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Calcium-dependent cleavage of the Na^+/Ca^{2+} exchanger by m-calpain in isolated endoplasmic reticulum

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We have recently demonstrated the localization of associated m-calpain and calpastatin in the endoplasmic reticulum (ER) of bovine pulmonary artery smooth muscle. Herein, we sought to determine the role of m-calpain on calcium-dependent proteolytic cleavage of Na^+/Ca^{2+} exchanger (NCX) in the ER. Treatment of the ER with Ca^{2+} (5 mM) dissociates m-calpain-calpastatin association leading to the activation of m-calpain, which subsequently cleaves the ER integral transmembrane protein NCX1 (116 kDa) to an 82 kDa fragment. Pre-treatment of the ER with calpain inhibitors, calpeptin (10 μM) or MDL28170 (10 μM), or Ca^{2+} chelator, EGTA (10 mM) does not cleave NCX1. *In vitro* cleavage of the ER purified NCX1 by the ER purified m-calpain also supports our finding. Cleavage of NCX1 by m-calpain in the ER may be interpreted as the main cause of intracellular Ca^{2+} overload in the smooth muscle, which could be important for the manifestation of pulmonary hypertension.

Keywords: calpastatin/endoplasmic reticulum/m-calpain/Na^+/Ca^{2+} exchanger/pulmonary smooth muscle.

Abbreviations: DHPC, 1,2-diheptanoyl-sn-phosphatidylcholine; DOPC, dioleoyl-phosphatidylcholine; ER, endoplasmic reticulum; NCX, Na^+/Ca^{2+} exchanger; WGA, wheat germ agglutinin.

Ca^{2+} plays a role as a second messenger in many biochemical and physiological events (*1*). An increase in Ca^{2+} level *in situ* caused by a variety of agonists is due to an influx of extracellular Ca^{2+} and/or release of Ca^{2+} from its suborganelle stores (*1*, *2*). The major pathway for the increase in the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) is the entry of extracellular Ca^{2+} through various types of Ca^{2+} channels (*3*). Endoplasmic reticulum (ER) sequesters a considerable amount of mobilizable Ca^{2+} and plays a pivotal role in regulating Ca^{2+} dynamics under stimulated conditions (*1*, *2*). Various mechanisms are involved in the return of cytosolic Ca^{2+} to the resting level. These mechanisms include Ca^{2+} uptake by organelles such as ER (*3*). The presence of Na^+/Ca^{2+} (NCX) in ER and NCX-mediated Ca^{2+} uptake in ER have been reported (*4*, *5*). As Na^+-dependent Ca^{2+} uptake (via NCX) and ATP-dependent Ca^{2+} uptake (via Ca^{2+} ATPase) are involved in Ca^{2+} sequestration in ER of contractile tissues (*1*, *2*), any defect in their activities could lead to an increase in $[Ca^{2+}]_i$ *in situ* (*1*, *2*). An increase in the mobilization of Ca^{2+} in pulmonary smooth muscle has been suggested to be an important mechanism for pulmonary hypertension and oedema (*6*). In cardiac muscle, the sarcolemmal NCX plays an important role in regulating $[Ca^{2+}]_i$ and changes in the activity of NCX modulate the force of contraction in the heart (*7*).

An increase in $[Ca^{2+}]_i$ causes an increase in calpain activity, which subsequently degrades some intracellular proteins by limited proteolysis (*8*). This effect could be more damaging than other reversible calcium-dependent processes such as phosphorylation and dephosphorylation (*8*). Nicotera *et al.* (*9*) suggested that inhibition of ER Ca^{2+} uptake could manifest a sustained intracellular Ca^{2+} elevation and the demise of cells by activating Ca^{2+}-dependent hydrolytic enzymes including members of the calpain protease family.

Calpains are a family of Ca^{2+} activated cysteine proteases including ubiquitous and tissue-specific isoforms that cleave their substrate proteins at discrete sites to modulate activity (*10*). While calpains are a 14 members' family, the best characterized and predominant calpains are the classical m- and μ-calpains, both of which are ubiquitously expressed enzymes (*11*). Their physiological roles have not been fully elucidated but include cell motility, cell differentiation, membrane fusion, platelet activation and signal transduction (*12*). The interaction of the endogenous inhibitor calpastatin with calpain prevents both activation and catalytic activity of calpain (*12*). *In vitro* studies have shown that calpain and calpain fragments bind to calpastatin and that calpastatin fragments bind to calpain in the presence of Ca^{2+} (*13*). Consequently, the level of calpastatin is considered to play a critical role in preventing calpain-mediated catabolism (*14*). The level of calpastatin, which is also a suicidal substrate of calpain, is decreased by calpain-mediated proteolysis (*15*).

Production of hydrogen peroxide (H_2O_2) during oxidative phosphorylation (*16*), inflammation (*17*) and ischaemia–reperfusion injury to cells and tissues (*18*) can cause oxidative stress and subsequently increase the Ca^{2+} concentration in the systems. The elevated $[Ca^{2+}]_i$ in turn activates calcium-dependent protease, calpain (*19*). Bano *et al.* (*20*) demonstrated that in brain ischaemia, an elevated production of m-calpain

cleaves plasma membrane NCX. In cardiac ischaemia–reperfusion, m-calpain has been shown to make unstable the Na-pump by the cleavage of its structural proteins, ankyrin and fodrin (*21*). These observations raise an important question about the role of m-calpain on the proteolytic cleavage of NCX in the ER.

In the present study, we report that treatment of ER isolated from bovine pulmonary artery smooth muscle with Ca^{2+} causes dissociation of the associated m-calpain and calpastatin in the ER membrane and consequently activates the m-calpain, which cleaves the ER integral transmembrane protein NCX1 (116 kDa) to an 82 kDa fragment. To the best of our knowledge, this is the first report regarding calcium-dependent cleavage of NCX1 by m-calpain in the ER. We suggest that the calcium-dependent proteolysis of NCX1 by m-calpain in the ER plays an important role in intracellular Ca^{2+} overload, which could be important for the manifestation of pulmonary hypertension (*1*, *2*).

Materials and methods

Materials

Bicinchoninic acid (BCA) protein assay kit was purchased from Pierce (Rockford, IL, USA). Casein was purchased from U.S. Biochemical Corp. (Cleveland, OH, USA). CGP-37157 was from Tocris (Ellisville, MO, USA). The flurogenic calpain substrate, SLLVY-AMC, was purchased from Bachem (King of Prussia, PA, USA). 1,2-diheptanoyl-sn-phosphatidylcholine (DHPC) and dioleoyl-phosphatidylcholine (DOPC) were obtained from Avanti polar lipids (Alabaster, AL, USA). Bio-Beads SM-2 was from Bio-Rad (CA, USA). Rabbit polyclonal voltage-dependent anion channel (VDAC) antibody was the product of Affinity Bioreagents (Golden, CO, USA); rabbit polyclonal anti-mannosidase II was obtained from U.S Biological (Swampscott, MA, USA); mouse anti-calnexin was the product of BD Transduction Laboratories (San Jose, CA, USA). Calpeptin, mouse anti β1-integrin and mouse anti Lamp-1 were the products of Calbiochem (San Francisco, CA, USA) and MDL28170 was obtained from Hoechst Marion Roussel (Bridgewater, NJ, USA). Bovine skeletal muscle m-calpain and polyclonal anti-lactate dehydrogenase (LDH) (host goat) were the products of Fitzerald Industries International, Inc. (Concord, MA, USA). Ribosomal protein S6 (RPS6) antibody was the product of Cell Signalling Technology (Boston, MA, USA). Rabbit polyclonal antibody of m-calpain was kindly donated by Professor K.K.W. Wang, Brain Institute, University of Florida, Gainesville, FL; rabbit polyclonal antibody of calpastatin was kindly donated by Professor J. Takano, Laboratory of Proteolytic Neuroscience, RIKEN Brain Science Institute, Saitama, Japan. Mouse monoclonal NCX1 (R3F1) antibody was kindly donated by Professor Kenneth D. Philipson, Department of Physiology, University of California, Los Angeles. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG and anti-rabbit IgG secondary antibodies were purchased from Zymed (San Francisco, CA, USA) and Jakson Immunoresearch (West Grove, PA, USA), respectively. DEAE cellulose, phenylsepharose and Mono Q HR (5/5) were the products of GE Healthcare Bio-Sciences Corp. (Piscataway, NJ, USA). DEAE-TSK (ToyoPearl) 650S was purchased from Supelco (Bellefonte, PA, USA). Reactive Red agarose and all other chemicals and reagents used were obtained from Sigma Chemical Co. (St Louis, MO, USA).

Methods

Isolation of pulmonary artery smooth muscle tissue. Bovine pulmonary artery smooth muscle tissue was collected according to our previously described method (*22*).

Isolation and subfractionation of rough endoplasmic reticulum

Isolation of RER. Rough endoplasmic reticulum (RER) was isolated from the bovine pulmonary artery smooth muscle tissue as previously described (*22*, *23*). Briefly, the smooth muscle tissue was homogenized with a cyclomixer in ice-cold homogenization medium A (A: 78 mM KCl, 4 mM $MgCl_2$, 8.37 mM $CaCl_2$, 10 mM EGTA, 50 mM Hepes/KOH, pH 7.0, 1 mM PMSF and complete protease inhibitor mixture without cysteine protease inhibitor). The tissue homogenate was centrifuged at 20,000*g* for 20 min to remove nuclei, cell debris, mitochondria and lysosomes. The 20,000*g* supernatant was applied to the top of a sucrose gradient composed of 1 ml of 600 mM sucrose and 15 mM CsCl layered over 2 ml of 1.3 M sucrose and 15 mM CsCl and centrifuged at 100,000*g* for 1 h in an ultracentrifuge (Beckman, USA). The 600 mM sucrose and 1.3 M sucrose interface contains smooth endoplasmic reticulum (*22*, *23*). The pellet (RER) was suspended in medium B (B : 250 mM sucrose and 100 mM Tris–HCl buffer, pH 7.4) (*22*, *23*). The RER was used immediately for the present studies. All operations were carried out at 4°C.

Removal of ribosomes from RER. Ribosomes were removed from RER according to our previously described procedure (*22*, *24*). Briefly, to the RER suspension (~0.25 mg protein), 15 mM sodium pyrophosphate was added and the pH was adjusted to 8.3 with the addition of KOH, and centrifuged at 120,000*g* for 1 h in an ultracentrifuge (Beckman, USA). The pellet was washed in medium B by resuspension and centrifugation. The resulting pellet was again resuspended in medium B and considered as ribosome-free ER (*22*, *24*). To a part of the ribosome-free ER suspension, Triton X-100 (1%) was added, incubated in a rotary shaker for 1 h and then centrifuged at 100,000*g* for 1 h. The supernatant was considered as the Triton-extracted ER lysate. All operations were carried out at 4°C.

Isolation of the ER membranes by Na_2CO_3 treatment. The ribosome-free ER suspension was diluted with 100 mM sodium carbonate and pH was adjusted to 11.5 to bring the protein concentration to ~0.5 mg ml^{-1} and incubated at 0°C for 30 min. The resulting suspension was centrifuged at 150,000*g* for 1 h in an ultracentrifuge (Beckman, USA). The supernatant was decanted and the pellet (membrane) was washed in medium B by resuspension and centrifugation. The pellet was resuspended in medium B and considered as ER membrane (*22*, *24*). To a part of the ER membrane suspension, Triton X-100 (1%) was added, incubated in a rotary shaker for 1 h and then centrifuged at 150,000*g* for 1 h. The supernatant was considered as the Triton-extracted ER membrane lysate (*22*, *24*). All operations were carried out at 4°C.

Electron microscopic study. Electron microscopic (EM) studies of RER, ribosome-free ER and the ER membrane were performed by following the procedure of Baudhuin *et al.* (*25*), with some modifications as described by Samanta *et al.* (*22*).

Protein assay. Protein concentration was estimated by the BCA protein assay kit by following the procedure of Smith *et al.* (*26*) using bovine serum albumin (BSA) as the standard.

SDS–PAGE and western blot. Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis was performed according to the procedure of Laemmli (*27*). The protein bands were visualized by the silver staining method (*28*). Western blot was performed according to the procedure described by Towbin *et al.* (*29*).

Co-Immunoprecipitation of m-calpain and calpastatin. Of the m-calpain antibody (or calpastatin antibody), 5 µg was incubated with 50 µl of protein A/G agarose beads for 40 min at 4°C as described previously (*22*). The calpain antibody (or calpastatin antibody) was substituted with IgG in controls. The protein A/G agarose-anti-m-calpain (or anti-calpastatin) complex was washed three times with PBS containing 0.1% Triton X-100 and then incubated overnight at 4°C with the sample (~1 mg protein). The beads were then washed three times with PBS containing 0.1% Triton X-100. The immunoprecipitate was subsequently subjected to western blotting using calpastatin (or m-calpain) antibody to assess co-immunoprecipatation with m-calpain or calpastatin.

Measurement of Ca^{2+} level in the ER vesicles and determination of NCX1 activity. The Ca^{2+} concentration in the ER vesicles was determined by following the previously described procedure (*30*, *31*) with some modifications. Briefly, to the ER vesicles, 10 μM Fura-2 AM was added and incubated for 10 min at 30°C. This was then diluted with ice-cold 180 mM KCl, 50 μM EGTA and then centrifuged at 100,000*g* for 15 min in a TLA100.3 rotor (Beckman Optima TLX) to wash and sediment the Fura-2 loaded ER vesicles. The sedimented ER vesicles were resuspended in a small volume of the ice-cold medium to obtain a concentrated stock suspension of Fura-2 loaded ER vesicles. The ER Ca^{2+} level was determined by measuring the Fura-2 fluorescence with fluorescence spectrophotometer (Hitachi F-7000). The fluorescence of Fura-2 loaded ER vesicles (1 mg) was measured at 37°C and the Ca^{2+} concentration in the ER vesicles was determined. The sample was excited at wavelengths alternating between 340 and 380 nm and the fluorescence emission at 510 nm was recorded, and the Ca^{2+} concentration in the ER was determined by following the method of Grynkiewicz *et al*. (*32*).

For determination of Na^{+}-dependent Ca^{2+} uptake, the ER vesicles were pre-treated with or without CGP-37157 (NCX inhibitor) (20 μM) or calpeptin (10 μM) or MDL28170 (10 μM) (calpain-specific inhibitors) for 10 min followed by addition into the NaCl (140 mM)/MOPS (20 mM) buffer (pH 7.4) at 37°C for 30 min, which were then taken into 1 ml of the Ca^{2+} uptake medium containing 140 mM KCl, 20 mM MOPS, 0.4 μM valinomycin and 100 μM $CaCl_2$ (pH 7.4) (NCX assay condition). After10 min, the reaction was stopped by the addition of ice-cold 0.03 ml stopping solution containing 140 mM KCI, 1 mM $LaC1_3$ and 20 mM MOPS (pH 7.4). The sample was washed twice with ice-cold washing solution containing 140 mM KC1, 0.1 mM $LaCl_3$ and 20 mM MOPS (pH 7.4). Then Fura-2 was loaded to those ER vesicles. The ER Ca^{2+} level was determined by measuring Fura-2 fluorescence as described above.

Spectrophotometric assay of m-calpain. m-Calpain activity was assayed spectrophotometrically using the calpain-specific substrate, SLLVY-AMC (Bachem) as described by Atsma *et al*. (*33*) with some modifications. Briefly, before and after the Ca^{2+} treatment, the ER was diluted in the buffer containing 130 mM KCl, 9 mM Tris–PO_4, 4 mM Tris–HCl (pH 7.5) and in the presence of 50 μM SLLVY-AMC. Activity was measured under linear conditions as a function of AMC hydrolysis using excitation and emission wavelengths of 355 and 444 nm, respectively. ER, incubated in the absence of substrate, exhibited the same fluorescence as the buffer alone. m-Calpain activity was measured in the ER preincubated with the calpain-specific inhibitors, calpeptin (10 μM) or MDL28170 (10 μM) for 20 min followed by treatment with 5 mM $CaCl_2$. m-Calpain activity was also measured upon pre-treatment of the ER with the Ca^{2+} chelator, EGTA (10 mM), separately for 20 min followed by the addition of 5 mM $CaCl_2$.

Determination of the localization of NCX1 in the ER

Carbonate treatment of the ER. Sodium carbonate treatment has been used to determine the association of membrane-associated proteins with the outer surface of membrane either peripherally or integrally (*24*). The ER suspension was subjected to carbonate treatment, which has been used to prepare ER membrane as described earlier in the method.

Immunogold labelling and electron microscopy. Immunoelectron microscopic studies were performed according to our previously described procedure (*31*). Briefly, soon after collection of the fresh bovine pulmonary artery smooth muscle tissue from slaughter house, the tissue samples were fixed for 24 h at 4°C with 8% paraformaldehyde in 120 mM phosphate buffer (pH 7.3), washed for 20 min with phosphate buffered saline (PBS) and saturated with 2 M sucrose in PBS (4 × 5 min) for cryoprotection, frozen in liquid nitrogen. Ultrathin ~50-nm thick cryosections were cut on a ultramicrotome and collected onto EM grid then incubated with 5% foetal calf serum in PBS for 30 min at room temperature to eliminate non-specific reactions, washed 2 × 5 min in PBS and incubated for 40 min with appropriate antibodies and diluted with 10% foetal calf serum in PBS. After washing with PBS (4 × 5 min), the sections were incubated for 30 min with secondary goat-anti-rabbit antibody labelled with 5 nm gold particles. After washing with PBS (6 × 5 min) and redistilled water (5 × 2 min), the samples were impregnated for 10 min with a mixture of 2% methylcellulose and 3% uranyl acetate (9 : 1) examined in a FEI Tecnai G^2 Transmission Electron Microscope (with SIS Protem programme).

Proteinase K treatment of the ER. Proteinase K (PK) cleaves proteins present in the outer surface of a membrane (*34*). To 2 ml (0.25 mg protein) of the ER suspension, 0.5 μg ml^{-1} of PK was added. The sample and control (ER suspension) were incubated with rotation at 37°C for 20 min. After incubation, the reaction was quenched with 1 mM PMSF to inhibit PK and then the resulting suspension was spun at 100,000*g* in a Beckman Optima TLX ultracentrifuge with TL-100.3 rotor for 1 h at 4°C. The pellet was washed with the isolation medium (250 mM sucrose and 100 mM Tris–HCl buffer, pH 7.4) by resuspension and centrifugation. The resulting pellet was resuspended in a buffer containing 70 mM sucrose, 220 mM D-mannitol, 2 mM HEPES and 0.5 mg ml^{-1} BSA, pH was adjusted to 7.4 with KOH.

Purification of m-calpain. m-Calpain was purified from bovine pulmonary smooth muscle ER membrane according to Thompson *et al*. (*35*) with some modifications. Briefly, the Triton X-100 (1% Triton X-100) solubilized ER membrane fraction was centrifuged at 20,000*g* for 30 min. The supernatant was saturated with 50% ammonium sulphate and the precipitated protein was collected by centrifugation at 45,000*g* for 1 h. The precipitate was redissolved in the TEM (20 mM Tris–HCl, pH 7.5; 1 mM EDTA, 0.1% 2-mercaptoethanol) buffer and then it was dialysed against several changes of TEM and clarified by centrifugation at 100,000*g* for 1 h. Then the solution was applied onto a series of chromatographic columns: DEAE-cellulose, phenylsepharose, reactive red and DEAE-TSK anion exchange columns. Finally, the DEAE-TSK anion exchange column was eluted with a linear gradient from 135 to 275 mM KCl in 20 mM Tris–MES (pH 6.5), 1 mM EDTA, 0.1% 2-mercaptoethanol and m-calpain containing fractions were pooled and concentrated by ultrafiltration (Amicon YM-10 membrane; molecular weight cutoff 10 kDa).

The purity of the m-calpain from the ER fraction was determined by SDS–PAGE, western blot and N-terminal amino acid sequence analyses.

Purification and reconstitution of NCX1

Purification of NCX1. Freshly prepared 300 mg of ER membrane was solubilized in a buffer containing 20 mM MOPS–Tris (pH 7.4), 2 M glycerol, 40 mM sucrose, 1 mM DTT and 1 mM EGTA to obtain the desired final detergent concentration of 6 mg ml^{-1} in the solubilizing buffer. After centrifugation at 150,000*g* for 15 min the extracted proteins were taken for fractionation as previously described (*36*) with some modifications. Briefly, the solubilized sample was applied to the DEAE-sepharose column (0.6 ml bed volume in a 1.5 cm diameter column pre-equilibrated with 140 mM NaCl, 0.5 mM decylmaltoside). The column was eluted with 1.4 ml of 200 mM NaCl, 0.5 mM decylmaltoside, and then the fraction enriched in NCX1 activity was eluted with 2.4 ml of 500 mM NaCl, 3 mM 3-[(3-cholamidopropyl)dimethylamonio]-1-propanesulfonate (CHAPS). This fraction was applied twice to the wheat germ agglutinin (WGA)-agarose column (0.4 ml bed volume in a 0.8 cm diameter column pre-equilibrated with 140 mM NaCl, 1 mM CHAPS). The WGA column was washed with 0.8 ml of 500 mM NaCl, 1 mM CHAPS, and then eluted with 1.5% Triton X-100, 500 mM NaCl, 20 mM MOPS–Tris (pH 7.4). The eluted fraction was concentrated by Amicon Ultra filtration cell with YM10 membrane (molecular weight cutoff 10 kDa). Both columns were run at room temperature. Purified and concentrated pool was analysed by SDS–PAGE, western blot and N-terminal amino acid sequencing. In order to determine the Ca^{2+} transport activity of the purified protein, it was reconstituted into proteoliposomes.

Reconstitution of purified NCX1 into DOPC. Reconstitution experiments were performed by following our previously published protocol (*37*). Briefly, 50 mg of DOPC in 2 ml of buffer [50 mM MOPS (pH 7.4), 0.25 M sucrose, 1 M KCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 1 mM DTT, 0.025% sodium azide] containing the detergent, Triton X-100 to give a final lipid:detergent ratio of 1 : 1.5 (w/w) was vortex mixed vigorously for ~60 s to disperse the lipid and left at room temperature for 90 min. The suspension was clarified by

sonication for 1–2 min with a Soni Prep model 150 sonifier/cell disrupter. Purified NCX1 was solubilized at a ratio of 0.6 : 1 (w/w; detergent:protein) (*37*) by vortexing for 60 s and left at room temperature for 10 min followed by further incubation on ice for an additional 90 min. The solubilized NCX1 was centrifuged at low speed to remove insoluble material and aggregates. The clear sample was then mixed with pre-solubilized exogenous lipid to a desired molar ratio of lipid to NCX1 (1500:1) (*37*), and vortex mixed for 30 s and left at room temperature for 15 min. This mixture was incubated at 4°C for 90 min. At the end of the incubation, the detergent was removed by adsorption on Bio-Beads SM-2 in two batches of 700 mg for 1 h at 4°C. The reconstituted vesicles were separated from the Bio-Beads by low-speed centrifugation. The proteoliposomes were then purified on a discontinuous sucrose gradient.

Measurement of the Ca^{2+} level in the proteoliposomes and determination of NCX1 activity. Ca^{2+} concentrations in the proteoliposomes under normal condition and under NCX assay condition with or without CGP-37157 (NCX inhibitor) (20 μM) pre-treatment were measured as described previously (*31*), and Na^+-dependent Ca^{2+} uptake was determined. Fluorescence of Fura-2 loaded proteoliposomes was measured as described previously (*31*, *37*) and the Ca^{2+} concentration in the proteoliposomes was determined by following the method of Grynkiewicz *et al*. (*32*).

For determination of Na^+-dependent Ca^{2+} uptake, the proteoliposomes pre-treated with or without CGP-37157 (NCX inhibitor) (20 μM) for 10 min were added into the NaCl (140 mM)/MOPS (20 mM) buffer (pH 7.4) at 37°C for 30 min, which were then taken into 1 ml of the Ca^{2+} uptake medium containing 140 mM KCl, 20 mM MOPS, 0.4 μM valinomycin and 100 μM $CaCl_2$ (pH 7.4) (NCX assay condition). After 10 min, the reaction was stopped by the addition of ice-cold 0.03 ml stopping solution containing 140 mM KCI, 1 mM $LaCl_3$ and 20 mM MOPS (pH 7.4). Sample was washed twice with ice-cold washing solution containing 140 mM KC1, 0.1 mM $LaCl_3$ and 20 mM MOPS (pH 7.4). Then fluorescence of Fura-2 loaded Ca^{2+}-treated proteoliposomes was measured as described in the previous section.

Determination of amino-terminal sequences of the purified m-calpain and NCX1. The purified m-calpain (80 kDa large and 28 kDa small subunits) and NCX1 (116 kDa) were subjected to SDS–PAGE (10% for m-calpain and 7.5% for NCX1). Then the gels were electroblotted onto PVDF membranes. The protein bands of interest were cut out and their N-terminal amino acid sequences were determined according to the procedure described by Matsudaira (*38*).

In vitro cleavage of ER NCX1. The purified NCX1 (116 kDa) was suspended in a buffer containing 50 mM NaCl, 10 mM EGTA, 0.1% Triton X-100 and 100 mM HEPES, pH 7.5 (calpain buffer). To it, 0.5 μg of the purified m-calpain and $CaCl_2$ (5 mM final concentration) were added and incubated for 20 min. The reaction was stopped by adding 20 μl of the sample buffer (8 M urea, 0.5 M DTT, 5% SDS, 5 mM EDTA, 50 mM Tris–HCl, pH 8.0 and bromophenol blue) (*20*). The sample was separated by SDS–PAGE and western blot study was performed using the NCX1 monoclonal (R3F1) antibody.

Statistical analysis. The appropriate ANOVA was performed for each data set by using SigmaStat statistical software. Individual means were compared with Fisher's protected least-significant difference test.

Results

Characterizations of the RER vesicles, ribosome-free ER vesicles and ER membrane

Characterization of RER vesicles. We ascertained the purities of the RER vesicles by electron microscopic and western blot studies as previously described (*22*).

Removal of ribosomes from the RER and characterization of ribosome-free ER vesicles. The purities of the ribosome-free ER vesicles were determined by electron microscopic and western blot studies as previously described (*22*).

EM study of the ER membrane. The characteristic trilaminar structure of the ER membrane was determined by electron microscopic study as described previously (*22*).

Identification and determination of activity of NCX1 in the ER

Identification of NCX1 in the ER. Western blot of the Triton-extracted ER lysate with NCX1 monoclonal antibody (R3F1) showed a band at 116 kDa (Fig. 1A), which indicated the presence of NCX1 in the ER. Alternative splicing of an intracellular loop of NCX1 can lead to a number of smaller isoforms (*39*). The observed band having an apparent molecular mass of 116 kDa (Fig. 1A) appeared to correspond to the reported size of NCX1.3 isoform (*39*).

Determination of NCX1 activity in the ER. Figure 1B indicated the Na^+-dependent Ca^{2+} uptake by NCX1 in the ER, which appeared to be 57 ± 2.56 $[(354 \pm 11.03) - (297 \pm 9.28)]$ nmol/10 min/mg protein. But, in presence of NCX inhibitor, CGP-37157 (20 μM), no appreciable Na^+-dependent Ca^{2+} uptake in the ER has been detected (Fig. 1B). In the presence of calpeptin or MDL28170 (calpain specific inhibitors), there is no change in Na^+-dependent Ca^{2+} uptake by NCX1 in the ER (Fig. 1B), which indicated that

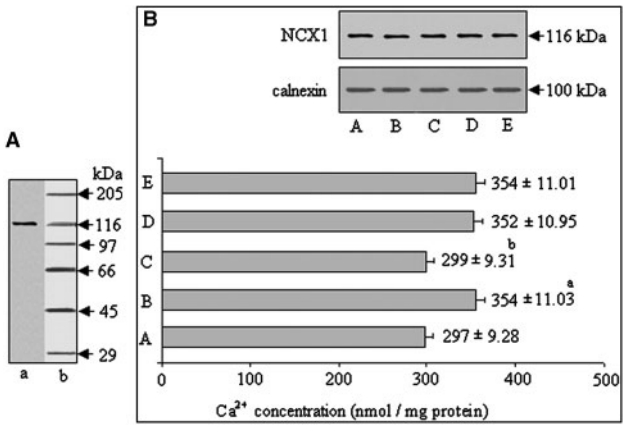

Fig. 1 Identification and determination of activity of NCX1 in the ER. (A) Western blot study of the ER lysate with NCX1 monoclonal antibody (carried out on 7.5% SDS–PAGE); lane a, ER lysate; lane b, molecular weight standards. (B) Determination of NCX1 activity by measuring the Ca^{2+} level in the ER vesicles. A, ER vesicles under normal condition (not NCX assay condition); B, ER vesicles under NCX assay condition; C, CGP-37157 (NCX inhibitor) (20 μM) pre-treated ER vesicles under NCX assay condition; D, calpeptin (calpain specific inhibitor) (10 μM) pre-treated ER vesicles under NCX assay condition; E, MDL-28170 (calpain specific inhibitor) (10 μM) pre-treated ER vesicles under NCX assay condition. Data are mean ± SE ($n = 4$); $^aP < 0.001$ compared to the basal condition (under normal condition); $^bP < 0.001$ compared to NCX assay condition. In all the above conditions (A, B, C, D and E), NCX1 (116 kDa) remained intact, which was shown in the western blot of NCX1 in the inset [lanes A, B, C, D and E; equal amount (50 μg) of protein was loaded in each lane using calnexin, ER membrane marker protein as the loading control].

m-calpain has no role upon Na^+-dependent Ca^{2+} uptake by NCX1 in the ER and NCX1 remain intact in all the assay conditions that was shown in the western blot of NCX1 in the inset of the Fig. 1B, wherein calnexin (ER membrane marker protein) has been used as the loading control.

Proteolytic cleavage of NCX1 by m-calpain in the ER

In order to determine the proteolytic cleavage of NCX1 by m-calpain, the isolated ER vesicles were incubated in a buffer (130 mM KCl, 9 mM Tris–PO_4, 4 mM Tris–HCl, pH 7.5) at 37°C for 10 min supplemented with 5 mM $CaCl_2$. When desired, ER was pre-treated with calpeptin (10 μM) or MDL28170 (10 μM) for 20 min. Similarly, ER was pre-treated with EGTA (10 mM) for 20 min. The dose and time of treatments of the agents used were determined to be optimum in separate experiments (data not shown).

After treatment of the ER with 5 mM Ca^{2+}, we have performed the western blot with NCX1 monoclonal (R3F1) antibody. We found an 82-kDa fragment in the western blot (Fig. 2A, lane b). On the other hand, no band having a molecular mass of 82 kDa was observed in the western blot when the ER was pre-treated with the calpain-specific inhibitor, calpeptin (10 μM) or MDL28170 (10 μM) or with the Ca^{2+} chelator, EGTA (10 mM) (Fig. 2A, lanes d, f and h).

It was our interest to determine whether the m-calpain and calpastatin were associated in the ER in Ca^{2+} (5 mM)-treated condition. We, therefore, performed the co-immunoprecipitation study of m-calpain and calpastatin on the Ca^{2+} (5 mM)-treated ER. The result revealed that there was no association between m-calpain and calpastatin in the Ca^{2+} (5 mM)-treated ER (Fig. 2B and C), indicating that dissociation of m-calpain and calpastatin has occurred in the presence of Ca^{2+} (Fig. 2B and C). Thus, in the presence of Ca^{2+} (5 mM), the m-calpain is activated, which leads to the cleavage of NCX1 in the ER (Fig. 2A, lane b).

In order to determine the m-calpain activity under the Ca^{2+} (5 mM)-treated condition, we measured the m-calpain activity with the fluorogenic calpain substrate SLLVY-AMC. In the presence of Ca^{2+},

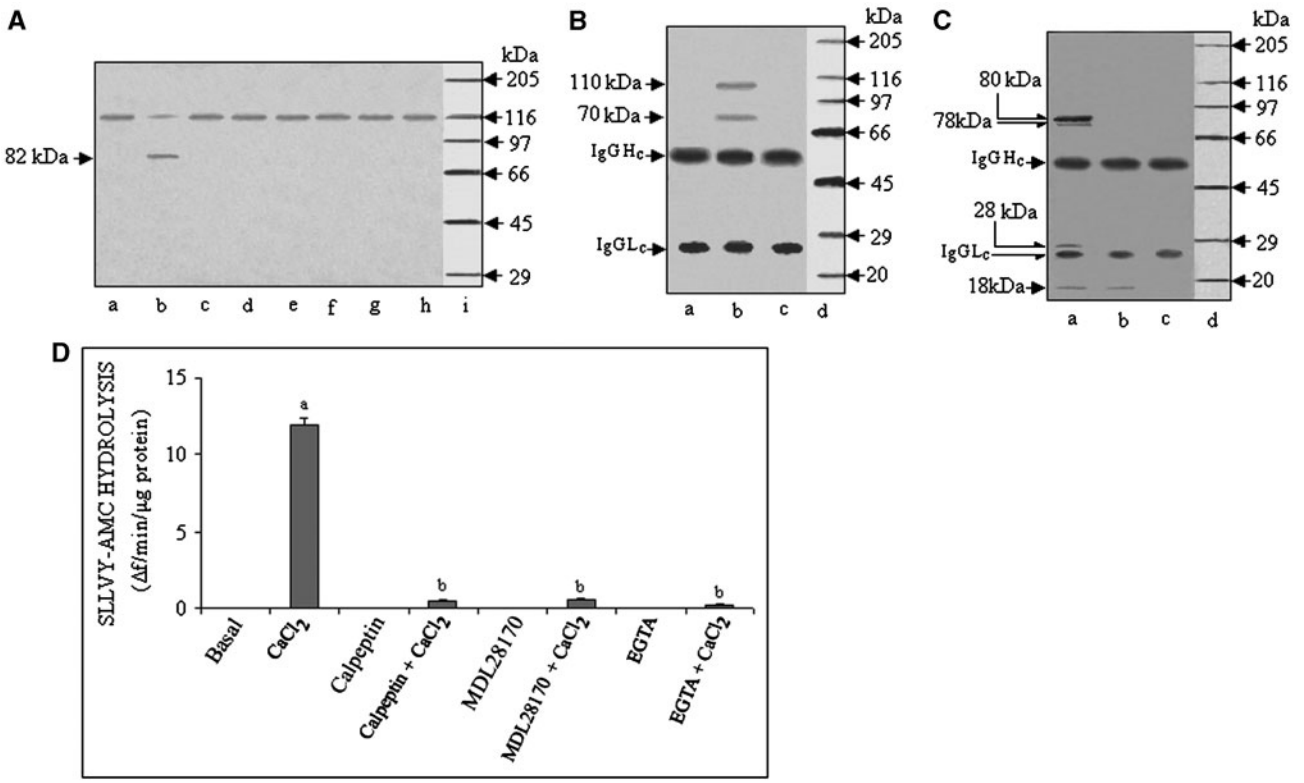

Fig. 2 Effect of treatment of Ca^{2+} (5 mM) on m-calpain activity and cleavage of NCX1 in the ER. (A) Western blot of NCX1 (carried out on 7.5% SDS–PAGE). Lane a, ER suspension (control); lane b, Ca^{2+} (5 mM) treated ER suspension; lane c, calpeptin (calpain specific inhibitor) (10 μM) treated ER suspension; lane d, ER was pre-treated with calpeptin (10 μM) followed by treatment with Ca^{2+} (5 mM); lane e, MDL 28170 (calpain specific inhibitor) (10 μM) treated ER suspension; lane f, ER was pre-treated with MDL 28170 (10 μM) followed by treatment with Ca^{2+} (5 mM); lane g, EGTA (Ca^{2+} chelator) (10 mM) treated ER suspension; lane h, ER was pre-treated with EGTA (10 mM) followed by treatment with Ca^{2+} (5 mM); lane i, molecular weight standards. (B and C) Immunoprecipitation (IP) of the Ca^{2+} (5 mM) treated ER suspension with polyclonal m-calpain and calpastatin antibodies followed by western blotting with the calpastatin and m-calpain antibodies, respectively (carried out on 7.5% SDS–PAGE). (B) Lane a, IP m-calpain blotted with the calpastatin antibody; lane b, IP calpastatin blotted with the calpastatin antibody; lane c, IgG control blotted with the calpastatin antibody; lane d, molecular weight standards. (C) Lane a, IP m-calpain blotted with the m-calpain antibody; lane b, IP calpastatin blotted with the m-calpain antibody; lane c, IgG control blotted with the m-calpain antibody; lane d, molecular weight standards. (D) Effect of different calpain inhibitors, calpeptin (10 μM) and MDL28170 (10 μM) and Ca^{2+} chelator, EGTA (10 mM) on Ca^{2+} (5 mM) induced m-calpain activity in the ER. Data are mean ± SE ($n = 4$); [a] $P < 0.001$ compared to the basal condition; [b] $P < 0.001$ compared to Ca^{2+} (5 mM) treatment.

m-calpain cleaved the SLLVY-AMC and we obtained m-calpain activity, but in the absence of Ca^{2+}, no m-calpain activity was detected (Fig. 2D).

Pre-treatment of the ER with calpain-specific inhibitors, calpeptin (10 μM) and MDL28170 (10 μM) elicited, respectively, 96% and 95% decrease in the m-calpain activity caused by Ca^{2+} (Fig. 2D).

Similarly, pre-treatment of the ER with EGTA (10 mM) almost completely inhibited the m-calpain activity caused by Ca^{2+} (Fig. 2D).

Determination of the localization of NCX1 in the ER

Carbonate treatment. In order to determine the localization of the NCX1 in the ER, we performed Na_2CO_3 treatment. Treatment of the ER with Na_2CO_3 removed the ER luminal protein calreticulin (Fig. 3A, lane c). Removal of the ER lumenal protein was confirmed by the presence of calreticulin in the Na_2CO_3 supernatant isolated subsequent to ultracentrifugation (Fig. 3A, lane c). In contrast, calnexin, a transmembrane protein associated with the ER was not removed by carbonate and did not appear in the carbonate supernatant (Fig. 3A, lane c).

Similar experiments were done to determine the localization of NCX1 in the ER vesicle after carbonate treatment as described. We observed the presence of NCX1 in the carbonate pellet (Fig. 3A, lane b) but not in the supernatant (Fig. 3A, lane c). These results indicate that the NCX1 is an integral membrane protein or localized in the ER similar to integral membrane proteins.

Immunogold labelling and electron microscopy. In order to further confirm the localization of NCX1, we performed immunoelectron microscopic study of the ER isolated from pulmonary artery smooth muscle tissue as described under the 'Materials and methods' section with the appropriate antibodies of the ER membrane marker protein, calnexin and ER luminal marker protein, calreticulin. Figure 3B and C represented the localization of calnexin in the ER membrane and calreticulin in the ER lumen. Similarly, Figure 3D confirmed localization of NCX1 in the ER membrane.

Determination of the orientation of the NCX1 with the ER membrane. ER vesicles were treated with proteinase K (PK), which enzymatically cleaves proteins on the cytoplasmic face of ER (*34*). ER transmembrane protein calnexin and ER luminal protein calreticulin subsequent to the treatment served as controls (Fig. 3E). Treatment with PK cleaved the cytosolic domain of calnexin, generating an 80-kDa fragment (Fig. 3E, lane c). Calreticulin, an ER luminal protein was resistant to PK treatment (Fig. 3E, lane c). NCX1 was not completely removed from the ER vesicles after PK treatment. We found a 56-kDa fragment of NCX1 in the PK supernatant (Fig. 3E, lane c) and ~60 kDa

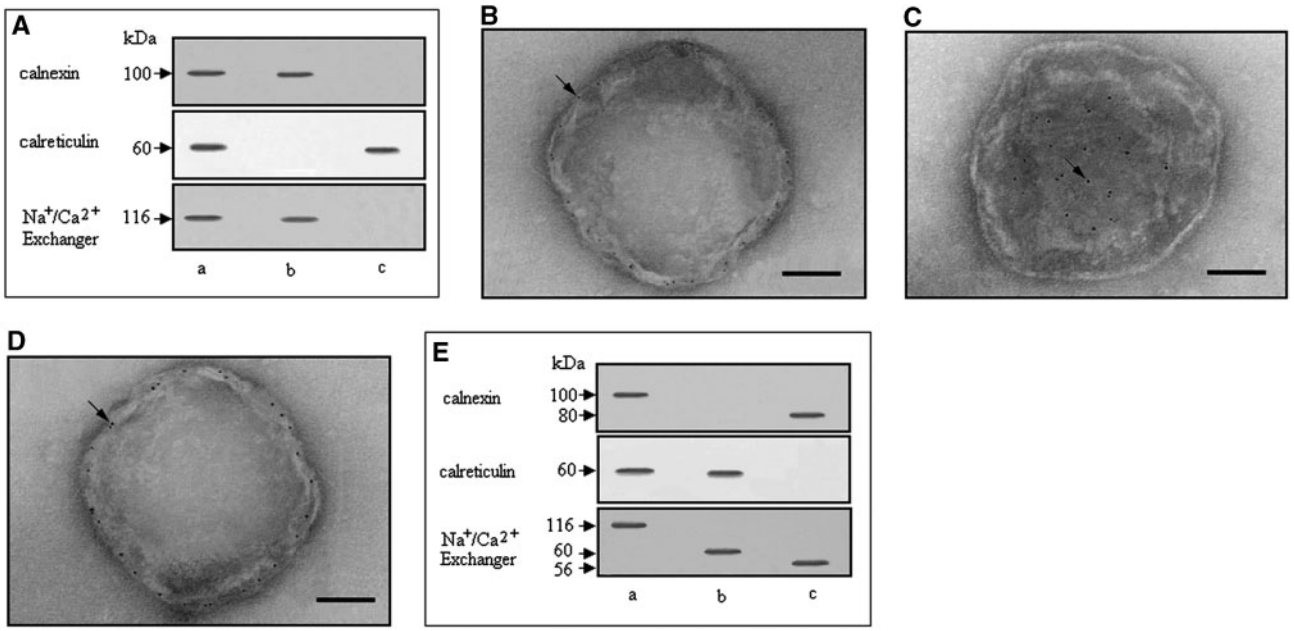

Fig. 3 Localization of NCX1 in the ER. (A) Treatment of the ER with Na_2CO_3 to determine integral association of NCX1 with the ER membrane. ER suspension (0.25 mg) was incubated with 0.2 M Na_2CO_3, (pH 11.5) for 30 min at 0°C and subjected to SDS–PAGE (10% for calreticulin and 7.5% for calnexin and NCX1) and western blot analysis was done using antibodies to calnexin and calreticulin as control proteins of ER membrane and lumen, respectively, and to NCX1. Lane a, ER suspension; lane b, Na_2CO_3 pellet; lane c, Na_2CO_3 supernatant. (B) Immunoelectron microscopy of ER membrane marker protein calnexin; scale bar 0.5 μm × (60,000). Arrow indicates the ER membrane localization of calnexin. (C) Immunoelectron microscopy of ER luminal marker protein calreticulin; scale bar 0.5 μm × (60,000). Arrow indicates the ER luminal localization of calreticulin. (D) Immunoelectron microscopy of NCX1. Immunocomplexes visualized with 5 nm gold particle present in the ER membrane; scale bar 0.5 μm × (60,000). Arrow indicates the ER membrane localization of NCX1. (E) Determination of the orientation of NCX1 in the ER membrane. ER suspension (0.25 mg) was incubated with PK (0.5 μg ml^{-1}) for 20 min at 37°C and subjected to SDS–PAGE (10% for calreticulin and 7.5% for calnexin and NCX1) and western blot analyses were performed using antibodies to calnexin and calreticulin (as control proteins of ER membrane and lumen, respectively) and NCX1. Lane a, ER suspension; lane b, PK pellet; lane c, PK supernatant.

fragment of NCX1 in the PK pellet (Fig. 3E, lane b), indicating that the NCX1 is a transmembrane protein and exposed towards cytosolic face of the ER membrane.

Purification of the ER m-calpain and NCX1 and in vitro cleavage of NCX1

Purification of m-calpain and NCX1 from the ER. In order to ascertain the m-calpain-mediated cleavage of the NCX1 of ER, we have purified the m-calpain and NCX1 from the ER. Figure 4A and B depicted the SDS–PAGE and western blot profiles of the purified m-calpain.

Figure 4C and D depicted the SDS–PAGE and western blot of the purified NCX1. In order to ascertain the functional characteristics of the ER NCX1, we have incorporated the purified 116 kDa protein into the proteoliposomes and the Na^+-dependent Ca^{2+} uptake in the proteoliposomes was determined. Figure 4E indicated the Na^+-dependent Ca^{2+} uptake mediated by the reconstituted 116 kDa protein in the proteoliposomes, which appeared to be 69 ± 3.11 $[(143 \pm 10.53) - (74 \pm 5.50)]$ nmol/10 min/mg protein.

For further confirmation of the purified proteins, we determined the N-terminal amino acid sequences of the purified m-calpain large (80 kDa) and small (28 kDa) subunits and also of the purified 116 kDa NCX1.

The N-terminal amino acid sequence of first 25 amino acid residues of the purified bovine ER m-calpain large (80 kDa) and small (28 kDa) subunits was determined and compared with that of human m-calpain (*40*, *41*) (Tables 1 and 2). The bovine ER m-calpain large subunit has 88% sequence identity with the corresponding human m-calpain large subunit (*40*) (Table 1) and the bovine ER m-calpain small subunit has 84% sequence identity with the corresponding human m-calpain small subunit (*41*) (Table 2).

Similarly, we have also determined the N-terminal amino acid (first 10) sequence of the purified bovine ER 116 kDa NCX1 and compared with the amino acid sequence of human NCX1 (*42*) (Table 3). The result showed that the purified NCX1 has a similar sequence with that of human NCX1 (*42*) (Table 3).

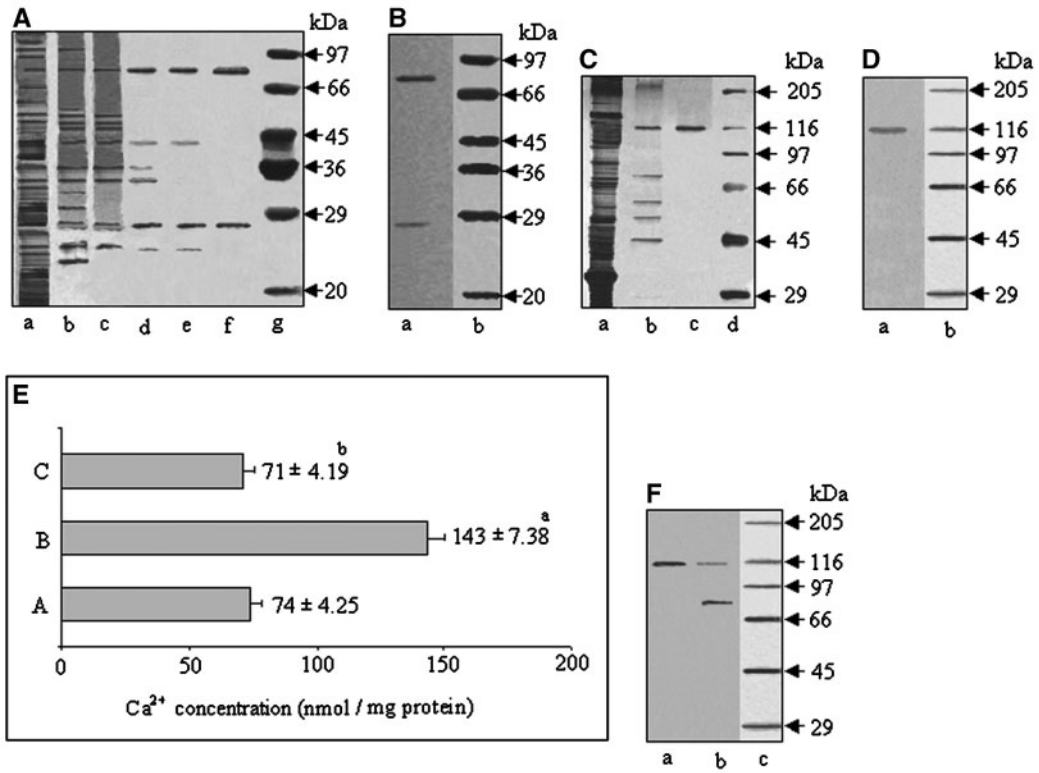

Fig. 4 Purification of ER m-calpain and NCX1, determination of the activity of the purified NCX1 in the proteoliposomes and *in vitro* cleavage of the purified NCX1 by the purified m-calpain. (A) 10% SDS–PAGE protein purification profile for m-calpain. Lane a, solubilized ER membrane; lane b, 0–50% ammonium sulphate precipitate; lane c, DEAE cellulose eluate fraction; lane d, phenyl sepharose eluate fraction; lane e, reactive red eluate fraction; lane f, DEAE-TSK eluate fraction; lane g, molecular weight standards. (B) Western blot of the purified m-calpain with rabbit polyclonal m-calpain antibody (carried out on 10% SDS–PAGE). Lane a, purified m-calpain; lane b, molecular weight standards. (C) 7.5% SDS–PAGE protein purification profile for NCX1. Lane a, solubilized ER membrane; lane b, DEAE sepharose eluate fraction; lane c, WGA agarose eluate fraction; lane d, molecular weight standards. (D) Western blot of the purified NCX1 with the NCX1 monoclonal antibody (carried out on 7.5% SDS–PAGE). Lane a, purified NCX1; lane b, molecular weight standards. (E) Determination of NCX1 activity by measuring Ca^{2+} level in the proteoliposomes. A, proteoliposomes under normal condition (not NCX assay condition); B, proteoliposomes under NCX assay condition; C, CGP-37157 (NCX inhibitor) (20 μM) pre-treated proteoliposomes under NCX assay condition. Data are mean ± SE ($n = 4$); $^{a}P < 0.001$ compared to the basal condition (under normal condition); $^{b}P < 0.001$ compared to NCX assay condition. (F) The ER purified 116 kDa NCX1 was incubated with purified m-calpain as described in experimental procedures. The degradation product of NCX1 was detected by western blot analysis with NCX1 monoclonal antibody. Lane a, the purified NCX1; lane b, the purified NCX1 treated with purified m-calpain; lane c, molecular weight standards.

Table 1. N-terminal amino acid (1–25 residues) sequence of a bovine ER m-calpain large (80 kDa) subunit as compared with the human m-calpain large (80 kDa) subunit sequence (*40*).

Amino acid sequence of an 80 kDa large subunit of bovine ER m-calpain	M-A-G-I -A-A-K-L-A-K-D-Q-E-A-A-D-G-L-G-S-H-E-R-G-I
Amino acid sequence of an 80 kDa large subunit of human m-calpain[a]	M-A-G-I-A-A-K-L-A-K-D-R-E-A-A-E-G-L-G-S-H-E-R-A-I

[a]Taken from reference (*40*).

Table 2. N-terminal amino acid (1–25 residues) sequence of a bovine ER m-calpain small (28 kDa) subunit as compared with the human m-calpain small (28 kDa) subunit sequence (*41*).

Amino acid sequence of a 28 kDa small subunit of bovine ER m-calpain	M-F-L-V-N-S-F-L-K-G-G-G-G-G-G-G-G-G-G-L-G-G-G-L-G
Amino acid sequence of a 28 kDa small subunit of human[a]	M-F-L-V-N-S-F-L-K-G-G-G-G-G-G-G-G-G-G-G-L-G-G-G-L

[a]Taken from reference (*41*).

Table 3. N-terminal amino acid (1–10 residues) sequence of bovine ER NCX1 as compared with the human NCX1 sequence (*42*).

Amino acid sequence of bovine ER NCX1	M-Y-N-M -R-R-L-S-L-S
Amino acid sequence of human NCX1[a]	M-Y-N-M -R-R-L-S-L-S

[a]Taken from reference (*42*).

In vitro cleavage of the ER NCX1. To determine the *in vitro* cleavage of NCX1 of the ER, the purified NCX1 (116 kDa) was resuspended in the calpain buffer and subsequently 0.5 μg of the purified m-calpain was added to this suspension as described under the 'Materials and methods' section. Western blot analysis of the suspension with the NCX1 monoclonal antibody detected the fragment of about 82 kDa, indicating proteolytic cleavage of the ER NCX1 by ER m-calpain (Fig. 4F).

Discussion

The concentration of Ca^{2+} in the extracellular space is between 1 and 10 mM, whereas the Ca^{2+} in the cytosol $[Ca^{2+}]_i$ is in the order of 0.1 μM thus creating a large inwardly directed electrochemical gradient forcing Ca^{2+} entry across the plasma membrane (*43*). Cells have several mechanisms for maintaining a low $[Ca^{2+}]_i$, which at the same time also ensues that the appropriate transient peak levels of Ca^{2+} are reached during activation. Notable among these are accumulation of the ion into the ER by the Na^+-dependent Ca^{2+} uptake via NCX (*44*). The ER NCX is one of the main Ca^{2+} transport mechanisms in the pulmonary smooth muscle, which apparently plays an important role in regulating $[Ca^{2+}]_i$ homoeostasis and subsequently pulmonary vascular tone (*6*).

In the present study, western blot (Fig. 1A) and N-terminal amino acid sequence (Table 3) analyses along with the assays of ER NCX1 activity (Fig. 1B) and the ER purified and reconstituted NCX1 activity (Fig. 4E) confirmed the presence of NCX1 in bovine pulmonary artery smooth muscle ER. In the western blot experiment, the apparent molecular mass of the NCX1 was determined to be 116 kDa (Fig. 1A). Bradley *et al*. (*39*) reported that 120 kDa is the apparent molecular mass of NCX1.1, while that of NCX1.3 was slightly lower, since it contains 36 fewer amino acids. The existence of NCX1.3 in the smooth muscle was demonstrated by Lee *et al*. (*45*). During the measurement of Na^+-dependent Ca^{2+} uptake in the ER vesicles, no ATP was present in the NCX assay medium indicating that the increase in the Ca^{2+} concentration in the ER vesicles with respect to the basal value was only due to Ca^{2+} uptake by the NCX1 in the ER but not by sarco(endo)plasmic reticulum Ca^{2+}ATPase (SERCA). Moreover, we obtained similar result when we performed the experiment (Ca^{2+} uptake) in the presence of SERCA antagonist, thapsigargin (data not shown), which further confirmed that the increase in the Ca^{2+} concentration was due to ER NCX1. Figure 1B indicated the Na^+-dependent Ca^{2+} uptake by NCX1 in the ER, which was 57 ± 2.56 $[(354 \pm 11.03) - (297 \pm 9.28)]$ nmol/10 min/mg protein. But, in the presence of NCX inhibitor, CGP-37157 (20 μM), no appreciable Ca^{2+} uptake in the ER has, however, been observed (Fig. 1B). CGP-37157, a benzothiazepine derivative of clonazepam is a calcium antagonist, which inhibits the Na^+/Ca^{2+} exchanger activity of the isolated mitochondria (*46*). In our present study, we have shown that CGP-37157 also inhibits the Na^+/Ca^{2+} exchanger activity of the isolated ER (Fig. 1B). Malli *et al*. (*47*) reported that inhibition of mitochondrial Na^+/Ca^{2+} exchanger with CGP-37157 increases $[Ca^{2+}]_{mito}$ and abolishes the ability of mitochondria to buffer subplasmalemmal Ca^{2+}, resulting in an increase in the activity of large conductance Ca^{2+}-activated K^+ channels (BKCa channels) and a decrease in the capacitative Ca^{2+} entry (CCE) and hence CGP-37157 also reversibly inhibits ER refilling during cell stimulation (*47*). However, the mode of inhibition of CGP-37157 on the NCX activity of the isolated ER remains to be resolved.

Our present study indicates that treatment of the pulmonary artery smooth muscle ER with Ca^{2+} (5 mM) causes dissociation of the associated m-calpain and calpastatin (Fig. 2B and C). In our previous report (*22*), by co-immunoprecipitation study, we have demonstrated that the associated m-calpain-calpastatin is localized in the cytosolic side of the ER membrane of the smooth muscle. In the co-immunoprecipitation study, the 18 kDa band is the autolysed product of 28 kDa small subunit of m-calpain (*22*). The 18 kDa autolysed fragment contains domain VI, the calmodulin like domain of the

28 kDa subunit of m-calpain, which binds calpastatin (*13*, *22*). Our present study indicates that NCX1 is localized in the cytosolic face of the ER membrane (Fig. 3). Such localization of m-calpain and NCX1 in the ER membrane provides opportunity of activated m-calpain to act on NCX1 as substrate. Our data suggest that m-calpain activation occurred due to dissociation of the associated m-calpain and calpastatin in the ER membrane in the presence of Ca^{2+} (5 mM). In some instances calpastatin behaves as a substrate of calpain (*48*) and diminution of calpastatin eventually caused activation of calpain (*49*). In the present study, we find that the activated m-calpain cleaves the NCX1 to an 82 kDa fragment (Fig. 2A; lane b). Bano *et al.* (*20*) demonstrated that in brain ischaemia, the astrocyte cell membrane NCX1 was degraded to an 82 kDa fragment by m-calpain. The following evidences support the contention that m-calpain causes cleavage of NCX1 in the ER: (i) pre-treatment with the calpain-specific inhibitors, calpeptin or MDL28170, or with the Ca^{2+} chelator, EGTA, prevents the cleavage of the NCX1 in the Ca^{2+}-treated ER (Fig. 2A; lane b) and (ii) treatment of the purified NCX1 with the purified m-calpain resulted in an 82 kDa fragment (Fig. 4F).

We have used 5 mM of Ca^{2+} for *in vitro* activation of m-calpain in the ER membrane, which is far higher than the cytosolic Ca^{2+} concentration (100–300 nM) (*43*, *50*). In general, several cellular events or additional factors such as binding to phospholipids or activation proteins play an important role for *in vivo* activation of calpain in the presence of a low Ca^{2+} level (*34*, *51*). In different pathophysiological conditions, for example, ischaemia–reperfusion injury, there is an increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$) up to 1 μM (*49*, *52*), which along with the other activation factors appeared to be sufficient for *in vivo* activation of m-calpain (*51*).

In heart sarcolemmal vesicles, the Na^+-dependent Ca^{2+} uptake has maximum transport velocity that was nearly 30 times higher than that produced by the Ca^{2+} pump system (*53*). Na^+-dependent Ca^{2+} uptake along with Ca^{2+} pump is believed to play a role to sequester Ca^{2+} in the ER of pulmonary artery smooth muscle (*1*, *2*). A decrease in Ca^{2+} sequestration via inhibition of Na^+-dependent Ca^{2+} uptake would be expected to prolong the duration of the free Ca^{2+} transients produced by vasoconstrictors (*54*, *55*). NCX has been shown to control the contractility of smooth muscle in different systems (*54*, *55*).

Intracellular Ca^{2+} is a critical signal transduction element in regulating pulmonary smooth muscle contraction, proliferation as well as expression of a variety of gene responsible for pulmonary hypertension (*56*). Cytosolic Ca^{2+} dynamics is regulated, at least partly, by the ER, which takes up when cytosolic Ca^{2+} levels are high and releases it when cytosolic Ca^{2+} levels are low (*57*). A variety of agents increase intracellular free Ca^{2+} levels, which in turn up-regulates Ca^{2+}-dependent protease including calpain. Under some pathological conditions, for example, ischaemia–reperfusion injury, pulmonary smooth muscle circumvents Ca^{2+} overload. NCX1 in ER plays an important role in regulating $[Ca^{2+}]_i$ and inhibition of the exchanger produces disregulation of $[Ca^{2+}]_i$ dynamics (*58*). Thus, calcium-dependent cleavage of NCX1 by m-calpain in the ER, as evidenced from the present study, appears to be an important mechanism for the intracellular Ca^{2+} overload, which could be important for the manifestation of pulmonary hypertension (*1*, *2*).

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Conflict of interest

None declared

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