copper sulphate solution (light path about 2 cm) to filter out light of <300 nm, multiple additions of calbindin being required to raise the labelling efficiency. Cleavage of the calbindin from ATPase was achieved by 2 h incubation at room temperature with three additions of 100 mM sodium dithionite, followed by dialysis against 10 mM phosphate buffer, 1% Triton X-100 and 100 mM dithionite (with 50000 kDa cut off dialysis tubing).

A preparation of labelled ATPase (5 mg) was reduced and alkylated with iodoacetate in 6 M guanidinium chloride as described in [21]. Excess reagent and guanidinium chloride were removed by dialysis against several changes of 1% Triton X-100. Subsequently the ATPase was precipitated with trichloroacetic acid (final concentration 8%) and washed first with acetone/HCl and then with cyclohexane to remove lipids and detergents. The ATPase was solubilized completely by sonication in 70% formic acid and digested with CNBr under nitrogen at room temperature for 24 h in the dark. The reagents were removed by blowing with a stream of dry nitrogen at 40°C to achieve a final volume of 200 µl and then 5 ml of water were added, the procedure being repeated 3 times. The digest (2 ml) was then centrifuged to remove particulate matter and injected onto a HPLC system. The reversed phase buffers used were (A) 0.1% TFA in water, (B) 100% *n*-propanol, (C) 50% acetonitrile and 0.1% TFA in water. Chromatography was carried out using a Machery and Nagel (Oensingen, Switzerland) C 18, 300 Å, 7 µm nucleosil reversed phase (250 × 10 mm) semi preparative column, and a Brownlee Laboratories (Foster City, CA, USA) C-8, 300 Å, 7 µm (30 × 2.1 mm) microbore column. The domain was also isolated by passing the CNBr digest dissolved in buffer A over a micro calmodulin column in the presence of 100 µM Ca^{2+}. The column (bed volume 200 µl in a pasteur pipette) was then washed in the same buffer and the peptide eluted with an 2 M urea solution containing 2 mM EGTA. The peptide was recovered by desalting on the C 8 column.

2.2.6. Protein determination

The protein concentration of the samples was determined with a modified [22] method of Lowry et al. [23], using bovine serum albumin as a standard.

3 RESULTS

3.1 *Cross-linking of calbindin 9K to the isolated erythrocyte Ca^{2+}-ATPase*

DJ-calbindin bound to the ATPase in the presence of µM concentrations of Ca^{2+}, but cross-linked only weakly in the presence of EGTA (Fig. 1). The binding was competitive with calmodulin, which evidently bound to the pump with greater affinity, since it prevented the binding of DJ-calbindin even when the latter was present in a 1000-fold excess (not shown). After labelling with DJ-calbindin the isolated Ca^{2+} pump after modification and digestion as detailed in Section 2 produced a single radioactive peptide. The latter was isolated on the semipreparative HPLC column, purified on the microbore and the micro-calmodulin columns (see Section 2) and sequenced. The HPLC peptide pattern repeated exactly that obtained during the identification of the calmodulin binding domain in the Ca^{2+} pump using DJ-calmodulin [13]. In agreement with this, the sequence obtained

E-L-R-R-G-Q-I-L-W-F-R-G-L-N-R-I-Q-T-Q-I-K

corresponded exactly to that of the calmodulin binding domain of the pump.

3.2. *Interaction of calbindin 9K with the synthetic calmodulin binding domain of the erythrocyte Ca^{2+}-ATPase*

The titration of dansyl-calbindin with the shorter

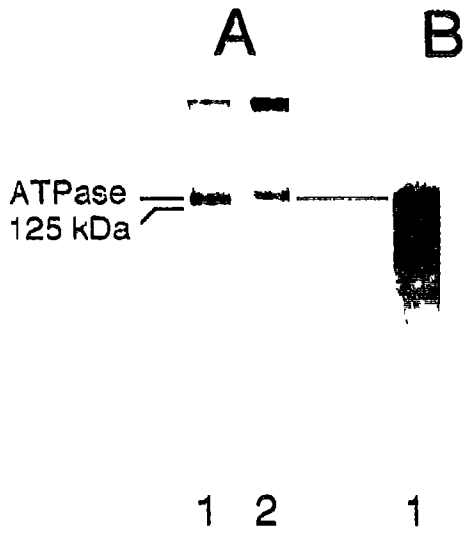

Fig. 1. Interaction of D1 calbindin with the erythrocyte Ca^{2+} pump. The details of the experiment are found in Section 2. Photolysis was carried out for 2 min using 1:1 molar ratio of ATPase to calbindin, and 200 μg of protein were loaded on each lane. Panel A shows a 7% SDS polyacrylamide gel stained with Coomassie blue. Two protein bands are visible: the main band (about 138 kDa) is the intact ATPase, the faintly visible band (about 124 kDa) is a calpain proteolysis product normally seen in purified ATPase preparations. Panel B is the ATPase photolysed together with D1 calbindin (Lane 1) in the presence of 100 μM Ca^{2+}, (lane 2) in the presence of 2 mM EGTA in buffer A (see Section 2).

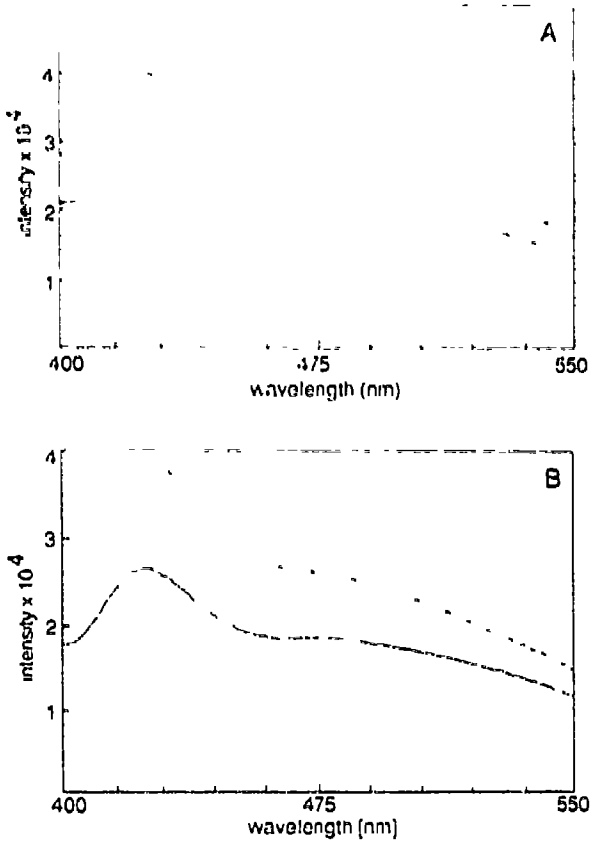

Fig. 2. (A) Emission spectrum of dansyl calbindin after complex formation with peptide C20W in the presence of Ca^{2+}. Excitation was performed at 340 nm. The figure shows the emission spectrum of Ca^{2+}-bound dansyl-calbindin (——) and of the peptide C20W/dansyl-calbindin complex in the presence of Ca^{2+} (– – –). The solution contained 165 nM dansyl-calbindin and 1.7 mM peptide C20W. (B) Emission spectrum of dansyl calbindin after complex formation with peptide C20W in EGTA solution (1 mM). The figure shows the emission spectrum of dansyl-calbindin (——) in EGTA solution, and of the peptide C20W/dansyl-calbindin complex in EGTA solution (– – –). The solution contained 165 nM dansyl-calbindin and 3.4 μM peptide C20W.

synthetic calmodulin binding peptide C20W is shown in Fig. 2. Previous work has shown that the affinity of peptide C20W for calmodulin (i.e. the K_d), although not as high as that of peptide C28W, is still in the nM range [19]. Since C20W is easier to handle, it was used preferentially. The titration was completed in the low μM concentration range: the maximum at 424 nm showed an enhancement factor of 1.4 (Fig. 2A). In the absence of Ca^{2+} the titration with this peptide could not be completed within the experimental limits for the titration, but the spectrum was essentially identical to that recorded in the presence of Ca^{2+} (Fig. 2B), showing an enhancement factor of 1.5 with 3.4 μM peptide. At a peptide concentration of 3.5 μM a small red shift of the maximum was observed. Peptide C28W produced essentially the same results, but the fluorescence enhancement at 424 nm was somewhat smaller (1.3).

3.3 *Determination of the affinity constants for the interaction of calbindin 9K with the synthetic calmodulin binding domain of the erythrocyte Ca^{2+}-ATPase*

Fig. 3A shows plots of the relative fluorescence of dansyl-calbindin incubated with various concentrations of the synthetic calmodulin binding domain in the two versions C28W and C20W, as expected, the interaction was Ca^{2+}-dependent. The points in the titration curve obeyed the equation discussed in Section 2, resulting in a straight line consistent with a 1:1 binding stoichiometry. Fig. 3 shows representative experiments from which K_ds of 17.8 and 46 μM for peptides C28W and C20W, respectively, were calculated. A series of experiments under identical conditions produced values of 20 ± 4 nM and 42 ± 6 nM for peptides C28W and C20W, respectively. Therefore, the affinity of the calmodulin binding peptides for calbindin is up to 10-fold lower than for calmodulin [19].

4. DISCUSSION

The plasma membrane Ca^{2+} pump is responsible for the maintenance of the Ca^{2+} homeostasis in eucaryotic cells. Although the Na^+-Ca^{2+} exchanger may also play a role in the process, and perhaps even take primacy in

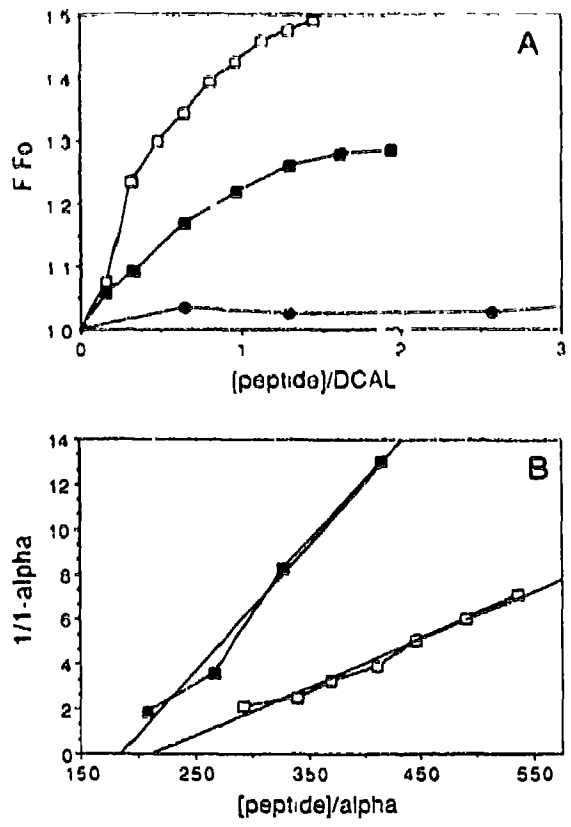

Fig 3 Titration of calbindin with the calmodulin binding peptides (A) Dansyl calbindin (165 nM) was titrated with the calmodulin binding peptides C20W (□—□) and C28W (■—■) in the presence of Ca^{2+} and with peptide C20W in the presence of EGTA (●—●) Excitation was performed at 340 nm The relative fluorescence intensities are plotted against the ratio between the total concentration of peptide and the total concentration of dansyl calbindin, as given by one representative titration experiment The data points were recorded as outlined in Section 2 (B) Titration curves of peptide C20W (□—□) and C28W (■—■) in the presence of Ca^{2+} 165 nM dansyl-calbindin was titrated with the corresponding peptides The data points were recorded and the fractional degree of saturation of dansyl-calbindin (alpha) was calculated as outlined in Section 2 The plot of 1/(1-alpha) against the free concentration of peptide (expressed as nM) divided by alpha results in a straight line if a 1 1 complex is formed and the titration endpoint is correctly estimated The zero intercept on the x-axis refers to the total dansyl-calbindin (165 nM), and serves as a control The reciprocal of the slope gives the affinity constant The plots describe one representative experiment out of 4 titrations The calculated K values were 46 nM for peptide C20W and 18 nM for peptide C28W

it in some tissues, i.e. excitable tissues, it is generally assumed that the Ca^{2+} pump is the prime agent in the ejection of Ca^{2+} during the normal life of most cells. This is clearly a task of vital importance, and it is thus not surprising that the pump should be modulated by the numerous means mentioned in Section 1 Of all these means calmodulin is considered to be the most important in vivo, although acidic phospholipids [24] and kinase-mediated phosphorylation [9] are also rapidly becoming very plausible agents for in situ modulation.

The matter of calbindin is more complex, since stimulation of the pump by it has only been seen so far with preparations of intestinal plasma membranes under special conditions [12]. In addition, preliminary experiments, in this laboratory (J.R Walters, unpublished) have failed to show stimulation of the purified Ca^{2+} pump of erythrocyte by calbindin 9K. Possibly, calbindin stimulation of the pump is specific for the intestine and could reflect the existence of one or more intestine-specific isoforms of the pump [25–29]

The work presented here has shown unambiguously that calbindin 9K interacts with the purified erythrocyte Ca^{2+} pump in the presence of Ca^{2+}, recognising the same domain in the pump which is recognized by calmodulin. The latter competes successfully with calbindin 9K for the binding domain, in line with the finding that the affinity of the synthetic calmodulin binding domain for calbindin 9K is 5–10 times lower than for calmodulin. Thus, unless the affinity of the putative intestinal isoform of the pump for calbindin is much higher, a role of calbindin 9K as an in vivo regulator of the pump would appear unlikely. On this, however, a word of caution is appropriate, since the concentration of calbindin in intestinal mucosa cells could be much higher than that of calmodulin [30]

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