Inhibitory Interaction of the Plasma Membrane Na\(^+\)/Ca\(^{2+}\) Exchangers with the 14-3-3 Proteins*

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The three Na\(^+\)/Ca\(^{2+}\) exchanger isoforms, NCX1, NCX2, and NCX3, contain a large cytoplasmic loop that is responsible for the regulation of activity. We have used 347 residues of the loop of NCX2 as the bait in a yeast two-hybrid approach to identify proteins that could interact with the exchanger and regulate its activity. Screening of a human brain cDNA library identified the ε and ζ isoforms of the 14-3-3 protein family as interacting partners of the exchanger. The interaction was confirmed by immunoprecipitation and in vitro binding experiments. The effect of the interaction on the homeostasis of Ca\(^{2+}\) was investigated by co-expressing NCX2 and 14-3-3ε in HeLa cells together with the recombinant Ca\(^{2+}\)-ATPase (PMCA) to facilitate its function, and, as a result, Ca\(^{2+}\) was the finding that the cardiac exchanger interacted with the C-terminal portion of calcineurin A. The membrane topology of the exchangers has been studied particularly in NCX1, but it is very likely shared also by the other isoforms. The predicted nine transmembrane segments (TMSs) can be divided into an N-terminal portion, composed of the first five TMSs, and a C-terminal portion composed of the last four TMSs. The two TMS portions are important for the binding and the transport of ions, and they are separated through a large intracellular loop of about 550 amino acids. Although this loop is not involved in Na\(^+\) and Ca\(^{2+}\) translocation, it is responsible for the regulation of activity. Several factors are responsible for the regulation (3), among them the two transported ions, Na\(^+\) and Ca\(^{2+}\) (13, 14), the intracellular pH (15), metabolic components (e.g. ATP, phosphatidylglycerol 4,5-bisphosphate (16), protein kinase A, and protein kinase C (17)), redox agents, hydroxyl radicals, H\(_2\)O\(_2\), dithiothreitol, O\(^{2-}\), Fe\(^{3+}\), Fe\(^{2+}\), Cu\(^{2+}\), and OH (18). So far, relatively little has become known about possible proteins that could interact with the exchanger and be involved in the regulation processes. One interesting early development in this context was the finding that the cardiac exchanger interacted with the cytoskeleton, specifically with ankyrin. The interaction was suggested as a possible mechanism responsible for the specialized localization of the exchanger to particular domains of the plasma membrane (19). More recently (20), the heart exchanger was found to interact with the C-terminal portion of calcineurin Aβ. The interaction was inhibitory and was enhanced in cardiomyopathic hamster hearts. Clearly, the matter of possible NCX interactors is potentially interesting. It was thus decided to perform a yeast two-hybrid screening to identify other proteins that could interact with the exchanger, regulate its function, and, as a result, Ca\(^{2+}\) signaling in the cell as well. It was decided to initiate the work with NCX2, by creating a “bait” construct made of a portion of the large cytoplasmic loop. A human brain cDNA library was screened, and the analysis of 2 × 10\(^6\) clones identified the 14-3-3ε and 14-3-3ζ proteins as possible interactors with NCX2; the interaction was further characterized using the 14-3-3ε protein. The effect of}

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2. The abbreviations used are: PMCA, plasma membrane Ca\(^{2+}\)-ATPase; NCX, Na\(^+\)/Ca\(^{2+}\) exchanger; PBS, phosphate-buffered saline; cytAEO, cytoplasmic targeted aequorin; KRB, Krebs-Ringer buffer; GST, glutathione S-transferase; InsP3, inositol 1,4,5-trisphosphate; TMS, transmembrane segment.
the interaction on the homeostasis of Ca$^{2+}$ in the living cells was examined by co-expressing the two proteins (NCX2 and 14-3-3ε) in HeLa cells together with the recombinant Ca$^{2+}$ probe aequorin. The experiments showed that the ability of the cells that had expressed both NCX2 and 14-3-3ε to clear off a Ca$^{2+}$ transient induced by an InsP3-producing agonist was substantially decreased. This indicated a reduction of NCX2 activity by the 14-3-3ε protein. The 14-3-3ε protein also inhibited the NCX1 and NCX3 isoforms, and it was found that all three NCX isoforms interacted with multiple 14-3-3 isoforms. The 14-3-3 protein was bound by both the phosphorylated and nonphosphorylated forms of NCX, but the phosphorylated form had much higher binding affinity.

**EXPERIMENTAL PROCEDURES**

**DNA Constructs**—The portion of the large cytoplasmic loop of NCX2 corresponding to residues 283–631 was amplified by PCR using the forward primer 5’-CGGATCCGGGAAAATGATGAAT-3’ and the reverse primer 5’-CGGGATCCGCACATTC-3’. The plasmids were completely sequenced. The expression plasmid of NCX3 was cloned as an EcoRI-SalI fragment from NCX2 recombinant protein, the portion of the large cytoplasmic loop of NCX1 (amino acids 289–637) and NCX3 isoforms, and it was found that all three NCX isoforms interacted with multiple 14-3-3 isoforms. To create glutathione S-transferase (GST)-NCX recombinant fusion proteins, the above portions of the large cytoplasmic loop of NCX1 (amino acids 289–637) and NCX3 (amino acids 288–636) were amplified by PCR using appropriate pairs of forward 5’-CGGATCCGGGAAAATGATGAAT-3’ (NCX1) and 5’-CGGATCCGGGAAAATGATGAAT-3’ (NCX3) and reverse 5’-CGTCGACACAGGGCTTTCTTTC-3’ (NCX1) and 5’-CGTCGACACAGGGCTTTCTTTC-3’ (NCX3) primers and subsequently subcloned into the BamHI-SalI sites of the vector pGilda to create a bait for the two-hybrid screening.

To create glutathione S-transferase (GST)-NCX recombinant fusion proteins, the above portions of the large cytoplasmic loop of NCX2 were amplified by PCR using the forward primer 5’-GGGATCCCGGAAAATGATGAAT-3’ and the reverse primer 5’-CGGGATCCGCACATTC-3’ and subcloned into the BamHI-SalI sites of the vector pGilda to create a bait for the two-hybrid screening.

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**Cell Cultures and Transfection**—Human neuroblastoma SH-SY5Y and HeLa cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 2 mM glutamine, penicillin (60 μg/ml) and streptomycin (120 μg/ml) in 75-cm² Falcon flasks at 37 °C. The cells were transfected using a calcium phosphate method. For the aequorin experiments, the cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 5% (v/v) horse serum, 2 mM glutamine, 100 μg/ml gentamicin, 7 μM p-aminobenzoic acid, 100 μg/ml pyruvate, and 100 micromolar/ml insulin at a density of 2.5-×-10⁶ cells/cm², in the presence of 5.3 mM KCl. After 24 h, 10 μM cytosine arabinofuranoside was added to inhibit mitotic cell growth. The cultures were maintained at 37 °C in a water-saturated 8% CO₂, 92% air atmosphere for 4 days.

**Co-immunoprecipitation**—Granule cells were cultured for 4 days at a density of 2.5-×-10⁶ cells/well in a 6-well plate at 37 °C. The cells were rinsed with PBS, and crude membrane proteins were prepared from the lysed cells. The cell lysates were centrifuged and solubilized in 10 mM Tris-HCl, pH 8.0, 1.0 mM EDTA, 0.5% SDS. NET buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.25% gelatin, 0.1% Nonidet P-40, 1.0 mM EDTA) was added to dilute the SDS to a final concentration of 0.2%. Triton X-100 and sodium deoxycholate were added to final concentrations of 0.3 and 0.5%, respectively. The mixture was incubated for 30 min at 4°C, and after centrifugation at 13,000 rpm the supernatant was incubated with the primary antibody (3 μl of anti-NCX2) (10) at 4°C on a rocking plate for 1–2 h. To recover the immunoprecipitates, 50 μl of protein A/G Plus-Sepharose (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were added to the mixture, which was incubated overnight at 4°C under gentle rocking. The protein A/G Plus-Sepharose-primary antibody complex was recovered by centrifugation for 1 min at 13,000 rpm and washed four times with 20 volumes of PBS. The material bound to protein A/G Plus-Sepharose was released by the SDS-PAGE sample buffer. The immunoprecipitates were analyzed by SDS-PAGE.

**Immunofluorescence Analysis**—HeLa cells were transfected with NCX1 or co-transfected with NCX1 and 14-3-3ε expression vectors. The cells were fixed with 3.7% formaldehyde in PBS for 20 min. After permeabilization of membranes with a 5-min incubation with 0.1% Triton X-100, the cells were washed with 1% gelatin (type B from bovine skin; Sigma) in PBS and immunostained with a primary antibody against NCX1 at a 1:100 dilution in PBS and the secondary antibody Alexa Fluor 594 (Molecular Probes). The images were acquired using an Olympus IX80 microscope (Olympus Optical Co., Ltd., Japan) with a 40× Plan Neofluar objective and a Photometrics cooled...
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\(Ca^{2+}\) Measurement with Recombinant Aequorin—Transfected cytAEQ was reconstituted by incubating HeLa or SH-SY5Y cells for 3 h with a 5 \(\mu M\) concentration of the aequorin prosthod group coelenterazine wild type (Molecular Probes) in Dulbecco’s modified Eagle’s medium supplemented with 1% fetal calf serum at 37 °C in a 5% CO\(_2\) atmosphere. Where indicated, the cells were loaded with 20 \(\mu M\) 5-(and 6-)-carboxyfluorescein diacetate (succinimidyl ester; Molecular Probes) for 20 min in a Krebs-Ringer solution (135 mM NaCl, 5 mM KCl, 0.4 mM KH\(_2\)PO\(_4\), 1 mM MgSO\(_4\), 20 mM Hepes, pH 7.4, at 37 °C) (Krebs-Ringer; KRB) supplemented with 1 mM CaCl\(_2\) and 0.1% glucose. The cells were then superfused with carboxyfluorescein-free solution for a further 10 min to allow carboxyfluorescein de-esterification and to wash off the residual compound in the bath solution. 13-mm round glass coverslips with the transfected cells were placed in a perfused, thermostatted (37 °C) chamber placed in close proximity to a low noise photomultiplier, with a built-in amplifier discriminator. The experiments were performed in the KRB physiological solution supplemented with 0.1% glucose and 1 mM CaCl\(_2\). The cytoplasmic \(Ca^{2+}\) concentrations were measured after the addition of the InsP3-generating agonists ATP (100 \(\mu M\)) in the KRB in the case of HeLa cells and 100 nM bradykinin (Sigma) in KRB in the case of SH-SY5Y cells. The experiments were terminated by lysing the cells with 100 \(\mu M\) digitonin (Sigma) in a hypotonic \(Ca^{2+}\)-rich solution (10 mM CaCl\(_2\) in \(H_2O\)) to discharge the remaining aequorin pool. The light signal from the discriminator was collected by a Thorn-EMI photon counting board and stored in an IBM-compatible computer for further analysis. The aequorin luminescence data were calibrated off-line into \([Ca^{2+}]_i\) values, using a computer algorithm based on the \(Ca^{2+}\) response curve of wild type aequorin as described in Ref. 21.

Western Blot Analysis—The antibodies used were a mouse monoclonal antibody for 14-3-3\(\beta\), a rabbit polyclonal antibody for 14-3-3\(\gamma\), 14-3-3\(\zeta\) (Santa Cruz Biotechnology), a rabbit polyclonal anti-NCX2 antibody (10), a rabbit polyclonal anti-NCX1 antibody (SWANT, Bellinzona, Switzerland), and a mouse monoclonal anti-GST (Santa Cruz Biotechnology). The intensity of the target bands was evaluated by an UN-SCAN-IT program, in which the region of interest was specifically identified from the negative control (Fig. 1B). Yeast EGY48 were co-transformed with the
expression vectors for NCX2 and protein 14-3-3, and the transformants were grown on a selective medium without leucine and further checked for /H9252-galactosidase activity. No interaction was detected between the identified proteins and a bait construct only encoding the LexA-binding domain. For further experiments it was decided to focus on the 14-3-3 isoform.

**Interaction of the 14-3-3 Protein with NCX2 in Rat Cerebellar Granule Cells**—To investigate whether the interaction between NCX2 and 14-3-3 occurred in a cell type where both proteins are endogenously expressed, co-immunoprecipitation experiments were performed on granule cells isolated from the cerebellum of 7-day-old rats, where the expression level of NCX2 is high. The cells were grown in the presence of physiological concentrations of KCl (5.3 mM) to prevent NCX2 degredation (10). The 14-3-3 protein was found to be associated with the NCX2 protein of the granules (Fig. 2). No immunoreactivity was detected among proteins that bind nonspecifically to the control antibody, indicating that the association of 14-3-3 with NCX2 was specific.

**Monitoring of the Cytosolic Ca²⁺ Concentration in HeLa Cells Overexpressing NCX2 and the 14-3-3 Protein**—The functional consequences of the interaction between 14-3-3 and NCX2 were studied by measuring the transport activity of NCX2. The overexpression of the exchanger increases the cell

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**FIGURE 1. Interaction between NCX2 and 14-3-3 proteins in a yeast two-hybrid system.** A, membrane topology and domain structure of NCX with the sequence of the portion of the large cytoplasmic loop (amino acids 283–631) of NCX2 used to create the “bait” construct. The topology scheme refers to NCX1 but can be assumed to be valid for NCX2 and NCX3 as well. B, specific interaction between NCX2 and the 14-3-3 and -ζ proteins in a two-hybrid screening. Yeast EGY48 cells were co-transformed with the “bait” construct, the 14-3-3 expression plasmid, and the reporter vector pSH18-34. Transformants were grown in the selective medium lacking histidine, uracil, tryptophan, and leucine (left) and checked for β-galactosidase activity in the same medium in the presence of X-gal (right) (for additional details, see “Experimental Procedures”).
ability to extrude Ca\(^{2+}\) and, consequently, leads to the reduction of Ca\(^{2+}\) concentration in the cytoplasm. The changes of Ca\(^{2+}\) concentrations were thus measured in the cytoplasm of HeLa cells transfected with the recombinant Ca\(^{2+}\)-sensitive photoprotein, aequorin (21), targeted to the cytoplasm (cytAEQ); co-transfected with cytAEQ and NCX2; or co-transfected with cytAEQ, NCX2, and 14-3-3\(\varepsilon\) expression vectors. The extrusion of Ca\(^{2+}\) was studied following the induction of a cytosolic Ca\(^{2+}\) transient obtained by applying to the cells 100 \(\mu\)M ATP, a purinergic receptor agonist that is coupled to the generation of InsP\(_3\). The rapid rise in cytosolic Ca\(^{2+}\) induced by the stimulation was followed by a gradual decline of the trace to a lower plateau. The decline comprises two components: the Ca\(^{2+}\) efflux from the cytoplasm mediated by the endogenous PMCA and sarcoplasmic reticulum Ca\(^{2+}\)-ATPase pumps and by NCX (when cells were transfected with NCX) and the Ca\(^{2+}\) influx into the cell via plasma membrane (capacitative) Ca\(^{2+}\) channels activated by the decrease of the Ca\(^{2+}\) concentration in the endoplasmic reticulum. The changes in the cytosolic Ca\(^{2+}\) transients induced by the overexpression of the proteins are shown in Fig. 3. The average peak value of 2.49 \pm 0.24 \(\mu\)M (\(n = 39\)) for the Ca\(^{2+}\) transient in control cells (only transfected with cytAEQ) was reduced to 1.77 \pm 0.17 \(\mu\)M (\(n = 25\)) in the cells expressing NCX2. However, in the cells expressing NCX2 and the 14-3-3\(\varepsilon\) protein, the peak of the Ca\(^{2+}\) transient was significantly higher (peak amplitude 2.31 \pm 0.12 \(\mu\)M; \(n = 25\)) than in those expressing only NCX2 and was followed by a slower declining phase. The data suggest an inhibitory effect of the 14-3-3\(\varepsilon\) protein on the function of NCX2.

Interaction between Recombinant GST-NCX Proteins and 14-3-3\(\varepsilon\)–It was then decided to examine whether 14-3-3\(\varepsilon\) interacted in vitro with NCX2 and the other two isoforms, NCX1 and NCX3, by using pulldown assays. To this aim, the portions of the large cytoplasmic loop of NCX1 and NCX3, corresponding to that of NCX2, which had been used to create the bait, and that of NCX2 were fused in frame with a GST protein using a pGEX4T1 expression vector. The GST-NCX1, GST-NCX2, or GST-NCX3 constructs were expressed in BL21 cells as described under “Experimental Procedures.” Fig. 4 shows that 14-3-3\(\varepsilon\) was bound by all three NCX isoforms. The binding of NCX2-GST to 14-3-3\(\varepsilon\) was compared with that of NCX1-GST and NCX3-GST. Equivalent amounts of 14-3-3\(\varepsilon\) protein were bound by all three NCX isoforms. No interaction was detected between 14-3-3\(\varepsilon\) and glutathione-Sepharose beads containing GST alone.
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Effect of 14-3-3e Overexpression on NCX1 and NCX3 Transport Activity in HeLa Cells—It was then examined whether the 14-3-3e protein had the same effect on the activity of NCX1 and NCX3 as it had on that of NCX2. HeLa cells were transfected with recombinant cytAEQ or co-transfected with cytAEQ and NCX1 (or NCX3) or with cytAEQ, NCX1 (or NCX3), and 14-3-3e expression vectors. Ca\(^{2+}\) transients were induced by stimulation with the InsP3-generating agonist ATP, as described above.

The changes in the Ca\(^{2+}\) transients are shown in Fig. 5. As expected from the results on NCX2, the 14-3-3e protein also inhibited the activity of NCX1 and NCX3. The peak of the Ca\(^{2+}\) transient, [Ca\(^{2+}\)]\(_{\text{p}}\), in the cells co-transfected with NCX1 (or NCX3) and 14-3-3e was significantly higher than in those transfected only with the exchangers. In the case of NCX1, the average peak value of the transient was increased from 1.24 ± 0.10 μM (n = 20) to 1.57 ± 0.13 μM (n = 20); in that of NCX3, it was increased from 1.16 ± 0.12 μM (n = 20) to 1.67 ± 0.11 μM (n = 20).

The Overexpression of 14-3-3e Does Not Affect the Level of Expression or the Membrane Targeting of Recombinant NCX—Experiments were performed to rule out the possibility that the inhibition of NCX activity by recombinant 14-3-3e was due to a decreased expression of NCX or to a change in its targeting to the plasma membrane. Fig. 6A shows a Western blot of membrane fractions of HeLa cells transfected with NCX1 or co-transfected with both NCX1 and 14-3-3e; the level of expression of NCX was the same in the two cell batches. Fig. 6B shows the immunofluorescence of recombinant NCX1 in HeLa cells transfected with the exchanger alone or co-transfected with the exchanger and 14-3-3e. The fluorescence in the plasma membrane was evaluated using the program described under “Experimental Procedures.” The histograms on the right of Fig. 6B show the equivalent levels of NCX1 in the plasma membrane of a large number of transfected and co-transfected cells. Fig. 6C shows that the recombinant exchanger and 14-3-3e co-localize at the plasma membrane.

NCXs Interact with Multiple 14-3-3 Isoforms—Next it was examined whether NCX1, NCX2, and NCX3 also interacted with 14-3-3 isoforms different from e. Pulldown assays were performed using GST-NCX1, GST-NCX2, and GST-NCX3 fusions incubated with HeLa cell extracts containing different isoforms of the 14-3-3 protein endogenously present in the cells. As shown in Fig. 7, other 14-3-3 isoforms (β, ξ, and θ) were retained by the three NCXs, indicating interaction of the exchangers with a domain common to the entire 14-3-3 protein family. No 14-3-3 binding to GST alone was observed.

Effect of 14-3-3e on the NCX Activity in HeLa Cells Loaded with Carboxyeosin—Previous work in the laboratory (22) had shown that PMCA4 was also inhibited by the 14-3-3e protein. PMCA4 is the plasma membrane Ca\(^{2+}\)-ATPase isoform present endogenously in HeLa cells (23). Therefore, it was necessary to eliminate the possibility that the overall inhibition of Ca\(^{2+}\) extrusion in HeLa cells co-transfected with NCX and the 14-3-3e protein was partially (or even totally) due to the inhibition of the endogenous PMCA4. The effect of 14-3-3e on the Ca\(^{2+}\) extrusion in HeLa cells expressing NCX was thus examined in the presence of the cell penetrant version of the inhibitor of PMCA ATPases, 5-(and 6-)carboxyeosin diacetate, succinimidyl ester (carboxyeosin). Control HeLa cells transfected with the cytAEQ expression vector, treated with carboxyeosin, and exposed to ATP showed a markedly slowed decay of the Ca\(^{2+}\) peak and a modestly higher [Ca\(^{2+}\)]\(_{\text{p}}\) level at the peak of the transient with respect to control cells. This can be logically attributed to the inhibition of the endogenous plasma membrane Ca\(^{2+}\)-ATPase. On average, carboxyeosin peak increased the half-time of [Ca\(^{2+}\)]\(_{\text{p}}\) decay by 100%, from 64 ± 8 to 125 ± 16 s, n = 10 (Fig. 8A). The height of the peak, by contrast, was markedly higher when cells were co-transfected with the NCXs and 14-3-3 with respect to those transfected only with the NCXs. This was in line with the accepted low affinity of NCX for Ca\(^{2+}\); it was expected that its efficiency of ejection would be higher when Ca\(^{2+}\) in the cell was at the peak level. For this reason, the effect of 14-3-3 on the height of the peak must be considered as a more significant parameter in the evaluation of the inhibitory effect of 14-3-3 on NCX.

HeLa cells were then co-transfected with cytAEQ expression vec-
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The Recombinant 14-3-3e Protein Inhibits Endogenous NCX in SH-SY5Y Cells—Figs. 3 and 5 have shown that recombinant 14-3-3e inhibits the activity of the three NCXs overexpressed in HeLa cells. It was decided to test whether recombinant 14-3-3e also inhibited the activity of endogenous NCX in cells that express it. SH-SY5Y were used for the experiments. Fig. 9 shows that the expression of 14-3-3e indeed inhibited the NCX activity of SH-SY5Y cells. The figure shows a representative experiment. The average height of the peak of the Ca\(^{2+}\) transient in the cytoplasm was 2.88 ± 0.06 μM (n = 7) in the controls and 3.3 ± 0.07 μM (n = 7) in the case of cells transfected with 14-3-3e. Control experiments were performed in the presence of carboxyossein to rule out the possible contribution of the inhibition of the PMCA pumps to the effect of 14-3-3e. Only a minor prolongation of the decay phase of the trace after the peak of the Ca\(^{2+}\) transient was observed, in keeping with the accepted minor contribution of PMCA pumps with respect to NCXs to the overall Ca\(^{2+}\) extrusion activity in excitable cells (not shown).
The 14-3-3 Protein Interacts with Both Phosphorylated and Nonphosphorylated NCX—14-3-3 proteins generally (albeit not invariably) bind to phosphorylated serine or threonine residues located in defined consensus motifs (24). The analysis of the primary structure of the large cytoplasmic loop of NCX failed to reveal sequences corresponding to the 14-3-3 binding motif. However, all of the NCX isoforms are known to be phosphorylated on some serine residues of the large cytoplasmic loop (17); these residues could conceivably still serve as binding sites for the 14-3-3 protein. Alternatively, 14-3-3 could bind NCX in a phosphorylation-independent manner (such cases have been described; see “Discussion”). Pull-down assays were thus performed with either the phosphorylated or dephosphorylated forms of the NCX protein. GST-NCX was phosphorylated in vitro in the HeLa cell lysates by supplementing these with \( [\gamma^{33}P]ATP/Mg^{2+} \) and the inhibitor of protein serine-threonine phosphatases, calyculin A. The dephosphorylated form of NCX was obtained by supplementing instead the HeLa cell lysates with 10 mM EDTA during the incubation. As shown in Fig. 10A, the incubation with Mg\(^{2+}/ATP\) led to the phosphorylation of GST-NCX by endogenous kinases in the lysates, whereas the incubation in the presence of EDTA totally prevented phosphorylation. Controls were run in which the samples were treated with alkaline phosphatase or with protein phosphatase-1 to rule out the possibility of nonspecific effects of EDTA. The results repeated those obtained with EDTA (not shown). The samples were then analyzed for binding of 14-3-3 by Western blotting; 14-3-3 bound to GST-NCX under phosphorylating conditions was significantly higher. The ability of NCX to bind 14-3-3 was then examined at different times of incubation with Mg\(^{2+}/ATP\) (Fig. 10B) (i.e. at presumably different levels of phosphorylation of Ser/Thr targets in the lysates). The amount of 14-3-3 bound to phosphorylated NCX increased between 0 and 1 min, remained approximately constant at 5 min, and then decreased progressively at 30 and 60 min. The late decrease could be due to competition for 14-3-3 binding by other proteins in the lysates, which would become phosphorylated under these conditions.

**Phosphorylated and Nonphosphorylated NCX Bind 14-3-3 with Different Affinities**—As mentioned, cases have been reported in which 14-3-3 may bind to ligands via a mechanism not involving phosphoserines or phosphothreonines (25, 26). However, the phosphorylation of the 14-3-3 ligands generally increases the affinity of 14-3-3 binding. Therefore, it was exam-
ined whether the phosphorylated and nonphosphorylated forms of NCX had different affinities for the 14-3-3 protein.

Bacterially expressed GST-NCX was incubated with different concentrations of HeLa extracts, which contain endogenous 14-3-3 proteins, under phosphorylating or dephosphorylating conditions. Immunoblotting tests (Fig. 10C) showed that after 1 h of incubation, the phosphorylated form of NCX bound the 14-3-3 protein even in the presence of the smallest amount of the lysate tested and in the presence of the presumed competition with other proteins of the lysate. By contrast, nonphosphorylated NCX was only able to bind 14-3-3 in the presence of much higher amounts of lysate.

**DISCUSSION**

The experiments described here were performed to identify possible regulatory protein partners of the NCXs. They have shown that various isoforms of the 14-3-3 protein family interact with NCX2 (but also with the other two exchangers) in a yeast two-hybrid system. The 14-3-3 protein family consists of at least seven isoforms (\(\gamma\), \(\beta\), \(\varepsilon\), \(\zeta\), \(\theta\), \(\sigma\), and \(\gamma\)), which are capable of homo- or heterodimerization, are highly conserved, and are abundantly expressed in a wide range of eukaryotes. They were logical candidates as potential regulators of NCX, since they play an important role in multiple signaling pathways and regulate the activity of numerous proteins, including some membrane proteins (27). The central groove of each subunit of the 14-3-3 dimer, which is the most highly conserved region both within and across species, is a ligand-binding site. Only small differences in ligand binding specificity thus exist among isoforms (28). The present work had shown that the large cytoplasmic loop of NCX, which contains \(\beta\) repeat sequence, is the binding site for the 14-3-3 proteins.

The interaction between the 14-3-3 proteins and NCX in HeLa and SH-SY5Y cells limited the ability of the exchangers to dispose of a cytosolic \(\text{Ca}^{2+}\) load induced by the stimulation of the cells with InsP3-generating agonists. Previous work (22) had shown that the 14-3-3 proteins had a similar inhibitory effect on at least one of the four isoforms of the other system for the extrusion of \(\text{Ca}^{2+}\) from cells, the PMCA pump. The relative contributions of the PMCA pumps and of the NCXs to the total \(\text{Ca}^{2+}\) ejection power vary from cell to cell, the exchanger being specially prominent in excitable cells like neurons. The model cells used in the experi-

**FIGURE 10. 14-3-3 binds both phosphorylated and nonphosphorylated NCX in vitro.** A, recombinant NCX-GST (25 \(\mu\)g of protein) was incubated with HeLa cell extract (200 \(\mu\)g of protein) under phosphorylating conditions or in the presence of 10 mM EDTA. For NCX phosphorylation, the lysate was supplemented with 25 \(\mu\)Ci of \([\gamma^{32}\text{P}]\text{ATP}, 2 \text{mM ATP, 15 mM Mg}^{2+}/\text{ATP}, 10 \text{mM calyculin and incubated at 37 °C for 1 h. For NCX dephosphorylation, the cell lysate was incubated at 37 °C for 1 h in the presence of 10 mM EDTA and 25 \(\mu\)Ci of \([\gamma^{32}\text{P}]\text{ATP. The probes were run on SDS-PAGE, and the gel was then dried and the NCX phosphorylation was analyzed on a STORM 820 PhosphorImager (Amersham Biosciences) or immunoblotted with an anti-14-3-3 monoclonal antibody (ab). The densities of the 14-3-3 bands were analyzed as described under "Experimental Procedures" and shown as histograms. B, NCX-GST was phosphorylated in the cell extract (20 \(\mu\)g of protein) supplemented with Mg\(^{2+}/\text{ATP/calyculin, and the phosphorylation was stopped by adding 10 mM EDTA after 0, 1, 5, 30, and 60 min of incubation at 30 °C. The samples were then analyzed for 14-3-3 binding. The histograms represent the density of the 14-3-3 bands on Western blot. C, GST-NCX was incubated with different concentrations of HeLa cell lysate under phosphorylating (Mg\(^{2+}/\text{ATP}) and dephosphorylating (10 mM EDTA) conditions for 1 h at 30 °C. The proteins were then run on SDS-PAGE and analyzed by immunoblotting for the binding of 14-3-3 protein. Additional details are found under "Experimental Procedures."**
tems described here contained the isoform of the PMCA pump (PMCA4) that is inhibited by the 14-3-3 proteins. Thus, as expected, a portion of the decrease in total Ca$^{2+}$ extrusion ability of cells overexpressing the NCXs and the 14-3-3 protein was related to the inhibition of PMCA4. However, the experiments with the PMCA pump inhibitor carboxyeosin have shown that the exchangers were still inhibited by the 14-3-3 protein. In heart cells or in neurons, where the contribution of PMCA to the total Ca$^{2+}$ extruding power is negligible, it is very likely that the inhibitory (PMCA4) that is inhibited by the 14-3-3 proteins. Thus, as the findings described here contained the isoform of the PMCA pump (PMCA4) that is inhibited by the 14-3-3 proteins. Thus, as the assembly of the 14-3-3 dimer may interfere with these secondary, phosphorylation-independent interactions, since it could be influenced by the high affinity binding of the phosphopeptide. Finally, the finding that the same interactor regulates the activity of PMCA and NCX is interesting and suggests that cells may have developed a common strategy to modulate their Ca$^{2+}$ extrusion activity.

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