A NOVEL LIPID BINDING PROTEIN IS A FACTOR REQUIRED FOR
MgATP STIMULATION OF THE SQUID NERVE Na⁺/Ca²⁺ EXCHANGER.

Graciela Berberiánᵃ,e, Mariana Bolloᵃ, Guillermo Montichᵇ, Gretel Robertsᵃ, Joseph A.
DeGiorgisᶜ,e, Reinaldo DiPoloᵈ,e and Luis Beaugéᵃ,e

From the Laboratorio de Biofísica, Instituto de Investigación Médica Mercedes y Martín
Ferreyra (INIMEC-CONICET)ᵃ, Departamento de Química Biológica (CIQUIBIC-
CONICET), Facultad de Ciencias Químicas, Universidad Nacional de Córdobaᵇ
Laboratory of Neurobiology, Biology Department, Providence College, Providence, RI,
USAᶜ, Laboratorio de Fisiología Celular, Instituto Venezolano de Investigaciones
Científicas, Cararas, Venezuelaᵈ and Marine Biological Laboratory, Woods Hole, MA,
USAᵉ.

Address correspondence to: Luis Beaugé, Instituto M. and M. Ferreyra, Casilla de Correo
389, 5000 Córdoba, Argentina. Fax: 54-351-469-5163; E-mail: lbeauge@immf.uncor.edu

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SUMMARY

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 \(\beta\)-strand in the secondary structure. Moreover, the predicted tertiary structure indicates ten \(\beta\)-sheets and two short \(\alpha\)-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.
INTRODUCTION

The Na⁺/Ca²⁺ antiporter is a ubiquitous structural plasma membrane protein in charge of exchanging Na⁺ and Ca²⁺ ions between the intra- and extra-cellular environments. As such, it is the major protein complex responsible for Ca²⁺ 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 Ca²⁺ 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 Ca²⁺ to that site; as expected, their effects are counteracted by increasing [Ca²⁺ᵢ]. In addition, there is a MgATP up-regulation which, by a yet unknown intimate mechanism, protects the Ca²⁺ 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}\text{P}]\text{ATP} was detected in the isolated fraction (lack of autokinase activity) but several bands, including one around 13 kDa incorporated \[^{32}\text{P}]\text{Pi} when squid nerve membrane vesicles were added; i.e. the responsible kinase/s is/are located in the plasma membrane. (ii) Staurosoporin, at 50-100 μM, did not prevent the \[^{32}\text{P}]\text{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 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.

**MATERIALS AND METHODS**
Identification of ReP1-NCXSQ

We used the 400,000 x 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 Mg.ATP stimulation of Na\(^+\)/Ca\(^{2+}\) exchange in squid membrane nerve vesicles remained in the flow-through of an anionic resin (1x8-400 Dowex) and was retained in a cationic resin (50x 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.

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).

**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<sub>600nm</sub> 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 6,000 x 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 x 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.

**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).

**Na⁺-gradient dependent \[^{45}\text{Ca}\]Ca\(^{2+}\) uptake in membrane vesicles**

[^{45}\text{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 4h before counting. Each experiment was run in triplicate and repeated at least once.

**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 x 3), incubated in secondary anti-rabbit IgG(H+L) antibody alkaline phosphatase conjugate (Promega) for 1 h, washed (TBST 5 min x 5, TBS 15 min x 1), and visualized by phosphorescence (ECF, Amersham-GE) with an image analyzer (Storm 840, Molecular Dynamics).

**Protein Phosphorylation**

Phosphorylation and detection of phosphoproteins were performed as described previously [12]. Briefly, the recombinant lipid binding protein (5 a 10 µg) or the proteins from the cytosolic 30-10 kD fraction (10-14 µg) were phosphorylated with 0.5 mM (500 cpm/pmol) $[^{32}\text{P}]\gamma$-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 $[^{32}\text{P}]$Pi into individual protein bands was detected with a Storm 840 Image Analyzer.

**In vitro Lipid Binding Assays**

Similar to an immunoblot analysis, Phosphoinositide (PtdIns) Lipid Strips, nitrocellulose membranes pre-spotted with various indicated lipid species (P-6002, Echelon, SaltLake
City, UT), were blocked in 3% (w/v) fat-free bovine serum albumin (BSA) for 60 min at RT, probed with the purified 6xHis-ReP1-NCXSQ lipid binding protein (0.5 ug/ml) in 3% (w/v) fat-free BSA in TBST for 1h at RT, followed by primary monoclonal antipolyHistidine Clone His-1 antibody (SIGMA, USA) and then by secondary anti-mouse antibody conjugated with alkaline phosphatase (Promega, USA) and finally ECF (Amersham-GE) detection and imaging (Storm 840, Molecular Dynamics). Unrelated recombinant protein expressed with 6-His flag (6xHis-14-3-3 kindly provided by Prof. Palgrem, Denmark [14] was used as negative control.

**Far UV-CD Circular dichroism**

The measurements were made with a Jasco J-810 spectropolarimeter using a 0.2 cm path length quartz cell. Each spectrum was an average of 6 scans. The protein concentration used was 10 μM. The contribution of the buffer was subtracted in all spectra. Scan speed was set at 50 nm/min, with a 2s response time, 0.2 nm data pitch and 2-nm bandwidth.

**Near UV-CD Circular dichroism**

Measurements were made using a 1 cm pathlength quartz cell. Each spectrum was an average of 6 scans. The protein concentration was 69 μM. The contribution of the buffer was subtracted in all spectra. Scan speed was set at 50 nm/min, with a 2-s response time, 0.2 nm data pitch and 2 nm bandwidth.

**Infrared Spectroscopy**
Infrared spectra were acquired in a Nicolet Nexus spectrometer. For the sample preparation, the protein was concentrated in a Microcon centrifugal filter device and the original buffer prepared with H$_2$O was replaced in the same device with a buffer prepared in D$_2$O. Before the acquisition of the spectra, the protein was incubated 24h at 10°C to allow deuterium exchange of the amide protons. The sample was placed in a demountable cell for liquid samples with CaF$_2$ windows and 70 µm teflon spacers. One hundred scans were collected both for the background and the sample at a nominal resolution of 2 cm$^{-1}$. The spectrometer was flushed with dry nitrogen to reduce the distortions of the spectra due to water vapor.

**RESULTS**

**Identification and primary structure of the cytosolic regulatory protein**

Peptide sequences obtained by mass spectroscopy of the 13 kDa protein band isolated from 30-10 kDa cytosolic fraction of squid optic ganglia were matched against a squid-expressed sequence tag (EST) database containing ~ 23,000 nucleotide sequences (DeGiorgis et al, manuscript in preparation). Four peptides matched the predicted amino acid sequence encoded by a single EST sequence with 100% sequence identify (Figure 1). This EST contains a 405 nucleotide full-length open reading frame and a putative 396 coding sequence that encodes 132 amino acids (Figure 1). By BLAST search of NCBI databases this amino acid sequence matches members of the lipocalin superfamily, a group thought to be transporters of small hydrophobic molecules including steroid hormones, bilins, retinoids and lipids (Figure 1).
The theoretical molecular weight is 14.8 kDa [15] which agrees with that of the SCRP. The theoretical pI of 5.85 coincides with an acidic nature previously described for SCRP [12]. As this protein is essential for MgATP regulation of the squid Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger we decided to name it Regulatory Protein of the Squid Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger (ReP1-NCXSQ). The number 1 implies the possibility of redundancy (other proteins may have a similar function). NCXSQ generalizes for more than one squid nerve exchanger because it was found that it acts at least in *Loligo pealei*, *Doritheutis plei*, *Loligo opalescens* and *Helix argentinus* (not shown here). In addition, Figure 1 shows the amino acid sequence used as antigen to obtain a polyclonal antibody against ReP1-NCXSQ.

**Expression and purification of ReP1-NCXSQ.**

ReP1-NCXSQ was expressed in BL21 *E. coli* and purified obtaining an average yield of 4 mg/L of original culture. The protein patterns during the different steps of the purification procedure are illustrated in Figure 2. Figure 2A displays a 12% SDS-PAGE stained with Coomassie Blue. Lanes 6 to 8 show that columns elution gives a single band at about 13 kDa. Therefore, the purity of the ReP1-NCXSQ in the elution samples can be assessed to be at least 95%. As expected, the single 13 kDa purified band was recognized by the polyclonal peptide antibody raised against ReP1-NCXSQ (Figure 2B).

**Data on secondary and tertiary structure of ReP1-NCXSQ.**

*Far-UV dichroism.* Figure 3A shows the far-UV CD spectra of ReP1-NCXSQ. We observed a broad negative band centered at 215 nm, which is characteristic of proteins with a large content of β-strand in the secondary structure [16]. Another positive band at 195 nm is also expected for proteins with β secondary structure. Because of the strong absorption of
the buffer used in our samples, we were not able to detect this band, although it is clearly suggested from the global spectral shape.

Near-UV dichroism. The anisotropy of the local environment of the lateral chains of amino acids in folded proteins with a defined tertiary structure, determine that the UV absorption of aromatic lateral chains be dichroic [17]. Figure 3B shows the CD spectrum of ReP1-NCXSQ in the aromatic region. Three well-defined groups of bands were observed. The spectral shape was remarkably similar to that observed for the intestinal fatty-acid binding protein (IFABP) [18]. These authors made a detailed study to assign the CD bands that can be used, based on the strong similarity, to propose the origin of the CD spectrum of ReP1-NCXSQ: the sharp bands between 250 and 275 nm can be assigned to the absorption of Phe residues, the peak at 279 to Tyr residues and the double peak above 280 nm to the absorption of Trp residues. The appearance of these highly-structured bands is indicative that the protein is in the native state with a well-defined tertiary structure.

Infrared spectroscopy. The infrared absorbance spectrum of ReP1-NCXSQ is shown in Figure 3C. The amide I’ band, between 1600 and 1700 cm\(^{-1}\), is mainly due to the C=O stretching of the peptide bonds and provides information on the secondary structure of the protein [19]. The spectral shape was similar to that observed for members of the fatty acid-binding proteins from human liver and heart [20], adipocytes [21] and avian liver [22]. We obtained the typical spectrum corresponding to a protein with a high content of \(\beta\)-strand structure. The Fourier self deconvolution [23] shown in the upper trace in Figure 3c, and the second derivative (not shown) enabled the different band components to be identified. Fitting of the components to the original (not deconvoluted) spectrum, performed according to the procedure described by [24] assuming a Gaussian shape, gave six components (Figure 3C). As in the case of avian liver [22], a remarkable feature was the band at 1626
cm$^{-1}$. This component is also observed in several proteins like concanavalin A, lipophilin, a peptide with hairpin structure, apo B-100 and a lentil lectin [22]. It has been assigned to extended $\beta$-chains in which part of the backbone hydrogen bonds are not formed with another $\beta$-chain. The appearance of this band is in agreement with the structure predicted on the basis of the sequence homology. The other bands were assigned according to the classic work of Byler and Susi [19]. The bands centered at 1639 and 1672 cm$^{-1}$ can be assigned to $\beta$-sheet chains. The band at 1651 cm$^{-1}$ is due to $\alpha$-helical structures. The bands at 1662 and 1684 cm$^{-1}$ can be assigned to turns and bends. To a very good approximation, the area under the component bands can be considered proportional to the amount of the corresponding secondary structures present in the protein. The total area of bands corresponding to $\beta$-structures (1626, and 1639 and 1672 cm$^{-1}$) sums 63% of the amide I’ area, while the $\alpha$-helix band corresponds to 18% of the total area.

In summary, the spectroscopy data agree with the expected conformation of a lipid binding protein and, what it more important, indicate that the recombinant protein is correctly folded.

**Biochemical and functional characterization of ReP1-NCXSQ**

*Detection of the native SCRP in cytosolic optic ganglia fraction with antibody raised against the ReP1-NCXSQ.* Figure 4 left side illustrates the protein patterns from the 30-10 kDa cytosolic fraction from squid optic ganglia samples (Lane 1) and nerve membrane vesicles (Lane 2) run on SDS-PAGE and stained with Coomassie blue. The immunoblot experiments, depicted in the right side of Figure 4, shows that a protein band of about 13 kDa of the 30-10 kDa cytosolic fraction (SCRP) reacts with a specific antibody against
ReP1-NCXSQ forming a doublet. The reason for this doublet remains unclear and several possibilities could account for it: unrelated contaminant proteins, other lipid binding protein, different conformation of the same protein, bound ligands and post-translational modifications [25]. The involvement of post-translational changes is supported by the fact that ReP1-NCXSQ expressed in a prokaryote always appears as a single band both with the Coomassie blue stain and when exposed to its specific antibody (Figure 2). Another important observation is that the same antibody failed to detect any protein in squid nerve membrane vesicles. These results strongly suggest that ReP1-NCXSQ is identical to the cytosolic component that promotes the MgATP stimulation of squid nerve Na\(^+/\)Ca\(^{2+}\) exchanger. Finally, it must be pointed out that the antibody against the recombinant protein was based on the amino acids sequence obtained from the squid gene data base. The fact that it detects a native protein in the cytosol of the squid nerve is further evidence that the gene and the protein are one in the same.

**Functional characterization of ReP1-NCXSQ.** In the next step we investigated whether ReP1-SQNCX could promote the MgATP up-regulation of Na\(^+/\)-gradient dependent Ca\(^{2+}\) uptake in squid nerve vesicles. In all cases, parallel experiments were run with native cytosolic fraction containing SCRP [12]. The assays were conducted in the presence of non-saturating, 1 μM extra-vesicular Ca\(^{2+}\) concentration. Figure 5 shows that in the presence of ReP1-NCXSQ Mg-ATP stimulation of the exchanger is similar to that observed with the SCRP. In addition, this figure shows that in both cases the MgATP effect is insensitive to 50 μM Staurosporin, a result already observed in dialyzed axons [2] and isolated nerve membrane vesicles [12].
In squid nerve vesicles MgATP up-regulation of the Na\(^+\)/Ca\(^{2+}\) exchanger coincides with the phosphorylation of the required cytosolic factor (SCRP) [12]. That allowed spotting a phosphorylated band around 13 kDa that was subjected to amino acids sequencing (Figure 1). If the expressed and purified lipid binding protein is indeed ReP1-NCXSQ it must become phosphorylated under conditions of ATP regulation of the exchanger. This point was investigated by running parallel experiments where either the 30-10 kDa cytosolic fraction or ReP1-NCXSQ were incubated for 10 s in the low Na\(^+\) medium used to measure Ca\(^{2+}\) fluxes containing 0.5 mM \(^{32}\)P-\(\gamma\)-ATP together with nerve membrane vesicles. Figure 6A and 6B illustrate typical results where the \(^{32}\)P incorporation in both preparations is similar in amount (Lanes A1 and B1) and in its insensitivity to Staurosporin (Lanes A2 and B2). Furthermore, both phosphoproteins remain stable after the centrifugation step carried out to separate them from the membrane vesicles (Lanes A3 and B3). In addition, as it happens with the SCRP [12] the phosphorylated recombinant ReP1-NCXSQ is able, without the need of ATP, to stimulate the Na\(^+\)-gradient dependent Ca\(^{2+}\) uptake in isolated squid nerve vesicles (Figure 7).

**Interaction of the recombinant ReP1-NCXSQ with lipids.** In mammalian heart MgATP stimulation of the Na\(^+\)/Ca\(^{2+}\)exchanger involves synthesis [8] and binding to the exchanger [9] of plasma membrane polyphosphoinositides, particularly PtdIns(4,5)P2. These interactions were not observed in the squid nerve Na\(^+\)/Ca\(^{2+}\) exchanger [26]. Moreover, the final metabolic path and/or the target for this up-regulation are not known in the squid. On the other hand, in oocytes expressing the squid exchanger, reverse Na\(^+\)/Ca\(^{2+}\) exchange current was stimulated by MgATP through the production of PtdIns(4,5)P2 and by the phosphoinositide itself [27]. Therefore, as ReP1-NCXSQ is a lipid binding protein, it
seemed worthwhile to explore with which membrane associated lipids it interacts. With that aim, nitrocellulose membranes pre-spotted with a variety of lipids, were incubated with the 6xHis-ReP1-NCXSQ recombinant. As a control we used the 6xHis-14-3-3, unrelated recombinant protein also expressed with a 6xHis flag. Figure 8 shows that 6xHis-ReP1-NCXSQ binds polyphosphoinositides such as PtdIns(4)P, PtdIns(4,5)P2 and PtdIns(3,4,5)P3. It also binds to Phosphatidic Acid but it does not seem to do so with Cholesterol, Sphingomyelin, Phosphoserine, Phosphoethanolamide nor to Phosphatidylinositol. The 6xHis-14-3-3 recombinant protein did not attach to any of the lipids investigated.

**DISCUSSION**

The present work shows that ReP1-NCXSQ, essential for the MgATP up-regulation of the squid nerve Na$^+$/Ca$^{2+}$ exchanger, is a new member of the fatty acid binding protein (FABP) multigene family, a distinct and more focused subgroup of the lipocalin supergene family. Comparison (NCBI BLAST) of the ReP1-NCXSQ amino acid sequence with human FABP family members shows 46% sequence identity with Myelin FABP (M-FABP or MP2), 45% with heart (H-FABP) and 44% with Epidermal (E-) FABP [28]. It is not known how many FABP family members are present in the squid, as the genome has not yet been completed. ReP1-NCXSQ contains the six highly conserved residues shared by P2 and other members of the lipid binding family proteins. These residues include amino acids Gly6, Asn15, Tyr20, Ile42, Gly46, Phe64, and Gly67 of MP2 [29].

LPB are cytosolic components of relatively low molecular weight (12-15 kDa) which allow solubilization and transport, within the cells, of long chain fatty acids molecules. These
proteins exhibit structurally conserved β barrel and a binding site formed by four loops linking the β sheets; these loops display high structural plasticity. Their specific functions are, in most cases, not well known; they are related to intracellular compartmentalization and storage of fatty acids, transport of fatty acids into the nuclei modulating the nuclear receptor activity and participating in the intracellular signal transduction systems [30, 31, 32]. In addition, brain LBP’s have been described as activating Na⁺-dependent but not Na⁺-independent uptake of amino acids, a process which seems to be due to the reversion of inhibition produced by oleic acid [33]. This work establishes, for the first time, a role of a LBP in the metabolic regulation of an ion transporting system representing an important step towards understanding structures and mechanisms related to regulation of membrane transporters.

To fulfill its role ReP1-NCXSQ must become phosphorylated. MgATP up-regulation of the squid nerve Na⁺/Ca²⁺ exchanger can be induced, in an identical manner, by MgATP-γ-S [2, 12]. ATP is the natural substrate of ATPases and also of kinases; on the other hand, ATP-γ-S is an excellent substrate for kinases but it does not support ATPases; then, some protein kinases/s must be involved in the metabolic regulation of the squid exchanger. In the experiments shown in Figure 7 phosphorylation of ATPases is ruled out by the presence of 300 µM vanadate; this indicates that protein phosphorylation patterns should result from kinases activity. Quite remarkably, this figure also shows that practically all phosphorylated bands disappear in the presence of 50 µM Staurosporin. Actually, only two bands of the membrane vesicles (around 60 kDa and 50 kDa), the 13 kDa band from the 30-10 kDa cytosolic fraction and the ReP1-NCXSQ show [³²P]Pi incorporation. Few kinases are insensitive to Staurosporin; among them CK2, CKI, MAP kinase, CSK and calmodulin-
dependent protein kinase III. Resistance to Staurosporin is related to the molecular structure of these enzymes [34]. This may become an important tool in the identification of the squid kinase/s implicated in MgATP modulation of this exchanger.

The intimate mechanism by which ReP1-NCXSQ participates in this regulatory process is still unknown. The interaction with the exchanger may be direct or indirect through other structure/s. One interesting possibility for a direct interaction could be the C-terminal part of the regulatory loop. In the cardiac exchanger this region has a strong hydrophobicity; this is such that part of it was initially considered a transmembrane segment [35]. And that segment is where the highest similarity (75 %) was found between the mammalian heart and the squid Na\textsuperscript{+}/Ca\textsuperscript{2+} exchangers [2]. The requirement for phosphorylation of ReP1-NCXSQ could be explained if that incorporation of inorganic phosphate is essential to acquire a configuration able to bind to its target. Lipocalins usually interacts with small molecules; however, human lipocalins with their binding sites mutated can attach to macromolecules [36]. This opens the possibility that native ReP1-NCXSQ, being so far in the evolution, can also bind to macromolecules such as the squid Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger. Another mechanism could be the simultaneous requirement of another structure; i.e. the exchanger protein is not the target for phosphorylated ReP1-NCXSQ. This would offer several possibilities, three of which are worth considering: (a) The existence of perphosphorylation where the phosphorylated LBP transfers its phosphate to another structure/s that eventually interact with the exchanger. An example of a functional consequence of this path is that of immunoglobulin E receptor signaling which produces mast cell activation by preventing an inhibitory effect of a lipid-protein interaction [37]. (b) A mechanism similar to that of LBP’s on the Na\textsuperscript{+}-dependent amino acid uptake in brain synaptosomes where they stimulate by removing inhibitory fatty
acids from the membrane [33]. (c) By allowing activatory lipid-exchanger interaction. In this regard is interesting that ReP1-NCXSQ binds polyphosphoinositides and phosphatidic acid but does not seem to do so to other membrane components such as Cholesterol, Sphingomyelin, Phosphoserine, Phosphoethanolamide or PtdIns (Figure 8).

Finally, the present results may have a broader physiological relevance. In dog red blood cells the Na$^+$/H$^+$ exchanger is up-regulated by ATP, a process that requires the presence of an additional cytosolic regulatory factor [38]. Consequently, it is not unlikely that other membrane transporters may be modulated by mechanisms of the type described here.

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REFERENCES


LEGENDS FOR FIGURES

Figure 1. Mass spectroscopy peptide analysis, schematic representation of the nucleotide sequence, domain structure, and primary structure of ReP1-NCXSQ. (A) Peptides obtained by mass spectroscopy of the 13 kDa band. For each peptide the amino acid sequence, mass and number of charged amino acids is listed. (B) Schematic representation of the ReP1-NCXSQ nucleotide and amino acid sequence. The expressed tag nucleotide sequence, which encodes the protein that matches to the peptides obtained by mass spectroscopy, contains a 396- nucleotide coding sequence that encodes a 132 amino acids sequence. The EST also contains elements of the 5’ and 3’ untranslated regions. The amino acid sequence used as an antigen to generate a polyclonal peptide antibody against ReP1-NCXSQ is listed. (C) The four mass spectroscopy peptides match to the full-length amino acid sequence of ReP1-NCXSQ (shaded amino acids). The peptides obtained by mass spectroscopy comprise 23% of the total ReP1-NCXSQ amino acid sequence.

Figure 2. Purification of the expressed 6xHis-ReP1-SQNCX. (A) Samples subjected to SDS-PAGE 12% and stained with Coomassie Blue. Lane 1: Molecular mass markers; Lane 2: Bacterial lysate before induction; Lane 3: Bacterial lysate after induction; Lane 4: Sonicated bacterial lysate after induction; Lane 5: Sample from the pass through; Lane 6: First ml of eluate (12 µg); Lane 7: Second ml of eluate (6 µg); Lane 8: third ml of eluate (2 µg). (B) Purified recombinant protein (2 µg) was run in a SDS-PAGE as indicated in (A), transferred onto PVDF membranes and immunodetected with primary antibody (Rabbit affinity purified anti-ReP1-NCXSQ, Gen Scrip Corp. USA). Lanes 1 and 2 are duplicate samples. See Materials and Methods for details.
Figure 3. Circular dichroism and Amide I’ infrared absorption spectrum analysis of ReP1-NCXSQ. (A) Far-UV Circular dichroism: The ordinate is the ellipticity calculated by mean residue molecular weight. The solution contained 10 μM ReP1-NCXSQ in 50 mM Tris-HCl (pH RT 7.3), 30 mM NaCl and 100 mM NMG-Cl. (B) Near-UV Circular dichroism: The ordinate is the measured ellipticity. The sample contained 69 μM ReP1-NCXSQ in 50 mM Tris-HCl (pH RT 7.3), 30 mM NaCl and 100 mM NMGCl. Note: (a) bands between 250 and 275 nm that can be assigned to the absorption of Phe residues; (b) the peak at 279 (b) associated to Tyr residues; (c) double peak above to 280 due to the absorption of Trp residues. The appearance of these highly structured bands is indicative that the protein is in the native state with a well-defined tertiary structure. (C) Amide I’ infrared absorption spectrum: The middle trace is the experimental spectrum. Upper trace is the Fourier self-deconvoluted spectrum using a bandwidth of 18 cm⁻¹ and a narrowing factor k = 2. The gaussian curves under the experimental spectrum are the band components obtained by Fourier self deconvolution and band fitting (see the text). The absorbance scale corresponds to the experimental spectrum.

Figure 4. Identification of a 13 kDa protein in the squid optic ganglia cytosolic fraction of 30-10 kDa (SCRp) by immunoblotting with an antibody against the ReP1-NCNSQ. (A) Coomassie blue stained SDS-PAGE (4-20% gradient gel, Invitrogen, USA). (B) Immunoblot decorated with a rabbit polyclonal antibody against ReP1-SQNCX (clone 76g04; Gen Scrip Corp. USA) and then with secondary antibody anti-rabbit IgG(H+L) antibody alkaline phosphatase conjugate (Promega, USA) and visualized by phosphorescence (ECF, Amersham-GE, USA) with an image analyzer (Storm 840). In A
and B: Lane 1: 30-10 kDa Cyt refers to the 30-10 kDa cytosolic fractions (14 µg); Lane 2: Membr. Vesic. refers to squid nerve membrane vesicles (40 µg). See Materials and Methods or details.

**Figure 5.** Comparison between the effects of SCRF and ReP1-NCXSQ alone or in combination with 1 mM MgATP, with and without Staurosporin, on the Na⁺ gradient-dependent Ca²⁺ uptake in squid nerve membrane vesicles. Vesicles were prepared as indicated in Materials and Methods and loaded with 300 mM NaCl, 20 mM Mops-Tris (pH 7.3 at 20°C) and 0.1 mM EDTA-Tris. Influx of ⁴⁵Ca²⁺ was measured during 10 s, incubating the vesicles at 20°C in the following solutions: 300 mM or 30 mM NaCl, 1 mM free Mg²⁺, 20 mM Mops-Tris (pH 7.3 at 20°C), 0.150 mM EGTA, 0.2 mM vanadate, and 1 µM Ca²⁺ without or with 1 mM ATP. Extravesicular NaCl was replaced with iso-osmolar concentrations of NMG-Cl. When present, Staurosporin was at 50 µM. The bars represent the Mean ± SE of triplicate determinations. *p ≤ 0.01; **p ≤ 0.001 (Student “t” test).

**Figure 6.** Phosphorylation by Mg[^32]P]ATP of the SCRP and ReP1-NCXSQ. SCRP (A) or ReP1-NCXSQ (B) were incubated for 10 s at 20°C with 0.5 mM [³²P]-γ-ATP, 1 µM Ca²⁺, 1 mM Mg²⁺, 200 µM vanadate, 260 mM NMG.Cl and 30 mM NaCl in the following conditions: Lane 1: Membrane vesicles of squid optic nerve. Lane 2: Membrane vesicles of squid optic nerve plus 50 µM Staurosporin. Lane 3: Membrane vesicles of squid optic nerve plus 50 µM Staurosporin but in this case the reaction was stopped with 4 mM Tris-EDTA-Tris and 1 mM Vanadate (pH 7.3 at 20°C) followed by 10 min centrifugation at 25 psi (100 000 x g) in a Beckman Airfuge centrifuge to remove all membrane proteins. The
arrows on the left indicate the positions of molecular weigh standards. Notice: (i) Staurosporin markedly reduces the number of phosphorylated bands in membrane vesicles; (ii) phosphorylation of the 13 kDa band in the 30-10 kDa cytosolic fraction and ReP1-NCXSQ are not affected by Staurosporin.

**Figure 7.** Previously phosphorylated SCRP and ReP1-NCXSQ can up-regulate the Na\(^+\)/Ca\(^{2+}\) exchanger in the absence of ATP. Before testing for their effects on Ca\(^{2+}\) uptake SCRP and ReP1-NCXSQ were incubated for 10 s with squid nerve vesicles as a kinase source in the absence and presence of 0.5 mM MgATP. The reaction was stopped with 4 mM EDTA-Tris and 1 mM vanadate (pH 7.3 at 20°C) and the membrane proteins were separated by centrifugation in a Beckman Airfuge for 10 min at 25 psi (100,000 x g). Aliquots of the supernatant (1/20 dilution) were used for \[^{45}\text{Ca}]\text{Ca}^{2+}\) uptake assays; the contaminants from previous incubations present in the uptake solutions were 187 μM EDTA-Tris, 45 μM Mg\(^{2+}\) and 50 μM Vanadate. In addition, those phosphorylated had 25 μM ATP; for that reason this amount of ATP was added to the uptake solutions containing unphosphorylated proteins. Bars are the means +/- SD of triplicate determinations. See [12] and Materials and Methods for details. * p ≤ 0.01 (Student “t” test). Note: (a) SCRP and ReP1-NCXSQ refer to unphosphorylated while P-SCR and P-ReP1-NCXSQ refer to previously phosphorylated proteins; (b) Under the conditions of these experiments 25 μM ATP does not significantly affect the Na\(^+\)/Ca\(^{2+}\) exchanger in squid nerve vesicles (DiPolo et al. 2000; also compare Figures 7 with Figure 5 in this work).

**Figure 8.** Interaction of several lipids with the recombinant ReP1-NCXSQ. (A) ReP1-NCXSQ-6His. (B) 14-3-3-6His recombinant protein. (C) Positions and Abbreviations of
the lipids contained in each spot: Triglyceride (TG), Phosphatidylinositol (PI), Diacylglycerol (DAG), PtdIns(4)P (PIP), Phosphatidic Acid (PA), PtdIns(4,5)P2 (PIP2), Phosphatidylserine (PtdS), PtdIns(3,4,5)P3 (PIP3), Phosphatidylethanolamine (PtdE), Cholesterol (CHO), Phosphatidylcholine (PtdC), Sphingomyelin (SM), Phosphatidylglycerol (PtdG), 3-sulfogalactosylceramide (Sulfatide) (3-SG Cer), Cardiolipin (CL) and Solvent Blank (Blank). The overlaying solution contained 0.5 µg/ml of either ReP1-NCXSQ-6His or 14-3-3-6His protein. Each spot in these commercial membrane strips had 100 pmoles of the corresponding lipid. See Materials and Methods for details.
A Mass Spectroscopy Analysis

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B Structure of Nucleotide and Amino Acid Sequences

5' UTR (108 nt)  Coding Sequence (396 nt)  3' UTR (80 nt)

Domain Structure (aa)

Lipocalin

Antigen Sequence (38-51 aa)

C Amino Acid Sequence and Mapping of Mass Spectroscopy Peptides

MAADLAGKWILESSENFDYYMKAVGVGMVMRKMMA
NAATPTQIEIKIDGDSWSIKTSTTFKTTDISFTIGQEFDDTTGDGRKIKTTCRVIDGNAMIQDQKGSFDSILSVREVKDGKMHMILKVNIDVVCVRITYKVRD
Figure 2

A) SDS-PAGE

B) Immunoblot

KDa

1 2 3 4 5 6 7 8

40- 20- 15-
Figure 6

A) SCRP

B) ReP1-NCXSQ

65kDa →

50kDa →

13kDa →

1 2 3
1 2 3
Figure 8

A B C

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