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A novel lipid binding protein is a factor required for MgATP stimulation of the squid nerve Na^+/Ca^{2+} exchanger

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ABSTRACT

Here we identify a cytosolic factor essential for MgATP up-regulation of the squid nerve Na^+/Ca^{2+} exchanger. Mass spectroscopy and Western blot analysis established that this factor is a member of the lipocalin super family of lipid binding proteins of 132 amino acids in length. We named it *Regulatory protein of the squid nerve sodium calcium exchanger* (ReP1-NCXSQ). ReP-1-NCXSQ was cloned, over expressed and purified. Far-UV circular dichroism and infrared spectra suggest a majority of β-strand in the secondary structure. Moreover, the predicted tertiary structure indicates ten β-sheets and two short α-helices characteristic of most lipid binding proteins. Functional experiments showed that in order to be active ReP1-NCXSQ must become phosphorylated in the presence of MgATP by a kinase that is Staurosporin insensitive. Even more, the phosphorylated ReP1-NCXSQ is able to stimulate the exchanger in the absence of ATP. In addition to the identification of a new member of the lipid binding protein family, this work shows, for the first time, the requirement of a lipid binding protein for metabolic regulation of an ion transporting system.

1. Introduction

The Na^+/Ca^{2+} antiporter is a ubiquitous structural plasma membrane protein in charge of exchanging Na^+ and Ca^{2+} ions between the intra- and extra-cellular environments. As such, it is the major protein complex responsible for Ca^{2+} extrusion from most cells in a variety of organisms [1,2]. An important characteristic of this exchanger is that it is highly regulated through a large intracellular loop within the protein. The experimental preparations that have provided most of the information on this point are the mammalian heart (either in its native state or with alien cells expressing the cloned exchanger) and the giant axon and nerve membrane vesicles of the squid [2]. These regulations are related to the existence, on the intracellular "regulatory" loop, of non-transporting Ca^{2+} regulatory sites that must be occupied, in order for any transport mode to take place. This was first described in the squid [3] and later in the mammalian heart [4]. Ionic (H^+_i and $H^+_i + Na^+_i$) inhibition acts by impairing the binding of Ca^{2+} to that site; as expected, their effects are counteracted by increasing $[Ca^{2+}]_i$. In addition, there is a MgATP up-regulation which, by a yet unknown intimate mechanism, protects the Ca^{2+} regulatory site from H^+_i and $H^+_i + Na^+_i$ inhibition [5,6,7].

Up to this point, the experimental data are strikingly similar in mammalian heart and squid nerve. However, a major difference arises regarding the metabolic pathways for MgATP regulation in both species: In the heart, this occurs through the production of phosphatidylinositol-4,5 biphosphate (PtdIns(4,5)P2) [8] that becomes bound to the exchanger [9]. On the other hand, PtdIns (4,5)P2 is ineffective in the squid [7] while MgATP up-regulation requires the presence of a soluble cytosolic protein present in the 30–10 kDa fraction of homogenates from squid axoplasm and optic ganglia. That unknown protein has been called Soluble Cytosolic Regulatory Protein (SCRP) [10,11]. The main findings that characterize SCRP are: (i) No phosphorylation from [^{32}P]ATP was detected in the isolated fraction (lack of autokinase activity) but several bands, including one around 13 kDa incorporated [^{32}P]Pi when squid nerve membrane vesicles were added; i.e. the responsible kinase/s is/are located in the plasma membrane. (ii) Stausosporin, at 50–100 μM, did not prevent the [^{32}P]Pi incorporation into the 13 kDa band nor did it have any effect on MgATP stimulation of the exchanger in dialyzed axons or nerve membrane vesicles. (iii) Heat denaturation of either the cytosolic fraction or the nerve vesicles prevented phosphorylation. (iv) The phosphorylated 30–10 kDa fraction was

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able to stimulate Na^+/Ca^{2+} exchange in nerve vesicles even in the absence of ATP. However, the simultaneous presence of Mg^{2+} was required. These results supported the suggestion that the protein band around 13 kDa was indeed involved in MgATP stimulation of the exchanger [12].

In this paper the 13 kDa band was isolated and subjected to mass spectral analysis. With that information we were able to identify this factor as a lipid binding protein of the lipocalin superfamily. This protein was cloned, expressed, purified and subsequently used for structural and functional studies. The results presented here not only identify a new member of the lipid binding protein family but show, for the first time, the requirement of a lipid binding protein for metabolic regulation of an ion transporting system.

2. Materials and methods

2.1. Identification of ReP1-NCXSQ

We used the 400,000×*g* supernatant of the optic ganglia of the squid from the Marine Biological Laboratory, Woods Hole, MA, USA and the Instituto National de Investigaciones Pesqueras (INIDEP), Mar del Plata, Argentina as starting material. The activity was recovered in a 30 kDa filtrated and was retained in 10 kDa filter. As was indicated in a previous paper [12], the protein that promoted MgATP stimulation of Na^+/Ca^{2+} exchange in squid membrane nerve vesicles remained in the flow-through of an anionic resin (1×8-400 Dowex) and was retained in a cationic resin (50×8-400 Dowex).

Coomassie blue stained SDS-PAGE bands of the ~13 kDa protein from the 30–10 kDa cytosolic fraction were excised and prepared for analysis by mass spectroscopy. Gel slices were digested with 5 mM trypsin diluted in water and subjected to MS/MS mass spectroscopy. Mass spectral data from this band were compared to mass spectral data of protein sequences in the NCBI database (National Library of Medicine, NIH, Bethesda, MD). Four peptides were identified and each of these peptides matched by FASTA search of a recently obtained EST database of squid (*Loligo pealei*). (DeGiorgis et al., manuscript in preparation). Comparison of peptides to sequences in the squid EST database identified a single EST sequence that contains a 396-nucleotide open reading frame and sequences in the 5′ and 3′ untranslated regions.

2.2. Cloning of ReP1-NCXSQ cDNA and construction of expression vector pET28-ReP1-NCXSQ

The synthetic and codon-optimized for *E.coli ReP1-NCXSQ* gene was cloned into the expression vector pET28a(+) using NcoI and XhoI restriction sites (GeneArt, Toronto, Canada).

2.3. Expression and purification of fusion protein 6xHis-ReP1-NCXSQ

A single colony of the *E. coli* strain BL21 transformed with pET28a-ReP1-NCXSQ was inoculated into 2 ml of LB containing 50 μg/ml Kanamycin (Kan) and incubated with shaking at 37 °C overnight. This was used to inoculate 1 l of LB (Kan). When $OD_{600\ nm}$ reached 0.6 the expression was induced by the addition of IPTG (250 μM), and the culture was incubated for an additional 4 h at 22 °C. The cells were harvested by centrifugation at 6000×*g* for 10 min and kept at −80 °C for at least 1 h. The cell pellet was resuspended in Buffer A (100 mM Hepes, 10 mM imidazole, 30 mM NaCl, pH 7.5, together with a cocktail of protease inhibitors. This cocktail included 200 μM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin and 1 μg/ml pepstatin). The cells were then lysed by sonication. The lysate was centrifuged at 65,000×*g* for 30 min and the supernatant collected. One milliliter of HisLink Protein Purification resin (Promega) was washed 3 times with Buffer A, mixed with the supernatant and incubated on a rotator for 2 h at 22 °C. The mix was transferred into a Bio-Rad Poly-Prep column and washed with 20 ml of Buffer A and 20 ml of Buffer B; in this case Buffer B contained 20 mM instead of 10 mM imidazole. The 6xHis-ReP1-NCXSQ was eluted with 5 ml of Buffer C (100 mM Hepes, 250 mM imidazole, 30 mM NaCl, pH 7.5), and 1 ml fractions were collected. The amount and purity of recombinant ReP1-NCXSQ were analyzed by absorbance at 280 nm and 12% SDS-PAGE. The fractions containing the highest amount of protein were dialyzed against 2 l of Buffer D (20 mM Tris-Cl, 30 mM NaCl, 100 mM N-Methyl-$_D$-glucamine, glycerol 10% (v/v), pH 7.3 at RT) and the aliquots kept at −80 °C.

2.4. Preparation of squid nerve membrane vesicles

Membrane vesicles from squid optic nerve (*Sepiotheutis sepioidea* and *Loligo pealei*) were prepared by differential centrifugation as described elsewhere [10,11] and loaded with 300 mM NaCl, 0.1 mM EDTA-Tris and 30 mM Mops-Tris (pH 7.3 at 20 °C).

2.5. Na^+-gradient dependent $[^{45}Ca]Ca^{2+}$ uptake in membrane vesicles

$[^{45}Ca]Ca^{2+}$ uptake in squid membrane vesicles [10,11] was measured at RT by incubating the vesicles (25–30 μg protein) for 10 s in media with high (300 mM) or low (30 mM) Na^+ (100 μl total volume). In addition, all extravesicular solutions contained 0.1 mM vanadate, 20 mM Mops-Tris (pH 7.3 at 20 °C), 0.15 mM EGTA-Tris (pH7.3 at 20 °C), 1 μM Ca^{2+}, 1 mM Mg^{2+} and the ATP concentration indicated in the Figures. In low Na^+ medium the osmolarity was compensated with NMG-Cl. The reaction was stopped with 0.5 ml of an ice cold-solution containing 20 mM Mops-Tris, 300 mM KCl and 1 mM EGTA and filtered through Whatman GF/F glass filters. The filters were washed with 5 ml of the same solution, immersed into 5 ml of scintillation fluid and counted in a liquid scintillation counter. In order to obtain steady counts, after addition of the scintillation fluid the filters were left for 4 h before counting. Each experiment was run in triplicate and repeated at least once.

2.6. Immunoblotting

Squid nerve membrane vesicles, the 30–10 kDa cytosolic fraction of squid optic ganglia and ReP1-NCXSQ were dissolved in Laemmli sample buffer [13], resolved by SDS-PAGE and stained with Coomassie blue or transferred onto polyvinylidene difluoride membranes (PVDF). The membranes were blocked for 60 min at RT (2% BSA (w/v) in TBST), incubated for 2 h with 1:2000 primary antibody (Rabbit affinity purified anti the recombinant lipid binding protein) (Gen Scrip Corp. USA), washed (TBST 5 min×3), incubated in secondary anti-rabbit IgG(H+L) antibody alkaline phosphatase conjugate (Promega) for 1 h, washed (TBST 5 min×5, TBS 15 min×1), and visualized by phosphorescence (ECF, Amersham-GE) with an image analyzer (Storm 840, Molecular Dynamics).

2.7. Protein phosphorylation

Phosphorylation and detection of phosphoproteins were performed as described previously [12]. Briefly, the recombinant lipid binding protein (5–10 μg) or the proteins from the cytosolic 30–10 kD fraction (10–14 μg) were phosphorylated with 0.5 mM (500 cpm/pmol) [^{32}P]-γ-ATP (Perkin Elmer, USA) in the same solution used for Na^+/Ca^{2+} transport experiments. The reaction was terminated by adding 5× Laemmli sample buffer [13] and the proteins were separated by SDS-PAGE (4–20% gradient gels, Invitrogen) and were transferred to PVDF by semidry electrophoretic transfer at 2.5 mA/cm^2 for 40 min. Prestained molecular mass markers were used to avoid staining and destaining of gels prior to imaging and quantification. The incorporation of [^{32}P]Pi into individual protein bands was detected with a Storm 840 Image Analyzer.