Cloning and expression of a cDNA encoding a new neurocalcin isoform (neurocalcin α) from bovine brain

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Neurocalcin (NC), a neuron-specific EF-hand Ca^{2+} -binding protein, purified from bovine brain [Terasawa, Nakano, Kobayashi and Hidaka (1992) *J. Biol. Chem.* **267**, 19596–19599] contains multiple isoforms. We previously cloned NC δ from bovine brain and showed high expression in neuronal tissues [Okazaki, Watanabe, Ando, Hagiwara, Terasawa and Hidaka (1992) *Biochem. Biophys. Res. Commun.* **185**, 147–153]. We report here the molecular cloning and expression of a cDNA encoding bovine brain NC α . The translated bovine protein is 191 amino acids long and shares 69.1% of its amino acid sequence with NC δ . Recombinant NC α migrates as a single 23 kDa band and exhibits a Ca^{2+} -dependent mobility shift on SDS/PAGE. Analysis of fluorescence emission spectra showed the Ca^{2+} -

induced peak at 337 nm. Interestingly, the mobility shift and the fluorescence intensity at 337 nm were larger for NC α than for NC δ . In Ca^{2+} -overlay experiments, however, the apparent affinity of NC α for $^{45}\text{Ca}^{2+}$ was similar to that of NC δ . Immunohistochemical analysis revealed NC α expression in the granular layer of the rat cerebellar cortex whereas NC δ was found in the Purkinje cell layer. In the rat olfactory bulb, NC α was located in external tufted cells, and NC δ was found in the periglomerular cells. These data demonstrate that NC isoforms differ in their tissue distribution and conformational changes induced by Ca^{2+} binding. Thus differential regulation of the two NC isoforms may be involved in control of neuron function.

INTRODUCTION

Ca^{2+} acts as a ubiquitous second messenger in cells, triggering a multitude of cellular processes such as secretion, contraction, metabolism, cell division and change in membrane permeability through a class of Ca^{2+} -binding proteins [1,2]. The latter exhibit a common structural feature, the EF-hand motif, which is composed of a helix– Ca^{2+} -binding loop–helix [3]. However, except for calmodulin, the exact biological functions of EF-hand protein remain unknown. Calmodulin antagonists, such as W-7 [*N*-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide], W-77 [(*S*)-*P*-(2-aminoethoxy)-*N*-[2-(4-benzoyloxycarbonylpiperazinyl)-1-(*P*-methoxybenzyl)ethyl]-*N*-methylbenzenesulphonamide dihydrochloride] and W-66 [*N*-(2-aminoethyl)-*N*-[2-(4-chlorocinnamylamino)ethyl]-5-isoquinolinesulphonamide], are useful tools for elucidating the physiological function of calmodulin and other Ca^{2+} -binding proteins [4–8].

In recent years, a new subfamily of EF-hand proteins predominantly expressed in neuronal cells has attracted attention [9]. It comprises more than 20 members with remarkably high sequence conservation between species homologues. This subfamily includes recoverin [10], visinin [11], S-modulin [12], hippocalcin [13], visinin-like protein [14], neural visinin-like Ca^{2+} -binding protein [15], neuronal Ca^{2+} sensors [16] and *Drosophila* neurocalcin [17]. Biochemical studies have demonstrated numerous physiological roles for these members, including inhibition of rhodopsin phosphorylation by rhodopsin kinase [G-

protein-coupled receptor kinase1 (GRK1)] [18–20]. We previously purified neurocalcin (NC), which belongs to this subfamily, from bovine brain using W-77 affinity chromatography [21]. Interestingly, native NC was found to comprise at least two isoforms (NC α and NC δ). To the best of our knowledge, there has been no study of isoform-specific physiological functions of NC, although NC δ cDNA has been cloned [22] and biochemical characteristics of NC δ *in vitro* have been reported. NC δ has a high Ca^{2+} -dependent affinity for S100B and tubulin β -chain [23] and may regulate microtubule assembly [24].

In the present study, we have isolated and sequenced a cDNA encoding a new isoform of bovine NC (NC α). We also describe biochemical characterization of NC α in comparison with that of NC δ , along with results for immunohistochemical studies of tissue distribution. In addition, it was demonstrated that NC has the ability to inhibit phosphorylation of muscarinic acetylcholine receptor m2 subtypes (m2) by GRK2 (β -adrenergic receptor kinase 1). The findings point to important physiological roles for these proteins in the control of neuron function.

EXPERIMENTAL

Cloning procedures

A λ gt11 bovine brain cDNA library (Clontech Laboratories Inc.) was screened with anti-NC serum, raised in a rabbit immunized with purified bovine brain NC [25]. Immunopositive

Abbreviations used: W-77, (*S*)-*P*-(2-aminoethoxy)-*N*-[2-(4-benzoyloxycarbonylpiperazinyl)-1-(*P*-methoxybenzyl)ethyl]-*N*-methylbenzenesulphonamide dihydrochloride; NC, neurocalcin; GRK, G-protein-coupled receptor kinase; m2, muscarinic acetylcholine receptor m2 subtypes; GST, glutathione S-transferase.

The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL and GenBank nucleotide sequence databases under the accession number AB006006.

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plaque was obtained and purified. Insert was amplified by PCR using two λ gt11 primers: the 3' primer contained an *Xba*I site, and the 5' primer contained a *Hind*III site. The PCR product was digested with *Xba*I and *Hind*III, and then subcloned into the pBluescript SK(–) plasmid vector. Sequencing was by an automated system with fluorescence-labelled deoxynucleotides (Applied Biosystems).

Expression and purification of NC α in *Escherichia coli*

The NC α cDNA clone was treated with *Taq* DNA polymerase and two synthetic oligonucleotide primers, which were added to create *Bam*HI and *Sma*I sites at the 5' and 3' ends respectively. The PCR product was subcloned into the *Bam*HI–*Sma*I sites of pGEX-4T-1 expression vector (Pharmacia LKB Biotechnology) to produce a glutathione S-transferase (GST) fusion protein. Competent *E. coli* DH5 α cells were transformed with the recombinant plasmid. Expression of fusion protein was induced by 1 mM isopropyl β -D-thiogalactoside for 3 h. After induction, the bacteria were pelleted, washed once with ice-cold TE (10 mM Tris/HCl, 1 mM EDTA, pH 8.0), and resuspended in lysis buffer [40 mM Hepes/KOH, pH 8.0, 1 mM EDTA, 100 mM potassium glutamate, 0.1 % (v/v) Triton X-100, 10 % (v/v) glycerol, 1 mM dithiothreitol]. Samples were sonicated for 5 min at 0.5 s intervals, and then centrifuged at 12 000 g for 20 min at 4 °C. After removal of the supernatant, the preparations were loaded on to a column of glutathione–Sephacrose 4B (Pharmacia LKB Biotechnology) and the GST fusion protein was eluted with 10 mM glutathione in 50 mM Tris/HCl, pH 8.0. Eluted GST fusion protein was incubated with human thrombin (1 unit/ μ g of protein; Calbiochem-Behring Corp.) for 10 min at 37 °C. The resulting solution was applied to a column of glutathione–Sephacrose 4B to remove GST and undigested fusion protein. Recombinant NC δ protein was purified as described [25]. Purified proteins were dialysed against distilled water to remove any trace of EGTA.

SDS/PAGE

Recombinant NC α and NC δ proteins were quantified by Bradford assay [26] using BSA (Sigma) as standard. NC α and NC δ proteins (1 μ g each) were dissolved in sample buffer: 25 mM Tris/HCl, pH 6.8, 0.2 % (w/v) SDS, 0.6 % (v/v) 2-mercaptoethanol, 3 % (v/v) glycerol and 0.001 % Bromophenol Blue containing either 1 mM CaCl₂ or 1 mM EGTA. SDS/PAGE was carried out by the method of Laemmli [27] using 15 % (w/v) polyacrylamide gels. Proteins were stained with Coomassie Brilliant Blue.

Tryptophan fluorescence

Emission fluorescence spectra were recorded with spectrofluorophotometer (Shimadzu). Tryptophan excitation was at 280 nm, with emission spectra monitored from 300 to 400 nm. The solvent used was made up of 50 mM Tris/HCl, pH 7.5, 150 mM KCl and 7.5 mM 2-mercaptoethanol. Fluorescence measurements were carried out on 1 μ M either NC α or NC δ at room temperature. EGTA (1 mM) and (CaCl₂) 1 mM were added to obtain the Ca²⁺-free and Ca²⁺-containing forms respectively. In titration experiments, fluorescence emission was recorded at 337 nm. Free Ca²⁺ concentration was adjusted by successively adding small aliquots of EGTA to protein solutions containing 1 mM EGTA and 1.05 mM CaCl₂. The total volume of EGTA added did not exceed 4 % of the initial volume.

Ca²⁺-binding assay

Ca²⁺ binding to NC α and NC δ on membranes was determined by the ⁴⁵Ca²⁺-overlay technique [28]. NC α and NC δ (2 μ g each) were diluted in sample buffer as described above, and electrophoresed on 15 % (w/v) polyacrylamide gel. The electrophoretic transfer of proteins from SDS/PAGE to the PVDF membrane (Millipore) was performed by the method of Towbin et al. [29]. After transfer, the membrane was washed with a buffer containing 60 mM KCl, 5 mM MgCl₂ and 10 mM imidazole/HCl, pH 7.0. The membrane was incubated in the same buffer containing 1 mCi/l ⁴⁵Ca (Amersham), 1 mM CaCl₂ and various concentrations of EGTA for 10 min, and rinsed with 50 % (v/v) ethanol for 2 min. Radioactivity was detected using a Fujix BAS2000 Bio-imaging analyser (Fuji Photo Film Corp. Ltd.). To estimate the amount of transferred proteins, the membrane was stained with Amido Black solution.

Preparation of polyclonal antibodies

Purified recombinant NC α protein (500 μ g) was emulsified with an equal volume of Freund's complete adjuvant and the emulsion was used to immunize a Japanese White female rabbit. After 4 weeks, the rabbit received a booster injection containing 500 μ g of antigen emulsified in Freund's incomplete adjuvant. At 2 weeks after the second injection, the animal was bled and antiserum was prepared. Anti-NC δ antibody was prepared as described by Okazaki et al. [30]. For the purification of NC α and NC δ antibodies, both were passed through affinity columns of the corresponding isoform-coupled Sepharose HP activated with normal human serum (NC–Sepharose columns), to eliminate antibodies that did not recognize NC α and NC δ . Bound antibodies were eluted from NC–Sepharose columns with 0.1 M glycine/HCl, pH 2.3, and collected into 1 M Tris base.

Immunohistochemistry

Wistar rats (body weight 200–300 g; male) were anaesthetized with sodium pentobarbital (50 mg/kg) and transcardially perfused with Zamboni's fixative [31]. The cerebellar cortex and olfactory bulb were removed and soaked in 30 % (w/v) sucrose in 0.1 M phosphate buffer, pH 7.2, at 4 °C overnight. The tissues were then embedded in OCT compound and frozen quickly. Sections (15 μ m thick) were cut on a cryostat and mounted on poly-L-lysine-coated glass slides. The sections were pre-incubated for 1 h in 10 % (v/v) normal goat serum, diluted with 10 mM PBS, pH 7.2, then allowed to react with anti-NC α (1:8000 dilution) or -NC δ (1:4000 dilution) antibodies overnight at room temperature. Subsequent steps were performed using a streptavidin/biotin/peroxidase kit (Histofine, Nichirei, Japan). Binding was visualized with a 0.03 % diaminobenzidine solution followed by washing in distilled water, dehydration and mounting in Entellan.

Phosphorylation assay

Human m2 and GRK2 were overexpressed and purified from Sf9 cells as described previously [32]. GRK2-mediated phosphorylation of m2 was carried out as described [33]. The phosphorylation reaction contained 7–11 nM m2 and 33 nM GRK2 in 20 mM Tris/HCl, pH 7.5, containing 5 mM MgCl₂, 10 μ M [³²P]ATP (4 $\times$ 10⁵ c.p.m./tube), 2 mM EDTA, 0.5 mM EGTA, 0 or 2 mM CaCl₂ and the indicated concentration of NC α or NC δ . Reaction mixtures were incubated at 30 °C for

60 min and stopped with SDS sample buffer. Samples were electrophoresed on a 12 % (w/v) polyacrylamide gel.

RESULTS AND DISCUSSION

Cloning and sequencing of NC α cDNA

A specific antibody against native NC was used to clone DNA encoding NC α from a bovine brain library. A clone with an 830 bp cDNA insert was studied (Figure 1). It contained a 573 bp open reading frame encoding a 191 amino acid with a calculated molecular mass of 22 142 Da. NC α has 69.1 % amino acid sequence identity with NC δ [22]. A consensus motif for N-terminal myristoylation was noted in NC α as for NC δ . Comparison of the amino acid sequences of NC α and NC δ with those of *Drosophila* NC [17], recoverin [10] and visinin [11] revealed 71.1, 46.3 and 38 % identity respectively for NC α and 88.4, 46 and 51 % respectively for NC δ . *Drosophila* NC is more similar to NC δ than to NC α .

Analysis of the Ca²⁺-induced mobility shift and fluorescence change

On SDS/PAGE, the electrophoretic mobility of NC α was slightly higher than that of NC δ in the presence of CaCl₂. When the same

1	AATTCGGGTGGGATTCCTCAATCTGCAGCAGAGATTACCCGAGCGTGTTCAGCGGCCGC	
61	TGGGCTTGCAGGGCGCAATCCAAGAGAGATTAAAGCAGCCGAGCTCTAGAAAAGGAG	
121	CGAGAGAGAACTCTGCAGAGAGCGTTAGCCGTTAGCCTGAATTAATATATATATTT	
181	TTTGAAGAAGCTGTGAGTTTATCATTTCTTAAAGTACGCTGCACAGCGCTATAACT	
241	GCAAAATGGGAAACAGAAATAGCAAACTGGCCCCAGAAAGTCTGGAGGACCTGGTGAAGA	
NC α	M G K Q N S K L A P E V M E D L V K	18
NC δ	M G K Q N S K L R P E V M Q D L L E	
301	GCACTGAGTTCAATGAGCATGAGCTTAAAGCAGTGGTACAAGGCTTCCTCAAGGACTGTC	
NC α	S T E F N E H E L K Q W Y K G F L K D C	38
NC δ	S T D F T E H E I Q E W Y K G F L R D C	
361	CGAGCGGAGGCTGAACCTCGAGGAGTTCCAGCAGCTCTATGTGAAGTTCTCCCTATG	
NC α	P S G R L N L E E F Q Q L Y V K F F P Y	58
NC δ	P S G H L S M E E F K K I Y G N F F P Y	
421	GAGATGCCTCAAGTTTCGCGCAGCAGCGCTTCGCAACCTTCGACAAGAACGGTGTGGCA	
NC α	G D A S K F A Q H A F R T F D K N G D G	78
NC δ	G D A S K F A E H V F R T F D A N G D G	
481	CCATCGATTTCGAGAGTTTCATCTGTGCTGTCCTATCAGCTCCAGAGGTAGCTTTGAGC	
NC α	T I D F R E F I C A L S I T S R G S F E	98
NC δ	T I D F R E F I I A L S V T S R G K L E	
541	AGAACTGAAGTGGGCTTCAACATGTATGATCTGGACGGTGCAGGCAAGATCACCCGAG	
NC α	Q K L N W A F N M Y D L D G D G K I T R	118
NC δ	Q K L K W A F S M Y D L D G N G Y I S K	
601	TGGAGATGCTGGAGATTATCGAGGCTATCTACAAAATGGTGGGCACCGTGATCATGATGA	
NC α	V E M L E I I E A I Y K M V G T V I M M	138
NC δ	A E M L E I V Q A I Y K M V S S V - - M	
661	AAATGAATGAGGATGGCCTGACGCTGAACAGCGGTTGACAAGATTTCAGCAAGATGG	
NC α	K M N E D G L T P E Q R V D K I F S K M	158
NC δ	K M P E D E S T P E K R T E K I F R Q M	
721	ATAAGAACAAAGATGACCAGATTACACTGGATGAATTCAGAAAGCTGCCAAGAGCGACC	
NC α	D K N K D D D Q I T L D E F K E A A K S D	178
NC δ	D T N R D D G K L S L E E F I R G A K S D	
781	CTTCCATTGTACTACTTCTGAGTGCGACATTGAGAAATGAGCGCGGAATT	
NC α	P S I V L L L Q C D I Q K	191
NC δ	P S I V R L L Q C D P S S A G Q F	

Figure 1 Nucleotide and deduced amino acid sequences of NC α

The NC α cDNA sequence is shown in the top lines, and the corresponding amino acid sequence in single-letter code is given in the middle lines. Amino acid sequences of NC α and NC δ (bottom lines) were compared. Asterisks denote identical residues. The extent of the classical EF-hand motifs is boxed.

Figure 2 Effects of Ca²⁺ on the electrophoretic mobility of NC α and NC δ

NC α and NC δ (1 μ g each) were separated by SDS/PAGE (15% gel) and stained with Coomassie Blue. The sample buffer contained either 1 mM CaCl₂ (+) or 1 mM EGTA (—) as indicated.

Figure 3 Effects of Ca²⁺ on the tryptophan fluorescence emission spectra of NC α and NC δ

(A) Fluorescence emission spectra of NC α (left) and NC δ (right) (1 μ M each) due to excitation at 280 nm were measured in 25 mM Tris/HCl, pH 7.5, with either 1 mM CaCl₂ or 1 mM EGTA as indicated. (B) Ca²⁺-dependence of the spectral changes of 1 μ M NC α (○) and NC δ (●) emission intensity measured at 337 nm. The data were expressed as a fraction of the maximal spectral changes in each protein. The experimental points represent means from three different experiments.

samples were run under identical conditions, except for the replacement of CaCl₂ with EGTA, migration was slower, with the change in mobility being larger in the case of NC α (Figure 2). This change in mobility probably reflects an alteration in the conformation of the protein.

Figure 4 Studies on binding of Ca^{2+} to NC α and NC δ

NC α and NC δ (2 μg each) were separated by SDS/PAGE and transferred to PVDF membrane. $^{45}\text{Ca}^{2+}$ binding to NC α or NC δ was determined at the indicated free Ca^{2+} concentration. Protein staining was performed using Amido Black as indicated.

NC α and NC δ have two tryptophan residues located at amino acid positions 30 and 103 (numbering based on positions within each NC isoform). The tryptophan fluorescence emission spectrum of recombinant NC α was measured and compared with that of NC δ (Figure 3A). The fluorescence intensity of NC δ increased slightly (1.2-fold) when the Ca^{2+} level was raised, whereas that of NC α was more markedly enhanced (2-fold) under the same conditions. Fluorescence peaks for NC α or NC δ were observed at 337 nm without any horizontal shift in the presence of either Ca^{2+} or EGTA. The data show that the two tryptophans of NC α are sensitive to conformational changes induced by Ca^{2+} binding. Likewise, the variation in fluorescence of NC α indicates that more dynamic movement occurs on Ca^{2+} binding to it than for NC δ . The results of titration experiments are shown in Figure 3(B). The half-maximum changes in fluorescence signal of NC α and NC δ were at 1.5 and 6.1 μM free Ca^{2+} respectively. These data indicate that Ca^{2+} -dependent conformational changes in NC α are more responsive than those in NC δ .

Ca^{2+} -binding assay

NC δ binds 2 or 3 mol of Ca^{2+} per mol of protein [34,35]. This discrepancy in the quantitative binding of Ca^{2+} to NC δ may be caused by assay conditions. NC α was compared with NC δ with respect to its ability to bind Ca^{2+} using the $^{45}\text{Ca}^{2+}$ -overlay technique (Figure 4). The apparent affinities of NC α for $^{45}\text{Ca}^{2+}$ were similar to those of NC δ . The difference in electrophoretic mobility and fluorescence intensity between NC α and NC δ (Figures 2 and 3A) is probably not due to their Ca^{2+} -binding affinities.

Immunohistochemical examination

We previously purified NC from bovine brain and showed high expression in neuronal tissues [25,34,36–39]. Affinity-purified anti-NC α and anti-NC δ antibodies only recognized the corresponding isoform and showed no cross-reaction on Western-blot analysis (results not shown). To determine the localization of NC α and NC δ in the rat cerebellar cortex and olfactory bulb, immunohistochemical analysis was performed. In the cerebellar cortex, anti-NC α antibody bound strongly to the granular

Figure 5 NC immunoreactivity in the adult rat cerebellar cortex

Details of the conditions are given in the Experimental section. Adult rat cerebellar cortex was stained with anti-NC α antibody (A) or anti-NC δ antibody (B). Note the prominent immunostaining of NC δ detected in the Purkinje cells. M, molecular layer; G, granular layer. Bar = 50 μm .

Figure 6 NC immunoreactivity in the adult rat olfactory bulb

Details of the conditions are given in the Experimental section. Adult rat olfactory bulb was stained with anti-NC α antibody (A) or anti-NC δ antibody (B). Note most NC α staining is restricted to external tufted cells, while periglomerular cells are positive for NC δ . Bar = 50 μm .

layer but not to the Purkinje cell layer. Cells in the granular layer demonstrated varying immunoreactivity. Interestingly, the most prominent immunostaining of NC δ was detected in the Purkinje cells. Granule cells and Purkinje cells are thought to be excitatory and depressor neurons respectively, indicating that NC α and NC δ may play roles in the control of neuron function (Figure 5). NC α and NC δ were also found to differ in their localization in the olfactory bulb (Figure 6). Although some co-localization was

Figure 7 Inhibitory effects of NC α and NC δ on GRK2 activity

m2 was phosphorylated by GRK2 in the presence or absence of NC α (4.5 μ M) or NC δ (3.3 μ M) as described in the Experimental section. Phosphorylation of m2 was analysed by SDS/PAGE (12% gel) followed by autoradiography.

observed, a large number of external tufted cells within the glomerular layer were NC α immunopositive. On the other hand, most NC δ -immunopositive staining was restricted to the periglomerular cells. Tufted cells are thought to be modified granule cells and could act as interneurons. The data indicate that NC α and NC δ may have specific physiological roles in tufted and periglomerular cells.

Inhibition of m2 phosphorylation

It has been shown that recoverin and some other members of the Ca²⁺ sensor protein family inhibit the phosphorylation of rhodopsin by GRK1 in a Ca²⁺-dependent manner [18,20]. GRK2 is a specific receptor kinase that is known to phosphorylate and induce desensitization of agonist-occupied forms of several G-protein-coupled receptors. By analogy with the reported effects on rhodopsin phosphorylation, we examined the inhibitory effects of NC α and NC δ on m2 phosphorylation *in vitro* by GRK2 (Figure 7). Phosphorylation of m2 by GRK2 was observed, with greatest efficiency in the presence of Ca²⁺ than in its absence. Addition of NC α (4.5 μ M) or NC δ (3.3 μ M) resulted in inhibition of m2 phosphorylation to 77 or 70 % respectively in the presence of Ca²⁺.

In the present study, we cloned, sequenced and expressed a cDNA for bovine NC α and used multiple approaches to clarify the possible functions of NC α and NC δ in neuron physiology. NC purified from bovine brain was found to contain at least two groups (α , β and γ 1, γ 2), with isoforms in each case sharing extensive homology (77 % and 90 %) [21]. The deduced amino acid sequence of cloned NC α is 95 % identical with that of NC α purified from bovine brain. NC δ has 85 % amino acid sequence identity with the γ 1 isoform. It is uncertain whether another NC isoform still remains to be cloned. The cDNA sequence shown in Figure 1 is highly homologous to the recently published sequences for chick visinin-like protein [14] (87.1 %) and rat [15] (96.3 %) and mouse (99.3 %) neural visinin-like Ca²⁺-binding protein in the coding region. However, the amino acid sequence of NC α is 100 % identical. NC α and NC δ show only about 69 % identity in their deduced amino acid sequences. Nevertheless, each sequence contains a number of similar recognition sites that correspond to functional domains for binding of Ca²⁺ and for myristoylation. The present biochemical characterization of NC α and NC δ indicates that the Ca²⁺-induced conformational changes in NC α occur in a different way from those in NC δ , rather than affinity for Ca²⁺. We previously showed a lack of immunoreactivity in periglomerular cells in the rat olfactory bulb using a specific antibody against native NC from bovine brain [36]. In this study,

however, most NC δ immunostaining was restricted to periglomerular cells, indicating that NC α may be the major component of native NC. Immunohistochemical approaches indicate that NC α and NC δ may differ not only in their localization but also in their neuron function. In addition, we find that NC α and NC δ are capable of inhibiting m2 phosphorylation *in vitro* when Ca²⁺ is present.

Although neither NC α nor NC δ has yet been functionally characterized, the biophysical features demonstrated indicate that these proteins could play roles as very precise cellular modulators in Ca²⁺-signal-transduction pathways.

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