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Presence of Sodium–Calcium Exchanger/GM1 Complex in the Nuclear Envelope of Non-neural Cells: Nature of Exchanger-GM1 Interaction*

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Previous studies have revealed the presence of Na^+/Ca^{2+} exchanger (NCX) activity associated with GM1 ganglioside in the nuclear envelope (NE) of neurons and glia as well as various neural cell lines. The nuclear NCX1 exchanger, unlike that in the plasma membrane, was shown to be tightly associated with GM1 and potentiated by the latter. One non-neural cell line, Jurkat, was found to contain no Na^+/Ca^{2+} exchanger of the NCX1, NCX2, or NCX3 types in either nuclear or plasma membrane. To determine whether such absence in the NE is generally characteristic of non-neural cells we have examined two more such cell lines in addition to human lymphocytes. RT-PCR showed NCX1 expression in both HeLa and NCTC cell lines and also NCX2 in the latter; NCX3, a subtype previously observed in NG108-15 cells, was not expressed in either. Immunocytochemical and immunoblot studies indicated NCX1 on the cell surface and nuclear envelope of both cell types. Some alternatively spliced isoforms of NCX1 in the nuclear envelope of both cell types were tightly associated with ganglioside GM1. Human lymphocytes, a mixed population of T and B cells, showed similar evidence for plasma membrane and nuclear expression in some but not all cells. The high affinity association between NCX1 and GM1, explored by reaction with base, acid, and proteases, was found to involve charge–charge interaction with a requirement for a positively charged moiety in NCX.

KEY WORDS: gangliosides; GM1 ganglioside; nuclear calcium; nuclear envelope; sodium–calcium exchanger.

INTRODUCTION

Sodium–calcium exchangers (NCX) provide one of the principal Ca^{2+} clearance mechanisms for

Abbreviations: Ab, antibody; BSA, bovine serum albumin; $[Ca^{2+}]_i$, intracellular Ca^{2+}; Ctx B, cholera toxin B subunit; DMEM, Dulbecco's modified Eagle's medium; FITC, fluoroisothiocyanate; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HPTLC, high performance thin-layer chromatography; HRP, horseradish peroxidase; IP, immunoprecipitation or immunoprecipitate; MAb, monoclonal antibody; MM, mixed membranes (including plasma- but excluding nuclear membranes); NCX, sodium–calcium exchanger; NE, nuclear envelope; PBS, phosphate-buffered saline; PM, plasma membrane; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; TM buffer, 20 mM Tris–Cl (pH 7.5) with 1 mM $MgCl_2$.

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maintenance of intracellular Ca^{2+} homeostasis. As plasma membrane (PM) components they function in the forward mode to extrude cytosolic Ca^{2+}, an uphill process driven by the high extra- to intracellular Na^+ gradient (1–3). As bi-directional exchangers they can, under certain conditions, catalyze net entry of Ca^{2+} in the so-called reverse mode (4). Na^+/Ca^{2+} exchangers are especially prominent in excitable cells such as neurons (5) and cardiomyocytes (6) that experience rapid, several-fold elevation of intracellular Ca^{2+}($[Ca^{2+}]_i$). They have also been detected in non-excitable cells such as astrocytes (7,8) and lymphocytes (9,10), these latter showing less robust exchange activity.

Whereas the PM has been the primary locus in which Na^+/Ca^{2+} exchangers have been studied, such molecules also occur in various intracellular compartments such as mitochondria (11,12) and nuclei (8,13). Cells in which such exchangers have been observed in the nuclear envelope (NE) include neurons, astrocytes, and the NG108-15 and C6 cell lines (8,13). As with PM, Na^+/Ca^{2+} exchangers in the NE include various isoforms of the NCX type, a multigene family consisting of the NCX1, NCX2, and NCX3 subtypes (3). A striking feature of some alternatively spliced isoforms in the NE is their tenacious association with GM1 ganglioside, as seen in survival of the association during immunoprecipitation (IP), sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblot analysis (8,13). One result of this association is potentiation of Na^+/Ca^{2+} exchange activity by GM1 (8,13). The nature of this association is not known and elucidation of its molecular basis was one purpose of this study.

The other objective of this investigation was to determine whether occurrence of NCX–GM1 complex(es) in the NE is unique to neural cells and cell lines, which has been our focus to date; all neural cell types we have studied show this phenomenon (8,13). This was even indicated in a neural cell line (C6) which had no observable NCX activity in the PM (8). In contrast Jurkat cells, the one non-neural cell type studied from that standpoint, showed no evidence of either NCX or GM1 in the NE (8). To determine whether Jurkat cells are characteristic in that regard of non-neural cells generally, we have examined the NE of HeLa cells, NCTC cells, and human lymphocytes. Further comparison was made to NG108-15 and Jurkat cells which served as positive and negative controls, respectively. Evidence for Na^+/Ca^{2+} exchange activity in PM of these non-neural cell lines is also described. Some aspects of this work have been presented in preliminary form (14).

EXPERIMENTAL PROCEDURES

Materials. The following materials were obtained from the sources indicated: Dulbecco's modified Eagle's medium (DMEM), heat inactivated fetal bovine serum (FBS), penicillin/streptomycin, gentamicin, and N2 chemically defined medium components from GIBCO (Grand Island, NY); cholera toxin B subunit (Ctx B), cholera toxin B subunit-horse radish peroxidase conjugate (Ctx B-HRP), cholera toxin B-fluoroisothiocyanate conjugate (Ctx B-FITC) from List Biological Labs (Campbell, CA); fura-2 AM, and sulfinpyrazone from Molecular Probes, Inc. (Eugene, OR); rabbit polyclonal anti-canine cardiac Na^+/Ca^{2+} NCX1 exchanger antibody (IgG) from Swant (Bellingzona, Switzerland); mouse monoclonal (MAb) anti-canine cardiac Na^+/Ca^{2+} exchanger antibody (C2C12, IgM), poly-l-lysine, bovine serum albumin (BSA), agarose beads linked to protein L, SIGMACOTE, and other chemicals from SIGMA-RBI (St. Louis, MO); RNeasy® Mini Kit and OneStep RT-PCR Kit from Qiagen. Stock solutions of fura-2 AM and sulfinpyrazone were prepared in dimethylsulfoxide.

Cell culture and whole cell immunocytochemical staining. NG108-15 neuroblastoma × glioma hybrid cells were a kind gift from Dr. Marshall Nirenberg (NIH, Bethesda, MD). The following cell lines were obtained from the American Type Culture Collection (Rockville, MD): Jurkat clone E6-1 (acute T-cell leukemia, human); HeLa (epitheloid carcinoma, cervix, human); NCTC clone 929 (connective tissue, mouse). Human peripheral blood lymphocytes were obtained from healthy volunteers by density-gradient centrifugation (15). Approval and informed consent were obtained from all subjects and the protocol was approved by the Institutional Review Board of UMDNJ-New Jersey Medical School. Hela, NCTC, Jurkat, and NG108-15 cells were routinely cultured in 10 cm Falcon dishes in DMEM supplemented with 10% FBS (5% for NG108-15), 50 U/ml penicillin, 50 μg/ml streptomycin and 50 μg/ml gentamicin in a 37°C incubator with 5%CO_2/95% humidified air. For immunocytochemical staining, cells were seeded on acid-cleaned glass coverslips coated with poly-L-lysine (0.2 mg/ml) that were placed in 6-well-plates. The cells were fixed in 4% paraformaldehyde in PBS for 30 min and stored at 4°C prior to cytochemical staining. Working at room temperature (for this and subsequent steps), the coverslip was washed 3× with PBS followed by incubation in 0.05% glycine/PBS for 30 min and then PBS with 0.05% Tween 20 plus 1% BSA for 1 h. The cells were exposed to monoclonal anti-Na^+/Ca^{2+} exchanger antibody (C2C12; 1:200 in PBS) followed by goat anti-mouse IgM conjugated to Texas Red. Finally, the coverslips were mounted on glass slides with glycerol/PBS (9:1, v/v) containing 2.5% DABCO. Observation and photography were carried out with a Nikon Diaphot microscope.

RNA isolation and RT-PCR. Total RNA was isolated with Qiagen RNeasy® Mini Kit , and RT-PCR was carried out with Qiagen OneStep RT-PCR Kit, both according to instructions of manufacturer (Qiagen). Forward and reverse primers (prepared by Dr. Robert Donnelly, director of the Molecular Biology Core Facility of this university) at 0.6 μM, and 1 μg of template RNA were employed. Reverse transcription was carried out for 30 min at 50 °C according to manufacturer's instructions. PCR was initi-

ated at 95°C for 15 min, followed by 30 3-step cycles. A 1% agarose gel impregnated with ethidium bromide was used to separate and identify PCR products that were revealed by UV light. The forward and reverse primers of exchangers and GAPDH are summarized in Table I.

Isolation and staining of nuclei. Cells grown as above in Falcon dishes were rinsed twice with PBS and quickly frozen at −80°C for 2 h. They were thawed on ice, scraped and collected in a minimum volume of TM buffer consisting of 20 mM Tris-Cl(pH 7.5) and 1 mM $MgCl_2$. Purified nuclei and a non-nuclear membrane mixture (MM) were isolated as described (13,16). Nuclei from parallel sets of cells were stained with monoclonal anti-Na^+/Ca^{2+} exchanger antibody (C2C12; 1:200 in PBS) followed by goat anti-mouse IgM-HRP, and by Ctx B-FITC (2.5 μg/ml Ctx B in PBS).

Immunoblot analysis. Each membrane fraction, including whole NE and non-nuclear membrane mixture (MM), which included PM, were dispersed in PBS containing 1% Triton X-100 and protease inhibitor cocktail. Gentle mixing was carried out at 4°C for 2 h followed by high speed centrifugation; the supernatants were subjected to protein determination as described (17). Immunoprecipitation, SDS-PAGE, electrophoretic transfer and immunoblot analysis were carried out as previously described (13); this utilized mouse monoclonal anti-canine cardiac Na^+/Ca^{2+} exchanger antibody C2C12 (diluted 1:50) for immunoprecipitation and polyclonal rabbit anti-canine cardiac Na^+/Ca^{2+} exchanger antibody (Swant, diluted 1:200) for Western blot on PVDF membrane; second antibody was goat anti-rabbit IgG antibody linked to HRP. A parallel blot was probed with cholera toxin B (Ctx B) linked to HRP. All blots were developed on Kodak BioMax film using Amersham ECL™ reagent according to manufacturer's instructions.

Measurement of plasma membrane Na^+/Ca^{2+} exchange activity. Intracellular Ca^{2+} ($[Ca^{2+}]_i$) measurements were carried out with cells initially grown in attachment as above for 2–3 days to confluence; cells cultured for shorter times responded poorly to stimuli. The cells were detached by gentle trituration, washed twice with DMEM, and incubated with 3 μM fura-2 AM in DMEM supplemented with 250 μM sulfinpyrazone for 30 min at 25°C. Aliquots of $1–1.5 \times 10^6$ cells were washed and transferred to a cuvette with 3 ml of MOPS-buffered solution (20 mM, pH 7.2) consisting of 120 mM choline chloride, 5 mM KCl, 1 mM $MgCl_2$, 10 mM glucose, 0.25 mM sulfinpyrazone and 20 mM NaCl (or 20 mM LiCl). Measurements were carried out with a RF-M 2001 Fluorometer (Photon Technology, International, Monmouth Junction, NJ) equipped with magnetic stirrer. Emission fluorescence intensity was recorded at 510 nm at 37°C, with excitation wavelength alternately at 340 and 380 nm.Gramicidin, a Na^+ ionophore, was added to facilitate Na^+ or Li^+ entry at the beginning of the experiment, final concentration being

Table I. Primers for RT-PCR

Rat GAPDH		
Forward	5′- ACC ACC ATG GAG AAG GCT GG-3′	Gibney et al. (33) (M17701 bases 310–729)
Reverse	5′- AAC ACG GAA GGC CAT GCC AG-3′	
Rat NCX1		
Forward	5′- AAC ACT GCC ACC ATA ACC-3′	Gibney et al. (33) (X68813 bases 1689–2381)
Reverse	5′- CTC CCC ACA TTC ATC ATC-3′	
Rat NCX2		
Forward	5′- GAC GAC GAA GAG TAT GAG AAG-3′	Gibney et al. (33) (NM_078619 bases 1789–2289)
Reverse	5′- AGT GAG CAG ACC AAT GAC C-3′	
Rat NCX3		
Forward	5′- CAG ACT GCA AGG AGG GTG TC-3′	Lu et al. (34) (NM_078620 bases 988–1326)
Reverse	5′- AAT CAC CAG CAA TGA ACC CG-3′	
Mouse GAPDH		
Forward	5′-ACC ACA GTC CAT GCC ATC AC-3′	(M7701, 312-764)
Reverse	5′-TCC ACC ACC CTG TTG CTG TA.-3′	
Mouse NCX1		
Forward	5′-ACA TGA AGG AGA CAG ACC AGC TTC-3′	Yamaji et al. (35) (AF004666 bases 922–1504)
Reverse	5′-CTC TGA TTT CCT TCT GGG TCT C-3′	
Mouse NCX2		
Forward	5′-ACT GCT CAC ACT GAT CTT CTT C-3′	Yamaji et al. (35) (NM_148946 bases 825–1339)
Reverse	5′-TGG GCT CAA AGA AGA TGC GAC T-3′	
Mouse NCX3		
Forward	5′-TTT TGA CCC ATG CTC TTA CCA G-3′	Yamaji et al. (35) (NM_078620,bases 2030–2306)
Reverse	5′-CTC TTC TAC ACG GAC ATT GCT C-3′	
Human GADPH		
Forward	5′- CCA CCC ATG GCA AAT TCC AT-3′	Dheenadayalu et al. (36) (NM_002046 bases 227–786)
Reverse	5′- AGT GGG GAC ACG GAA GGC CA-3′	
Human NCX1		
Forward	5′- CCT TGT GAC TCT CAG CAA T-3′	Van Eylen et al. (21) (NM_021097 bases 1553–2453)
Reverse	5′- GAA GGC AAA CAG GAC CTT C-3′	
Human NCX2		
Forward	5′- TCC GAC TAC GAG TAC AGC G-3′	Van Eylen et al. (21) (XM_038970 bases 1417–2273)
Reverse	5′-TGC ATC ACG TAG TCA AAG CAC-3′	
Human NCX3		
Forward	5′- GTT GAG CAA TGT CCG CAT AG-3′	Nicoll et al. (37) (NM_033262, bases 1661–2661)
Reverse	5′- CGT CTG CAT ATA CAT CCT GGA-3′	

1 μg/ml. After stabilizing the cells in the above MOPS buffer for 50 s, 30 nM thapsigargin was added to deplete intracellular Ca^{2+} store, followed by 5 mM $CaCl_2$ at 200 s. Measurement was continued for 200 s, $[Ca^{2+}]_i$ changes being recorded as $R_{340/380}$.

Study of GM1 binding to Na^+/Ca^{2+} exchanger of nuclear envelope. The first approach was to immunoprecipitate the complex by treating isolated NE from NG108-15 cells with Triton X-100 (1%) followed by C2C12 MAb plus protein L conjugated to agarose beads. After washing 2X with PBS the beads were subjected to the following treatments: (a) methanol alone, 1 h at 37°C; (b) 0.3 N KOH in PBS at 37°C for 20 or 40 min, then neutralized with HCl; (c) 0.1 N KOH at room temperature for 3 min, then neutralized with HCl; (d) 1% thrombin in Tris–HCl buffer (50 mM, pH 7.2), containing 1% mercaptoethanol and 25 mM KCl for 20 or 40 min at ~25 °C. The samples were mixed with 4 × sample buffer, boiled, and applied to 7% SDS-PAGE, transferred to two PVDF membranes and probed with Swant polyclonal anti-NCX Ab and Ctx-HRP.

The second approach was to carry out reactions on the intact NCX–GM1 complex attached to the PVDF membrane, following IP, SDS-PAGE and electrophoretic transfer. One PVDF lane was cut lengthwise and probed with Ctx B-HRP to identify the complexes at 70 and 220 kDa. The corresponding zones on the other six lanes were cut and the combined membrane cuttings treated in the following sequence: (a) methanol, 3 min; (b) chloroform–methanol (1:1, v/v), 3 h; (c) proteinase K (0.005%), 37°C, 24 h followed by chloroform–methanol (1:1). Each organic extract was dried, the residue dissolved in a small volume of chloroform–methanol and subjected to high performance thin-layer chromatography (HPTLC) on aluminum-backed silica gel plates, employing chloroform–methanol–2.5 M aqueous KCl (50:40:10, v/v/v) as developing solvent. Gangliotetraose gangliosides (including GM1) were revealed by neuraminidase treatment and Ctx B-HRP overlay as described (17,18). Additional reactions were carried out on PVDF zones after chloroform-methanol extraction to remove non-associated GM1 on

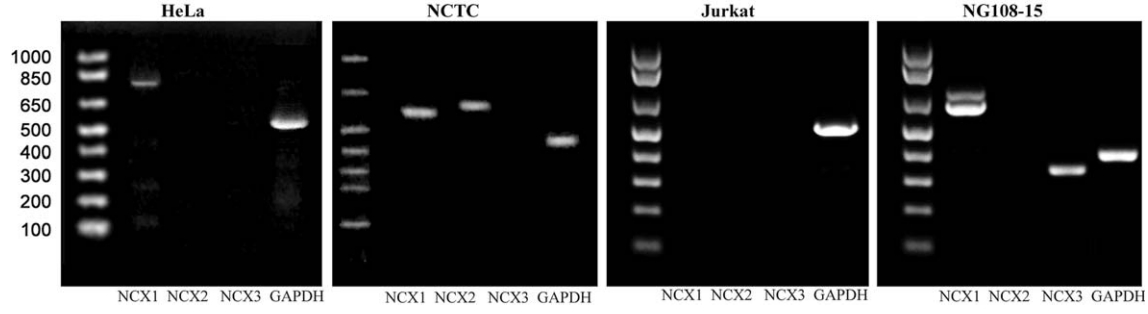

Fig. 1. RT-PCR Determination of NCX subtypes. RNA isolated from indicated cell lines was subjected to RT-PCR amplification with corresponding primers (Table I).The resulting products were developed in agarose gel and photographed. Housekeeper GAPDH gene was employed as positive control.Whereas Jurkat cells showed no NCX, the other three cell lines expressed NCX1 alone (HeLa) or together with NCX2 (NCTC) or NCX3 (NG108-15). The result for NG108-15 is reproduced from ref. 8 with permission.

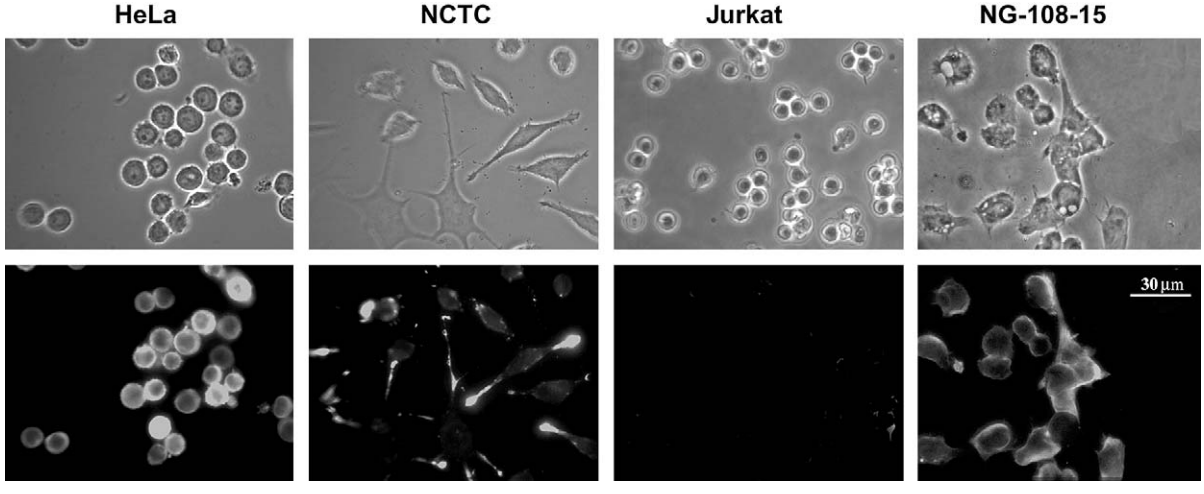

Fig. 2. Whole cell staining of NCX1. Cells were fixed with paraformaldehyde and subjected to immunostaining using anti-NCX1 MAb (C2C12) followed by second antibody linked to Texas red. In agreement with results of RT-PCR, Jurkat cells showed negative staining whereas HeLa and NCTC cells were NCX1 positive. NG108-15 cells provided positive control. Note that NCTC cells stained less prominantly than HeLa cells, with NCX limited to process extensions. In contrast NCX was well expressed in HeLa cell bodies. Magnification was the same for all fields. Upper panel is phase control corresponding to lower panel staining.

the membrane. These reactions included: (a) 0.25 N KOH in methanol at 37°C for 30 min followed by neutralization with HCl, (b) 0.3 N HCl in methanol at 37°C for 3 h followed by neutralization with KOH, and (d) trypsin (0.25%) at 37°C for 30 min. Samples from each treatment were extracted with chloroform-methanol (1:1) and each extract was evaporated, applied to HPTLC and analyzed for GM1 as described above.

RESULTS

NCX subtype determination. RNA was extracted and RT-PCR carried out with appropriate primers (Table I). HeLa cells expressed only the NCX1 subtype whereas NCTC cells expressed both NCX1 and NCX2 (Fig. 1). Jurkat cells showed no evidence of NCX expression, as previously reported (8). For comparison of cell lines, NG108-15 cells subjected to similar analysis expressed NCX1 and NCX3 (8). Human lymphocytes, representing both T- and B-cell populations, were shown to express NCX1 and NCX3 (Fig. 3A).

Whole cell immunocytochemistry. Cytochemical staining of cell lines with anti-NCX1 MAb (C2C12) revealed NCX1 on the surface of both HeLa and NCTC cells, whereas Jurkat cells showed no staining (Fig. 2). The less prominent staining of NCTC cells accorded with the limited immunoblot evidence for NCX1 in the PM (see below). For comparison, NG108-15 cells were strongly positive as indicated in earlier work (13). Similar treatment of the human lymphocyte preparation revealed only a portion of cells staining for NCX1 (Fig. 3b1–3b4). Phenotypic analysis of these lymphocyte preparations indicated $>90\%$ lymphocytes and $<10\%$ monocytes;

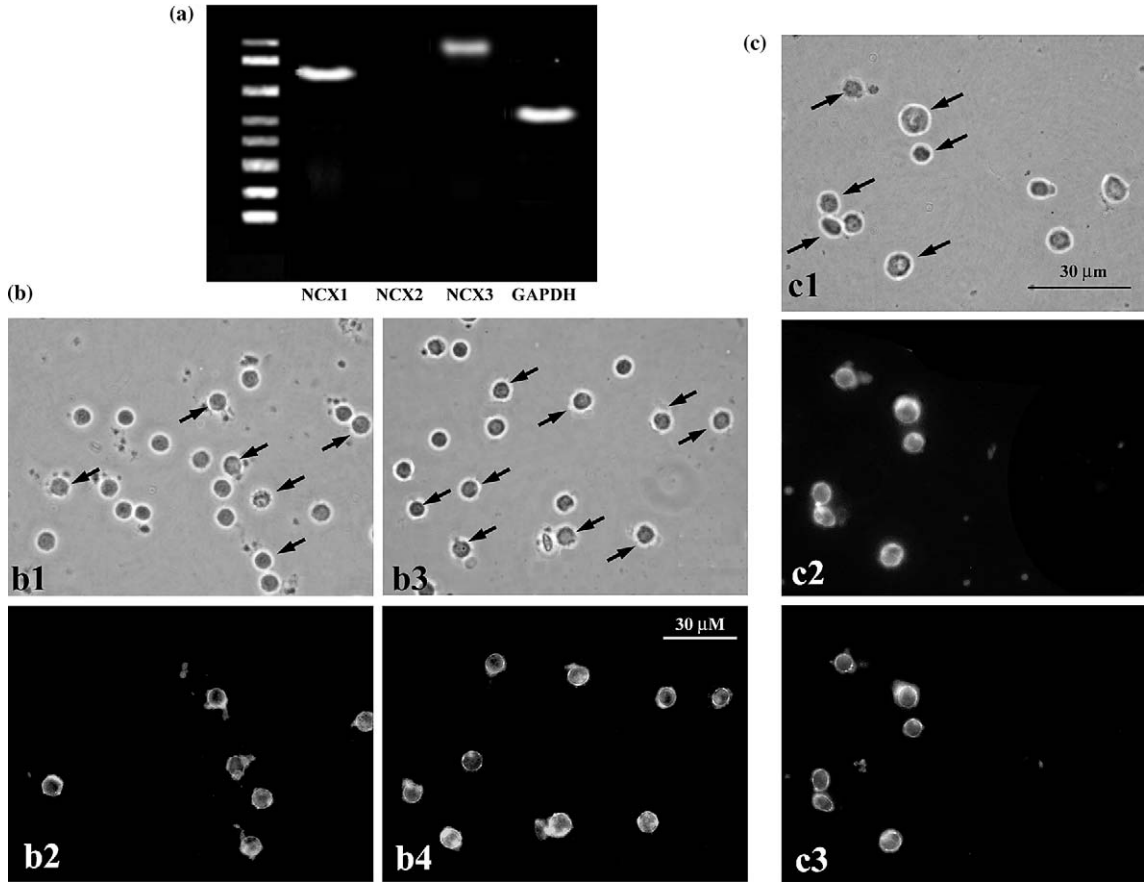

Fig. 3. NCX and GM1 in human lymphocytes. (a) RT-PCR assay of NCX gene expression was carried out on RNA from freshly isolated lymphocytes; use of human primers (Table I) revealed NCX1 and NCX3. (b) Freshly isolated lymphocytes fixed in paraformaldehyde were stained with anti-NCX1 MAb (C2C12) plus Texas red-linked 2nd Ab; only a portion of cells shown in phase contrast (b1, b3) expressed NCX1 in PM (b2, b4). (c) Double staining of isolated nuclei with anti-NCX1 (C2C12) plus 2nd Ab linked to Texas red (c2) and with Ctx B-FITC (c3); only a portion of nuclei seen in phase contrast (c1) co-stained for NCX1 and GM1. Arrows indicate positive staining cells or nuclei.

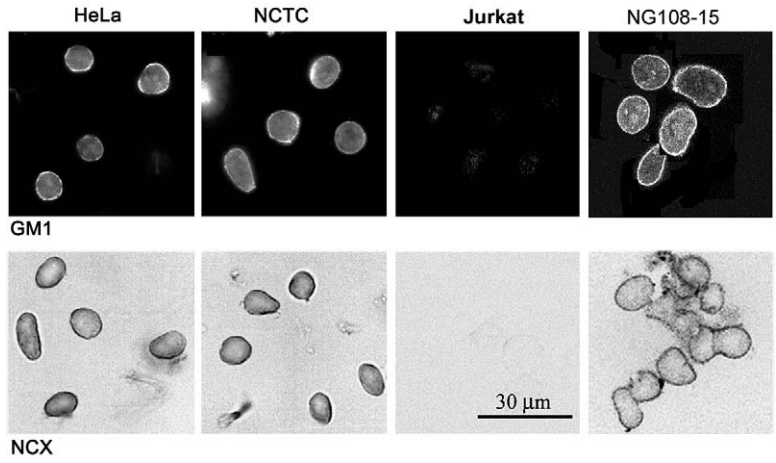

Fig. 4. NCX1 and GM1 immunostaining of isolated nuclei from cell lines.Isolated nuclei fixed with paraformaldehyde were stained with anti-NCX1 MAb (C2C12) followed by 2nd antibody linked to HRP. Separate fields of nuclei were stained with Ctx B-FITC. Hela and NCTC nuclei expressed both NCX1 and GM1 in the NE. NG108-15 nuclei served as positive control (reproduced from ref. 13, with permission) and Jurkat nuclei served as negative control (reproduced from ref. 8, with permission). Magnification is the same for all fields.

immunocytochemical analysis revealed that T-cells comprised the large majority of lymphocytes and B-cells the remainder (not shown). Detailed analysis of B-cells and T-cell subtypes is necessary to determine which lymphocyte types do and do not express NCX in the PM.

Cytochemical evidence for NCX and NCX–GM1 complex in the NE. Freshly isolated nuclei were fixed with paraformaldehyde, attached to poly-L-lysine-coated coverslips, and stained with Ctx B-FITC and mouse anti-NCX MAb (C2C12) followed by second Ab linked to HRP. Positive staining with both agents was evident in the NE of all cell lines except Jurkat, which showed no nuclear staining with either agent (Fig. 4). In the case of human lymphocyte mixture, positive staining was seen for NCX, but only in a portion of nuclei (Fig. 3c1–3c3). Those nuclei that stained for NCX also stained for GM1, again indicating the presence of NCX–GM1 complex. At this stage it is not possible to correlate nuclear staining with that in whole cells (PM).

Immunoblot evidence for NCX–GM1 complex in the NE. Following isolation of the NE from these nuclei, IP was carried out with MAb to NCX1 (C2C12) followed by SDS-PAGE and electropho-

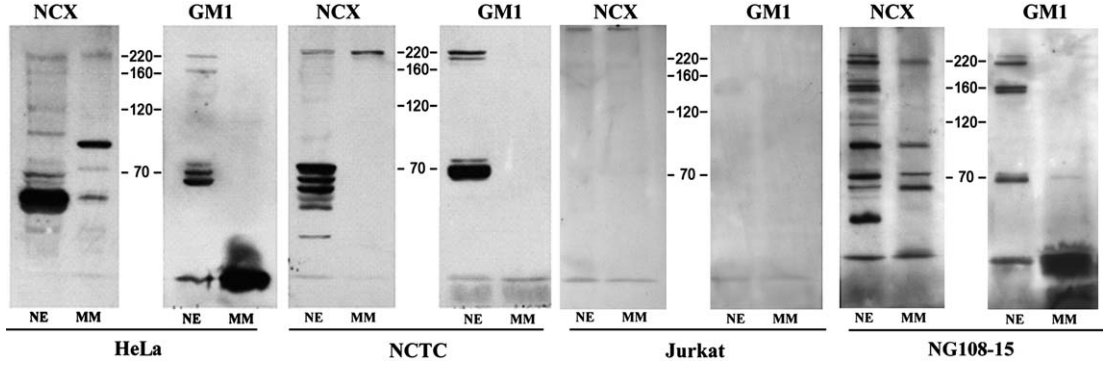

Fig. 5. Immunoblot evidence for NCX–GM1 complex in NE. Nuclear envelope (NE) and non-nuclear membrane mixture (MM) that included plasma membrane were immunoprecipitated and subjected to SDS-PAGE followed by immunoblot as described.The latter included parallel blots with anti-NCX1 polyclonal antibody plus 2nd antibody linked to HRP and Ctx B-HRP for GM1. Nuclear envelope preparations from HeLa and NCTC cells showed NCX1 positive bands in 220 and 70 kDa regions with parallel GM1 staining. NCX positive bands in MM were not associated with GM1. NG108-15 NE and MM served as positive control (reproduced from ref. 13 with permission) while Jurkat NE and MM served as negative control (reproduced from ref. 8 with permission).

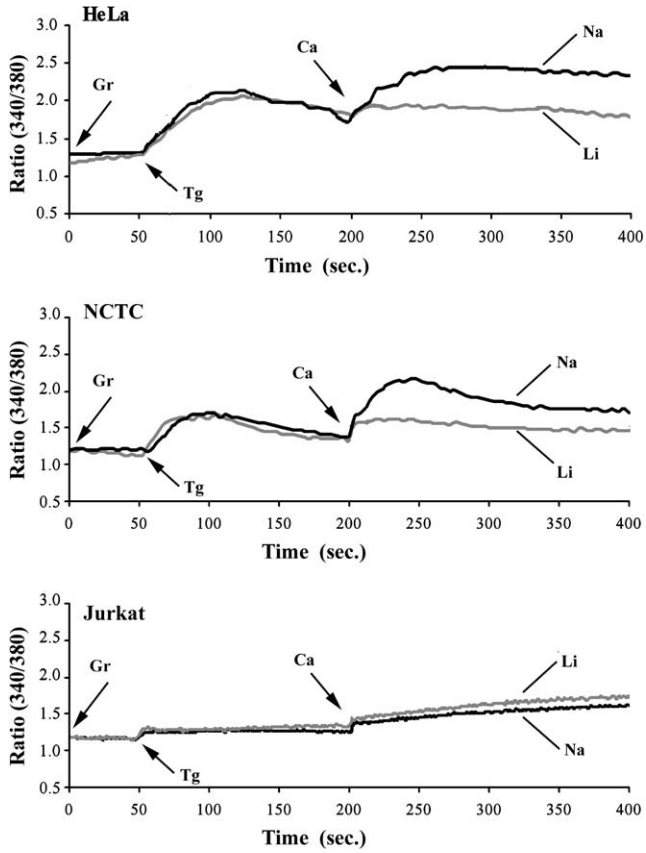

Fig. 6. NCX activity in plasma membranes of cell lines. Cells were loaded with fura-2 and suspended in Ca^{2+}-free MOPS-buffed solution containing 120 mM choline chloride plus 20 mM NaCl or LiCl. Gramicidine (Gr), thapsigargin (Tg) and Ca^{2+} were sequentially applied at indicated times. $[Ca^{2+}]_i$ was indicated ratiometrically as $R_{340/380}$, representing ratio of fluorescence emission at 510 nm following excitation alternately at 340 and 380 nm. Increase in $R_{340/380}$ in HeLa and NCTC cells on addition of Ca^{2+} was seen in cells exposed to Na but not Li, indicating NCX operating in reverse mode in the PM. For comparison, Jurkat cells deficient in NCX showed no difference between NaCl and LiCl. Each tracing is average of three independent experiments.

retic transfer to PVDF membranes. This was done with two parallel preparations, one PVDF membrane being stained with Ctx B-HRP and the other with rabbit polyclonal anti-NCX (Swant) followed by goat anti-rabbit IgG linked to HRP. The nonnuclear membrane mixture (MM), which included PM, was also processed for each cell type. All cell lines except Jurkat showed immunoblot evidence for both NCX1 and GM1 in the NE, although with differing patterns (Fig. 5). Nuclear envelopes from HeLa and NCTC cells showed major multiple bands staining for NCX1 in the region of 70 kDa and below; two or three of these showed parallel GM1 bands. Less prominent bands were evident at ~220 kDa with GM1 association for both cells. A few anti-NCX1 bands were barely discernible between these regions, and there seemed to be no indication of GM1 staining. Membrane mixtures from HeLa cells showed two major bands above and below 70 kDa with lighter bands at 70 and 220 kDa, while NCTC showed a single prominent band at 220 kDa. None of these presumably PM NCX molecules appeared to comigrate with attached GM1. Jurkat cells, in keeping with the RT-PCR result, showed no immunoblot evidence for NCX in either NE or PM; interestingly, GM1 also was not detected in the NE of that cell type. For comparison, the NE of NG108-15 cells showed clear evidence of NCX1, some isoforms of which were associated with GM1 as previously reported (13).

Study of Na^+/Ca^{2+} exchange activity in the plasma membrane. For ratiometric fluorescence measurement of $[Ca^{2+}]_i$, cells were loaded with fura-2AM as described and suspended in Ca^{2+}-free MOPS buffer contain 120 mM choline chloride and

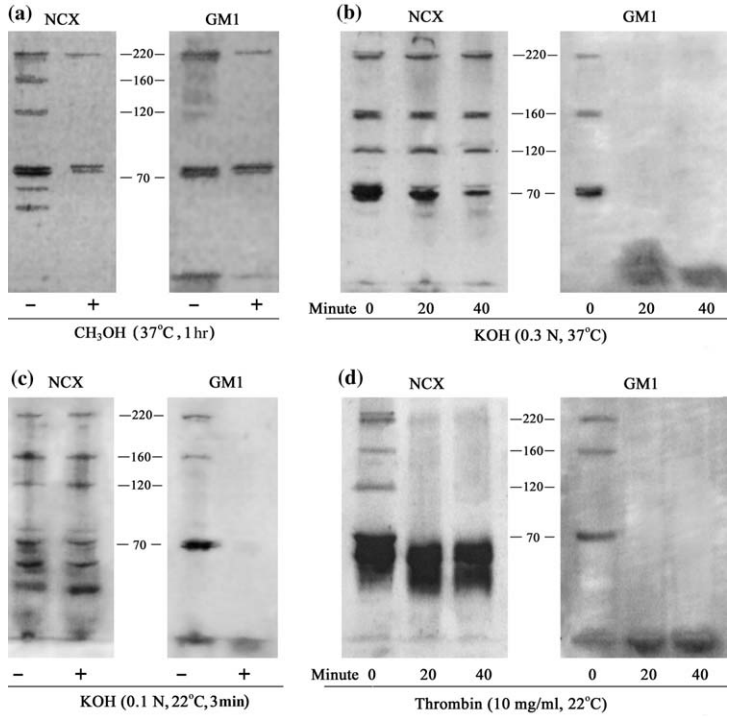

Fig. 7. Dissociation of NCX–GM1 complex. Immunoprecipitation of NCX–GM1 complex was carried out with NE from NG108-15 cells as described.While remaining attached to agarose–protein L beads, the complex was treated in separate runs with (a) methanol, (b) and (c) KOH, and (d) thrombin at the indicated concentration, temperature and time.The IP was then subjected to SDS-PAGE, transferred to PVDF membrane and blotted for NCX1 and GM1 as in Fig 5. Whereas methanol treatment failed to affect NCX–GM1 interaction, mild KOH treatment dissociated GM1 from NCX with no apparent change in the latter. Thrombin-catalyzed hydrolysis of NCX isoforms into multiple peptides (< 70 kDa) also released GM1 from the complex. These results suggested charge–charge interaction between NCX and GM1.

either 20 mM NaCl or 20 mM LiCl. These were exposed to gramicidin (1 μg/ml) at the beginning, and 50 s later to thapsigargin (30 nM) which induced moderate elevation of $[Ca^{2+}]_i$; subsequent addition of 5 mM $CaCl_2$ caused significant elevation of $[Ca^{2+}]_i$ in HeLa and NCTC cells exposed to Na^+ but not Li^+ (Fig. 6). This represented reverse mode exchange activity in the PM. Jurkat cells, in contrast, showed no difference between Na^+ and Li^+, consistent with absence of NCX activity in the PM.

NCX–GM1 interaction. The first approach, treating the NCX–GM1 complex from NE of NG108-15 cells on protein L–agarose beads following IP, showed that methanol alone did not remove GM1 from the 70 or 220 kDa isoform (Fig. 7a). However, similar treatment on the beads with 0.3 N KOH for 20 or 40 min at 37°C showed GM1 to be separated from all NCX isoforms (Fig. 7b). Since that treatment, although relatively mild, might have hydrolyzed an ester linkage between GM1 and NCX the reaction was repeated under very mild conditions (0.1 N KOH for 3 min at room temp), which also resulted in removal of GM1 from NCX (Fig. 7c). This suggested charge–charge interaction between NCX and GM1 that was disrupted by brief base treatment. Base did not destroy NCX, the subsequently determined isoform gel pattern remaining essentially unchanged. GM1 was similarly separated from NCX on treatment of the IP complex with thrombin, a mild and selective protease (Fig. 7d); in this case NCX proteins at 220, 160, 120, and 70 kDa were destroyed as expected, giving rise to multiple degradative peptides below ~70 kDa. GM1 was thereby removed, appearing as Ctx B-positive bands at the migration front.

The second approach involved sequential treatments of NCX–GM1 on the PVDF membrane, following SDS-PAGE and electrophoretic transfer; liberated GM1 was then identified by TLC. Following location of the NCX–GM1 complexes at 220 and 70 kDa, the combined zones of

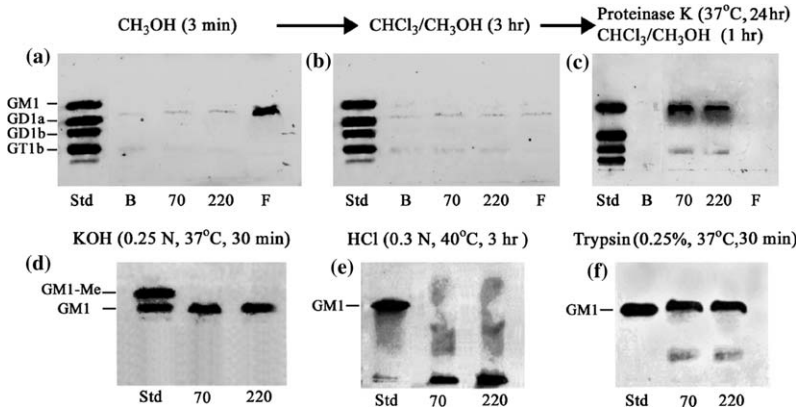

Fig. 8. Release of GM1 from NCX–GM1 complex. NE from NG108-15 cells was subjected to IP, SDS-PAGE and electrophortic transfer as described. Regions of the PVDF membrane corresponding to 70, 220 kDa, migration front (F) and blank PVDF region (B) were cut out and placed in tubes. These were treated in sequence with (a) methanol, (b) chloroform–methanol, and (c) proteinase K plus chloroform–methanol; each organic solvent extract was evaporated, the residue subjected to HPTLC, and GM1 revealed by Ctx B-HRP. Neither methanol nor chloroform/methanol extraction removed GM1 from NCX, whereas subsequent treatment with proteinase K did (c). Direct treatment with trypsin also removed GM1 (f). Treatment of the strips with KOH in methanol released GM1 without forming GM1-methyl ester (GM1-Me) (d), showing absence of transesterification (i.e., linkage of GM1 to NCX via sialic acid ester bond). Treatment of the strips with HCl did not release GM1 from NCX (e), indicating complex formation requires a positively charged amino acid in NCX. Std = bovine brain ganglioside standard.

each from several parallel PVDF strips were extracted briefly with methanol; those extracts showed no evidence of GM1 (Fig. 8a). On the other hand GM1 at the migration front, likely representing loosely bound or non-specific association of GM1 during IP, was readily extracted by methanol (Fig. 8a, lane F). Re-extraction of the PVDF zones with chloroform–methanol (1:1, v/v) similarly resulted in no GM1 removal (Fig. 8b). Subsequent treatment with proteinase K for 24 h at 37°C caused liberation of GM1 from both the 220 and 70 kDa complexes (Fig. 8c). Reaction of NCX–GM1 complexes on PVDF with base (0.25 N KOH in methanol for 30 min at 37°C also liberated GM1 (Fig. 8d); failure to form any methyl ester of GM1 (GM1-Me) indicated absence of base-catalyzed trans-esterification, a possible product had GM1 been attached to NCX via an ester linkage. In contrast to results with base, mild acid treatment of NCX–GM1 on PVDF caused no dissociation; chloroform–methanol (1:1) extraction removed the intact complex that remained at the TLC origin (Fig. 7e). Brief treatment of NCX–GM1 on PVDF with trypsin (0.25% for 30 min at 37°C) released GM1 from the complex. This series of experiments provided additional evidence for charge-based, non-covalent interaction of NCX with GM1 in which a positively charged moiety in NCX and conformational integrity of the latter are essential.

DISCUSSION

Presence of NCX subtypes in non-neural cells and cell lines revealed by RT-PCR. Na^+/Ca^{2+} exchangers in mammalian cells form a multigene family consisting of three subtypes: NCX1, NCX2, and NCX3. These have been cloned and shown to be products of distinct genes (3,19). All three subtypes, which share ~70% amino acid identity, are believed to have similar membrane topology with nine transmembrane segments and a large intracellular loop between the 5th and 6th segments. The present study, employing RT-PCR, showed both HeLa and NCTC cells to contain the NCX1 subtype, while NCTC also expressed NCX2. The human lymphocyte mixture employed here, which consisted predominantly of T cells plus lesser amounts of B cells and monocytes, showed evidence of both NCX1 and NCX3. As previously reported, NG108-15 cells used here as positive control express NCX1 and NCX3, while Jurkat T cells used as negative control express no subtypes of NCX (8).

NCX–GM1 complex occurrence in nuclei of non-neural cells. A principal finding of this study was evidence for the NCX–GM1 type complex in the

nuclei of HeLa and NCTC cells, as well as a subset of lymphocytes, thus establishing that this phenomenon is not limited to neural cells. This was based on cytochemical staining of GM1 and NCX1 in nuclei (Fig. 4) and immunoblot evidence for these in parallel Western blots following IP of NCX1 from isolated NE (Fig. 5). Such complexes migrated on SDS-PAGE in the regions of 70 kDa and 160–220 kDa, as was the case with C6 and NG108-15 cells as well as astrocytes (8,13). Additional light bands between those zones along with heavier bands below 70 kDa were revealed by anti-NCX1 Ab, but these appeared to be unassociated with GM1. Whether the bands below 70 kDa represent true NCX isoforms or products of proteolysis as previously suggested (20) remains to be determined. A functionally active 70 kDa exchanger was reported to occur as splicing variant of a larger protein (21). One NCX1 isoform of mature Na^+/Ca^{2+} exchanger migrates on SDS-PAGE with apparent M_r of 120 kDa (20) but shifts to 160 kDa gel position under non-reducing condition due to conformational change involving a disulfide bond (22). These forms were faintly evident in the NE of HeLa cells (Fig. 5). The doublet at ~220 kDa associated with GM1, also present in NE of NG108-15 cells (13) as well as astrocytes and C6 cells (8), possibly represents dimer(s) of the 120 kDa holoprotein or trimer(s) of the 70 kDa form. Additional as yet unidentified bands resulting from IP of nuclear NCX were seen in those studies.

It has been noted that one neural cell type (C6 glioblastoma) expresses the NCX–GM1 complex in NE but no NCX in the PM (8), suggesting nuclear expression of NCX as possibly more widespread than PM expression. Jurkat cells provide an example of a cell type lacking NCX function in both NE and PM, and the present study suggests that some types of lymphocytes may share that property. Cytochemical staining revealed only a portion of nuclei in the human lymphocyte cell mixture to contain the NCX–GM1 complex.

The carboxyl terminus of the large intracellular loop of NCX1 contains a variable region of 110 amino acids subject to alternative splicing, which gives rise to several isoforms. The genomic structure pertaining to this region contains six exons, two of which (A and B) are mutually exclusive; the remaining four exons (C–F) are cassette exons (23,24). The present study does not reveal which specific isoforms of the NCX1 class are present in the NE of the various cells. However, the results of previous work (8) and additional studies in progress (Wu et al., in preparation) suggest that at least some of the isoforms targeted to the NE contain members of the B exon family. NCX isoforms targeted to the nucleus reside on the inner membrane of the NE and are thus in position to transfer Ca^{2+} from nucleoplasm to NE lumen (8,13); association with GM1 was shown to significantly potentiate the exchange process. We have suggested that NCX at that locus, acting as a high capacity Ca^{2+} transporter, could provide protection against potentially damaging elevation of nucleoplasmic Ca^{2+}.

Owing to the specificity of the NCX Abs employed, these cells all showed cytochemical and immunoblot evidence for NCX1 expression in both the NE and PM. The subcellular location of NCX2 in NCTC cells and its possible association with GM1 are not known.

NCX occurrence in the plasma membrane of non-neural cells.The prominent cell surface staining of HeLa cells with anti-NCX1 Ab was in contrast to the noticeably weaker staining of NCTC cells (Fig. 2). This accorded with the limited immunoblot evidence for NCX1 in the PM of NCTC cells, a single band (or possibly doublet) being observed at 220 kDa (Fig. 3). Confirmatory evidence for occurrence of NCX in the PM of HeLa and NCTC cells was obtained by use of fura-2 to demonstrate Na^+/Ca^{2+} exchange (Fig. 6). The combination of Na gradient reduction and blockade of $[Ca^{2+}]_i$ buffering by thapsigargin-induced depletion of Ca^{2+} stores in the endoplasmic reticulum gave rise to influx of extracellular Ca^{2+} through reverse mode exchange along with elevation of $[Ca^{2+}]_i$. Similar phenomena were observed for astrocytes (7,8) and NG108-15 cells (25). Jurkat cells, in contrast, showed no evidence for Na^+/Ca^{2+} exchange activity in the PM (Fig. 6).

The results of this study revealed NCX1 expression in the PM of a subpopulation of lymphocytes (Fig. 3). It remains to be determined whether this represents B cells, T cells, or a subpopulation of the latter. If further study reveals subtypes of T cells to be deficient in NCX, this would be consistent with absence of NCX in the Jurkat T cell line. Previous studies with lymphocytes gave conflicting results on this question, some suggesting the presence of Na^+/Ca^{2+} exchangers in the PM of human peripheral T lymphocytes and/or Jurkat T cells (26,27) and others finding no such

evidence (28–30). Study of peripheral blood human lymphocytes by reverse mode Na^+/Ca^{2+} exchange produced convincing evidence for NCX expression, although the type of lymphocyte(s) showing such PM activity was not identified (9,10).

High affinity association of Na^+/Ca^{2+} exchanger with GM1. Strong binding of exchanger to GM1, as seen in the persistent association of GM1 with exchanger during immunoprecipitation, SDS-PAGE and Western blot, was a feature of the NE of all neural cells of previous studies (8,13) and some non-neural cells in the present study. As indicated, there was no evidence for such binding in the PM. The strength of this association in the NE suggested the possibility of covalent linkage, but this was discounted by the fact that dissociation occurred on very brief treatment with dilute base as well as various proteolytic enzymes. One of the latter, thrombin, cleaves selectively at Arg/Lys residues and would give rise to large peptide fragments, while trypsin and proteinase K would produce more degradation of NCX. That these all liberated GM1 unattached to peptide(s) or amino acid(s) suggests conformational interaction of GM1 with NCX. The same result was obtained whether reactions were carried out on IP beads or PVDF membrane following immunoblot. It should be noted that not all NCX1 bands in the NE showed GM1 association on Western blot, as was also the case for NG108-15 cells (13). Although not a widely reported phenomenon, high-affinity ganglioside–protein binding that survives SDS-PAGE has been observed in a few other systems (31,32). The structural features of the NCX alternatively spliced isoforms that mediate this interaction remain to be determined.

In summary, this study revealed the presence of Na^+/Ca^{2+} exchanger proteins in the NE (as well as PM) of non-neural cells, including the HeLa and NCTC cell lines as well as a subgroup of human lymphocytes whose identity remains to be determined. Jurkat cells were confirmed to contain no NCX in either membrane. Finally, the unusually tenacious association of GM1 with some isoforms of NCX in the NE was shown to result from charge–charge interaction with requirement for a positively charged unit in NCX and conformational integrity.

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