Evidence for Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger 1 Association with Caveolin-1 and -2 in C6 glioma cells
Evidence for Na\(^+\)-Ca\(^{2+}\) exchanger 1 Association with Caveolin-1 and -2 in C6 glioma cells

by

Yong-Ju Park

Department of Physiology and Biophysics

(Directed by Professor Chang Kook Suh)

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국문요약

세포 내 Ca²⁺의 변화는 여려 정보활동에서 중요한 역할을 하는데, Na⁺-Ca²⁺ 교환이동체 (NCX)는 Ca²⁺ 농도를 조절하며, 특히 세포 내 Ca²⁺ 향상성 유지에 중요한 역할을 수행한다. 또한 다양한 세포에서 정보활동 인자들은 caveolae 라고 불리는 부위에 집중분포 되어 있는데, caveolins 는 caveolae 를 구성하는 단백질로서 cardiac sarcolemmal vesicles 에서 caveolin-3 가 NCX1 와 연관되어 있음이 이미 보고 되었다. 따라서 본 저자는 caveolin-3 가 존재하지 않는 C6 교세포에서 분자 생물학적 실험법을 이용하여 NCX 1 과 caveolin 단백질들과의 연계를 조사하였다. RT-PCR 과 Western blotting 분석법을 통해 C6 교세포에서 NCX 와 caveolin 의 발현을 조사한 결과, NCX 의 isoforms 의 경우 NCX 1, 2, 3 가 모두 존재하였고 caveolin 의 경우 caveolin-1 과 -2 가 발현되었으나 caveolin-3 는 발현되지 않았다.

세포막분획 실험법을 이용하여 C6 교세포에서 NCX 1 이 caveolin-1, -2 와 같은 분획에 존재하는 것을 확인하였다. 또한 면역침강법 실험을 통해서는 NCX 1 과 caveolin-1, -2 가 서로 연계되어 있음을 확인하였다.

한편 caveolin 발현 억제가 NCX 활성도에 미치는 영향을 알아보기 위해 cholesterol chelating 계의 하나인 β-cyclodextrin 을 바탕한 C6 세포에 처리 하였더니 0 mM Na⁺ 용액 관류에 의한 세포 내 Ca²⁺ 유입이 감소되는 것을 확인하였다.
이상의 결과에서 caveolin-3 가 존재하지 않는 C6 교세포에서 NCX 1 은

caveolin-1 및 -2 와 연계되어 있음을 알 수 있었다. 따라서, caveolin-1, -2, -3
모두 NCX 1 의 정보활동 인자로서 역할을 수행하는 것으로 사료된다.

핵심 단어 : Na⁺-Ca²⁺ exchanger (NCX), caveolin, C6 glioma cells,
immunoprecipitaion, caveolae.
Abstract

The Na⁺-Ca²⁺ exchanger (NCX) is involved in regulation of intracellular calcium concentration via the forward mode (Ca²⁺ extrusion) or the reverse mode (Ca²⁺ influx). Because they play a role in cellular calcium homeostasis, NCX could be an important regulator for signaling factors. For many types of cells, the signaling factors are now known to be concentrated in microdomains called caveolae.

Caveolins are component proteins of caveolae. There is a report that NCX 1 associated with caveolin-3 in cardiac sarcolemmal vesicles. In this experiment, I investigate the interaction of NCX 1 with caveolin proteins in caveolin-3 null cells, C6 glioma cells confirmed by reverse transcriptase-polymerase chain reaction (RT-PCR) and western blotting technique. When the mRNA and protein expression of caveolin-1, -2 and 3 was investigated in C6 glioma cells, caveolin-1 and 2 was expressed high level in both mRNA and protein level. In contrast, no expression of mRNA and protein of caveolin-3 were observed.

Different with caveolin, mRNA expression of NCX 1, 2 and 3 was observed. Using the gradient fractionated glial cell membrane, NCX 1, caveolin-1 and caveolin-2 proteins located in fractions 4 and 5. In order to confirm the association of NCX 1 with caveolin, immunoprecipitaion experiments were performed. The presence of NCX 1, caveolin-1 and caveolin-2 was detected following precipitation with NCX 1 antibody. In addition, the presences of caveolin-1 and NCX 1 and
caveolin-2 and NCX 1 were detected following precipitation with each caveolin-1 and caveolin-2 antibody, respectively. Cholesterol chelation in C6 glioma cell decreased NCX 1 activity.

These results show that, in the case of lack of caveolin-3, NCX 1 bind caveolin-1 and/or caveolin-2 and suggest that all three types of caveolins might act as signaling factors of NCX 1.

Key words: Na⁺-Ca²⁺ exchanger (NCX), caveolin, C6 glioma cells, immunoprecipitaion, caveolae.
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I. Introduction

Intracellular free $\text{Ca}^{2+}$ can modulate the intracellular signaling in virtually all types of animal cells. Changes in intracellular $\text{Ca}^{2+}$ concentration ([Ca$^{2+}$]) can be occurred by the influx of $\text{Ca}^{2+}$ from extracellular fluid, via voltage-gated and receptor-operated Ca$^{2+}$-selective channels and Na$^+$-Ca$^{2+}$ exchanger (NCX) located in the plasma membrane. Ca$^{2+}$ can be also released from intracellular stores by Ca$^{2+}$-induced or inositol trisphosphate (IP$_3$)-activated mechanisms (Berridge, 1993; Bootman et al., 1995; Somlyo et al., 1994).

NCX, an ion transport protein, is expressed in the plasma membrane of many cell types. The NCXs comprise a family of three genes: NCX1 (Arlock et al., 1985), NCX2 (Blaustein, 1974) and NCX3 (Nicoll et al., 1996). NCX serves as one of regulators of [Ca$^{2+}$]$_i$ in various cells including neurons (Furman et al., 1993; Yip et al., 1992) and glial cells (Takuma et al., 1994 and 1996). Although NCX1, the cardiac type of NCX, is a dominant NCX gene expressed in the brain, previous studies (Linck et al., 1988; Li et al., 1994; Nicoll et al., 1996) have been identified two additional NCX genes (NCX2 and NCX3) that are also expressed in the central nervous system. Because NCX plays a role in cellular Ca$^{2+}$ homeostasis, NCX could be one of important regulators for signaling factors of cellular functions (Blaustein and Lederer, 1999).

NCX is involved in regulation of intracellular calcium concentration two modes,
via forward mode (Ca\textsuperscript{2+} extrusion) and reverse mode (Ca\textsuperscript{2+} influx) (Philipson and Nicoll, 1992; Philipson et al., 1998). It has been reported that NCX also functions as a regulator of intracellular calcium concentration in neuron and glial cells. Recent studies on the pathophysiology of ischemic brain injury have revealed that NCX in the reverse mode is responsible for Ca\textsuperscript{2+}-mediated cell injury (Matsuda and Baba, 1998).

Because they play a role in cellular Ca\textsuperscript{2+} homeostasis, NCX could be an important regulator for signaling factors (Blaustein and Lederer, 1999). For many types of cells the signaling factors are now known to be concentrated in microdomains called caveolae (Couet et al., 2001). Caveolae were first identified as an endocytic compartment in endothelial cells, where they appear to move molecules across the cell by transeftration. More recently, researchers have been found to be sites where small molecules are concentrated and internalized by a process called potocytosis (Anderson, 1992 and 1993). Caveolae are flask-shaped invaginations of the plasma membrane with a diameter of 50-100 nm and play a role in a variety of cellular functions, including signal transduction, lipid metabolism, cellular growth control and apoptotic cell death (Lisanti et al., 1994 and 1995; Okamoto et al., 1998).

The principal protein components of caveolae are the caveolin family (Peters et al., 1985; Rothberg et al., 1992). Caveolin, a 21-24 kDa integral membrane protein, and its isoforms have been identified, namely caveolin-1, -2 and -3 (Scherer et al., 1995, 1996; Way and Parton, 1995; Song et al., 1996; Tang et al., 1996). Caveolin
was originally described as a primay v-src tyrosine kinase substrate, and referred to as VIP21 (vesicular integral membrane protein of 21 kDa) (Glenney and SoppeL, 1992; Kurzchalial et al., 1994). Three caveolin genes have been cloned thus far and are termed caveolin-1, -2 and -3 (Scherer et al., 1996). Caveolin-1 and -2 are expressed in many cell types, but the expression of caveolin-3 is restricted to muscle cell types (Song et al., 1996). Caveolins interact directly with a number of caveolae associated signaling molecules to be concentrated within caveolae of plasma membranes (Okamoto et al., 1998).

Previous studies have provided evidences that caveolins are also expressed in brain (Cameron et al., 1997). But the functional significance of caveolins remains unclear. There is a report that NCX 1 associated muscle specific caveolin isoform, caveolin-3, in bovine cardiac sarcolemmal vesicle (Bossuyt et al., 2002). There are no previous reports of NCX 1 association with caveolins in C6 glioma cells. Therefore, in this study, I investigated the interaction of NCX1 with caveolin proteins in C6 glioma cells.
II. Materials and Methods

1. Cell culture

C6 glioma cells were cultured as monolayers in polystyrene dishes (60 mm and 100 mm in diameter) or onto coverslips coated with 0.01% poly-L-lysine (PLL). C6 glioma cells were grown in Dulbecco’s minimum essential medium (DMEM, Gibco) containing 10% heat inactivated fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were cultured in a humidified incubator at 37°C with 5% CO₂ atmosphere.

2. Preparation of cell extracts and Western blotting

C6 glioma cells were washed with PBS and scraped in a buffer (50 mM Tris/HCl (pH 7.5), 2 mM EDTA, 10 mM EGTA, 5 mM DTT (dithiothreitol), 250 mM sucrose and 1% Triton X-100), containing protease inhibitors (0.5 μg/ml leupeptin, 5μg/ml pepstatin, 1 mM PMSF, 1 mM benzamidine, 2 mM iodoacetamide, 1μg/ml aprotinin). Cells were sonicated and centrifuged at 10,000 x g for 15 min to remove the remaining insoluble materials. Protein samples were frozen in liquid nitrogen and stored at -70°C until use. The protein concentration of the supernatant was determined by the Bradford method. Protein samples were boiled at 100°C for 10 min and separated by 10 % SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel) The separated proteins were transferred to PVDF (polyvinylidene fluoride)
membrane. After transfer, PVDF membranes were subjected to immunoblotting. The immunoblots were developed by ECL reagent.

3. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total cellular RNA was extracted from cultured C6 glioma cells by using Trizol following the manufacturer’s instructions. Total RNA (500 ng) was subjected to the first-strand cDNA synthesis by reverse transcriptase. Sequence of used primer sets show in Table 1. The PCR products were resolved on 1% agarose gels stained with ethidium bromide, and photographed.

4. Isolation of caveolin-enriched membrane fractions

C6 glioma cells were washed with PBS, and scraped into 2 ml of 500 mM sodium carbonate (pH 11.0) followed by sonication. The homogenate was then adjusted to 45% sucrose by the addition of 2 ml of 90% sucrose in MES-buffered saline (25 mM Mes, pH 6.5, and 150 mM NaCl) and placed at the bottom of an ultracentrifuge tube. A 5 - 35% discontinuous sucrose gradient was formed above (4 ml of 5% sucrose / 4 ml of 35% sucrose; both in MES containing 250 mM sodium carbonate) and centrifuged at 170,000 x g for 18 hrs in an SW41 rotor. From the bottom of each gradient, 1 ml gradient fractions were collected to yield a total of 12 fractions. The fractions were added with 10% TCA (trichloroacetic acid) and centrifuged at 10,000
x g for 1 hr at 4°C. Gradient fractions were separated by SDS-PAGE, and subjected to immunoblotting.

5. Immunoprecipitation

C6 glioma cells were washed with PBS and scraped with solubilizing buffer (10 mM Tizma base, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% deoxycholate and 0.05% SDS, pH 8.0), containing 1 mM sodium vanadate, 1 mM PMSF, 1 μg/ml leupeptin and 1 μg/ml aprotinin. Cells were sonicated and centrifuged at 19,000 x g for 10 min at 4°C. Samples were precleared by incubation with an excess of the protein A sepharose beads and centrifugation. The cleared samples were incubated either with polyclonal anti-caveolin or polyclonal anti-NCX for 24 hrs at 4°C. Immunocomplexes were pulled down with protein A sepharose beads for 4 hrs at 4°C. Beads were washed with solubilizing buffer followed by low-speed centrifugation. Proteins were detected by SDS-PAGE and Western blotting.

6. Measurements of [Ca^{2+}]_i

C6 glioma cells cultured on glass coverslips were then washed twice with a normal Tyrode solution (140 mM NaCl, 4 mM KCl, 2.5 mM CaCl_2, 1 mM NaH_2PO_4, 5 mM HEPES and 5.5 mM glucose, pH 7.4). In the Na^+ -free solution, NaCl was isosmotically replaced by N-methyl-D-Glucamine (NMDG). Cells were incubated for 2 hrs at 37°C in a solution containing 10 μM fura 2-AM (the
membrane permeable acetoxyethyl ester of fura-2) and 2 μM cremophore. The cells were superfused with normal Tyrode solution for 15 min to wash away the remaining extracellular dye and also to allow time for the intracellular esterases to hydrolyse the fura-2 AM. The imaging system was designed around a Nikon Diaphot microscope optimized for ultraviolet transmission. The excitation light was supplied by high pressure Xenon UV lamp. The excitation wavelengths were selected by employing the 340 and 380 nm filters mounted on a processor-controlled rotating filter wheel between the UV lamp and the microscope. Fluorescent image was filtered by the light which wavelength of 510 nm (band with=10 nm) and was collected using a CCD digital camera (Photometrics PXL37, USA). Images were acquired at a rate of one image every 5 sec, and then the light was exposure for 300 msec. [Ca²⁺]ᵢ was presented as the ratio of collected fluorescence intensities excited at 340 and 380 nm To control filter changer and CCD digital camera, and analysis fluorescent imaging, I used Axon imaging workbench (Axon instrument, USA) installed on computer. Microcal Origin software was used for graphics.
Table 1. Nucleotide sequences of used PCR primers.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Nucleotide sequence</th>
<th>Size of PCR product (bp)</th>
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<tbody>
<tr>
<td>NCX 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sense</td>
<td>5’-ATGCTTCGACTAAGTCTCCCACCCAA-3’</td>
<td></td>
</tr>
<tr>
<td>antisense</td>
<td>5’-AAAGATGGGTCATGGGGTTCCCAA-3’</td>
<td></td>
</tr>
<tr>
<td>NCX 2</td>
<td></td>
<td></td>
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<tr>
<td>sense</td>
<td>5’-ATGGCTCCCTTGCTTGGTAGGGGT-3’</td>
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</tr>
<tr>
<td>antisense</td>
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</tr>
<tr>
<td>NCX 3</td>
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<td>sense</td>
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<tr>
<td>antisense</td>
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<tr>
<td>Caveolin-1</td>
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<td>sense</td>
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<td>antisense</td>
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</tr>
<tr>
<td>antisense</td>
<td>5’-TTCCCCACAGTTAGCCTTCCCTTC-3’</td>
<td></td>
</tr>
</tbody>
</table>
III. Results

1. The expression of Na\(^+\)-Ca\(^{2+}\) exchanger and caveolins in C6 glioma cells

I predicted location of NCX 1 caveolin - binding motifs (Figure 1). The hydropathy plot model of NCX 1 is based upon topological analysis. Cylinders represent transmembrane regions and black lines are extramembranal loops of NCX 1. Endogenous exchange inhibitory peptide (XIP) is shown as a black box (Nicoll, D.A. et al., 1999, J. Biol. Chem). XIP targets the NCX putative calmodulin-binding domain on the large cytoplasmic loop of NCX (Kleiboecker et al., 1992).

Two motifs (amino acids 200-210 and 905-915) are modeled to be in transmembrane region. And one motif (amino acids 255-263) is modeled to be in XIP region. The sequence data has been obtained from the protein / NCBI databases under accession number Q01728.

The expression of NCX 1, 2 and 3 in C6 glioma cells was examined by RT-PCR analysis as described materials and methods. Total RNA isolated from C6 glioma cells by using a TRI reagent following the manufacture’s instructions was subjected to RT-PCR analysis by using primers directed against the known sequence of NCX 1 2, and 3. RT-PCR analysis revealed that NCX 1, 2 and 3 were expressed in C6 glioma cells (Figure 2).

The expression of caveolin-1, -2 and -3 in C6 glioma cells has been shown in Fig. 3-4. Total RNA isolated from C6 glioma cells was subjected to RT-PCR analysis by
using primers directed against the known sequence of caveolin-1, -2, and -3 (Figure 3). Protein was extracted from C6 glioma cells and followed by SDS-PAGE and immunoblotting with antibodies directed caveolin-1, -2, and -3 (Figure 4). These results showed that caveolin-1 and -2 were expressed in C6 glioma cells. However, expression of caveolin-3 was undetected in cultured C6 glioma cells by RT-PCR and Western blotting analysis.
Figure 1. Predict location of Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger 1 caveolin-binding motifs.

Transmembrane regions and loops of extramembrane are represented by cylinders and black lines, respectively. Endogenous exchange inhibitory peptide (XIP) is shown as a shaded box. Arrows designate the approximate location of the NCX 1 caveolin binding motifs. Two motifs are existed in transmembrane region. The other one is in XIP region.
Figure 2. The expression of Na\(^+\)-Ca\(^{2+}\) exchanger 1, 2 and 3 in C6 glioma cells. Total RNA was prepared from C6 glioma cells. RT-PCR was performed using primers directed against the known sequence of NCX 1, 2, and 3. Products were separated on a 1 % agarose gel and then stained with ethidium bromide. M; Marker, NCX 1; 203 bp, NCX 2; 194 bp, NCX 3; 209 bp.
Figure 3. The expression of caveolin-1, -2 and -3 mRNAs in C6 glioma cells.

RT-PCR was performed using total RNA isolated from C6 glioma cells. The condition and sequence of used primer sets noted in method section. M; Marker, caveolin-1; 561 bp, caveolin-2; 268 bp.
Figure 4. The Western blot analysis of cavoelin-1, -2 and -3 in C6 glioma cells.

Protein was extracted from C6 glioma cells and subjected to SDS-PAGE and immunoblotting with antibodies directed caveolin-1, -2, -3.
2. Interaction of Na\(^+\)-Ca\(^{2+}\) exchanger 1 with caveolins in C6 glioma cells.

The expressions of caveolin family members and NCX 1 in plasma membrane fraction from C6 glioma cells were confirmed by immunoblot analysis as described materials and methods (Figure 5). Cell lysates were prepared from C6 glioma cells and were applied to 5-35% discontinuous sucrose gradient by following Song et al. (1996). Equal volumes of fractions collected across the gradients were separated by SDS-PAGE and immunoblotting with antibodies directed caveolin proteins and NCX 1 protein. The result showed that caveolin-1, -2 or NCX 1 were detected in caveolin-enriched membrane fractions, 4 - 5, but caveolin -3 was undetectable.

To elucidate the complex of caveolin proteins and NCX 1 protein in C6 glioma cells, immunoprecipitations were done with antibodies coupled protein A sepharose bead; non-immune antibody, anti-caveolin-1 antibody, anti-caveolin-2 antibody or anti-NCX 1 antibody. The immunoprecipitated proteins were detected using antibodies individually directed NCX 1, caveolin-1 and -2. Immunoprecipitation experiments showed that NCX 1 is associated to caveolin-1 and/or caveolin-2 (Figures 6 and 7).

And then I experimented the effect of β-cyclodextrin (CD) on NCX activity in C6 glioma cells. C6 glioma cells were pretreated with 10 mM of CD for 1h, and assayed for NCX 1 activity (Figure 8). C6 glioma cells were loaded with the fluorescent Ca\(^{2+}\) indicator fura-2AM. Data recordings of the fura-2 fluorescence ratio (Ratio\(_{340/380}\)) in
C6 glioma cells were shows in control cells and CD-pretreated cells. [Ca^{2+}]_i increased by 0 mM Na^+/ 2.5 mM Ca^{2+} solution and decreased by 140 mM Na^+/ 0 mM Ca^{2+} solution. These data indicates that the effect of 0 mM Na^+/ 2.5 mM Ca^{2+} solution showed a significant difference between control and CD-pretreated cells (P < 0.05).
Figure 5. Immunoblot analysis of the expression of caveolins and Na\(^+\)-Ca\(^{2+}\) exchanger 1 in detergent-resistant membrane fraction from C6 glioma cells.

Cell lysates were prepared from C6 glioma cells and were applied to 5-35% discontinuous sucrose gradient. Collected protein of membrane fractions were separated by electrophoresis and immunoblotted with respective antibodies. Fraction number is indicated horizontally.
Figure 6. The results of immunoprecipitation experiment of caveolin-1 and NCX 1 in C6 glioma cells.

C6 glioma cell homogenates were immunoprecipitated (IP) with either anti-caveolin-1 antibody or anti-NCX 1 antibody. Precipitated proteins (15 μg) were loaded onto each lane of a 10% SDS-polyacrylamide gel, and proteins were separated by electrophoresis as described materials and methods. After transferring to membrane, proteins were detected by respective antibody.
Figure 7. The results of immunoprecipitation experiment of caveolin-2 and NCX 1 in C6 glioma cells.

Immunoprecipitations were done with protein A sepharose beads coupled non-immune antibody, anti-caveolin-2 antibody or anti-NCX 1 antibody. The proteins were detected by antibodies directed caveolin-2 or NCX 1 as indicated in each column of data. Anti-caveolin-2 immunoprecipitates were probed with anti-NCX 1 antibody, and anti-NCX1 immunoprecipitates were probed with anti-nNOS antibody.
Figure 8. The effect of β-cyclodextrin on Na⁺-Ca²⁺ exchanger activity in C6 glioma cells.

C6 glioma cells were pretreated with 10 mM CD for 1hr, and assayed the activity of NCX 1. Data recordings of the fura-2 fluorescence ratio in C6 glioma cells were shown in (A) control cells and (B) CD-pretreated cells. (C) Summary of data shows as the changes in Ratio 340/380 induced by 0 mM Na⁺ solutions. *: P < 0.05.
Table 2. The effect of β-cyclodextrin on Na\(^+\)-Ca\(^{2+}\) exchanger activity in C6 glioma cells.

<table>
<thead>
<tr>
<th></th>
<th>Control cells (n=28)</th>
<th>CD-treated cells (n=15)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Basal level</strong></td>
<td>0.53 ± 0.01</td>
<td>0.60 ± 0.01</td>
</tr>
<tr>
<td><strong>Peak value</strong></td>
<td>1.75 ± 0.05</td>
<td>1.27 ± 0.04</td>
</tr>
<tr>
<td><strong>Change of Ratio\textsubscript{340/380}</strong></td>
<td>1.22 ± 0.05</td>
<td>0.67 ± 0.04</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, number of cells. Basal levels correspond to Ratio\textsubscript{340/380} before the perfusion of Na\(^+\)-free solution. Statistical analysis refer to comparison between control cells data and CD-treated cells data, respectively, and were determined by student’s t-test (P < 0.05).
VI. Discussion

1. The expression of Na\(^+\)-Ca\(^{2+}\) exchanger and caveolins in C6 glioma cells.

NCX plays a major role in regulating Ca\(^{2+}\) homeostasis in neuron and glial cells (Matsuda et al., 1998). Astrocytes, the most abundant glial cell type in the brain, are considered to have physiological and pathological roles in neuronal activities. Takuma and colleagues (1996) employed sodium nitroprusside (SNP) to generate nitric oxide (NO) and NCX activation and antisense-oligos to inhibit NCX activity in cultured astrocytes and investigated intracellular Ca\(^{2+}\) changes via NCX activation. The Ca\(^{2+}\) transients were attenuated by SNP and augmented by the antisense-oligos and by the NCX inhibitor, 3,4-dichlorobenzamil (Takuma et al., 1996; Matsuda et al., 1996). These results suggested that NCX plays a role in controlling the amplitude of Ca\(^{2+}\) transients in glia.

Reperfusion of cultured astrocytes after Ca\(^{2+}\) depletion causes Ca\(^{2+}\) overload followed by delayed cell death and the NCX in the reverse mode is responsible for this Ca\(^{2+}\)-mediated cell injury, Ca\(^{2+}\) paradox injury (Matsuda and Baba, 1998). The Ca\(^{2+}\) paradox injury of cultured astrocytes is considered to be an in vitro model of ischemia / reperfusion injury, since a similar paradoxical change in extracellular Ca\(^{2+}\) concentration is reported in ischemic brain tissue. Brain ischemia produces morphologic and biochemical alterations in astrocytes. They demonstrated that NCX is present in cultured neurons and astrocytes and that there are differences in their properties and distributional ratio of the isoforms between neurons and astrocytes.
They also found that Ca\(^{2+}\) depletion followed by reperfusion with Ca\(^{2+}\)-containing medium caused cell death in cultured astrocytes (Ca\(^{2+}\) paradox-like injury), but not in neurons. The study, carried out by the use of a specific antisense oligomer, provides direct evidence that Ca\(^{2+}\) paradox-like injury is mediated by NCX in the reverse mode.

Previous studies on the pathophysiology of ischemic brain injury have revealed that NCX is an important mediator of Ca\(^{2+}\) overload (Matsuda et al., 1996). Ca\(^{2+}\) entry via the NCX plays an important role in reperfusion-induced delayed glial cell death. A prominent feature of cerebral ischemia is the excessive intracellular accumulation of both Na\(^+\) and Ca\(^{2+}\), which results in subsequent cell death. NCX is involved in the anoxic injury to rat hippocampus and it may be one of the major ways leading to the anoxia-induced [Ca\(^{2+}\)]\(_i\) increase of hippocampal neurons. Studies examining the role of tetrodotoxin-sensitive ion channels in hypoxic-ischemic neuronal damage have concluded that sodium influx is an important initiating event. Reversal of NCX can occur during ischemia-reperfusion injury as a result of changes in intracellular pH and sodium concentration. Inhibition of NCX has been shown to be neuroprotective in vitro.

The expression of a caveolin-caveolae compartment in glial cells is similar to the one in peripheral tissues and cells (Cameron et al., 1997). Cameron et al. evaluated the expression of the caveolin-1 in primary culture of glial cells. Furthermore, Ikezu and colleagues (1998) detected expression of the different caveolin isoforms in
primary cultures of rat astrocytes. And Silva and colleagues (1999) demonstrated C6 glioma cells could be used as a model system to study the role of caveolae and caveolins in subcellular transport and signal transduction events in glial cells and the brain.

To investigate the expression of NCX and caveolins in C6 glioma cells, I used RT-PCR and Western blot analysis. The expression of NCX 1, 2 and 3 was observed in C6 glioma cells (Fig. 2). Of the known isoforms of caveolin, caveolin-1 and -2 was expressed in C6 glioma cells (Fig. 3-4). However, in contrast, expression of mRNA and protein of caveolin-3 were not observed. Therefore, in the absence of caveolin-3 I considered caveolin-1 and/or caveolin-2 also associate with NCX 1 and carried out further study of the interaction of NCX and caveolin-1 and/or caveolin-2.

2. Interaction between Na\(^+\)-Ca\(^{2+}\) exchanger 1 and caveolins in C6 glioma cells.

To elucidate the interaction of NCX with caveolin proteins, co-immunoprecipitation experiments of NCX and caveolins and Ca\(^{2+}\) imaging method to evaluate functional character of NCX were performed in C6 glioma cells. Using the gradient fractionated membrane, I found that NCX 1 was co-fractionated with caveolin-1 and -2 in fractions 4 and 5 (Fig. 5). The immunoprecipitation was subjected to Western blot analysis. Immunoprecipitation experiments showed that NCX 1 was co-precipitated with caveolin-1 and -2 (Fig. 6 and 7).

β-cyclodextrin (CD), one of cholesterol chelating agents, has been shown to
disrupt function and structure of caveolae (Li et al., 1996). Without caveolin in the membrane microdomain, caveolae cannot retain structural integrity. Because caveolins require cholesterol in the membrane, caveolae disappear by CD pretreatment (Hailstones et al., 1998). CD significantly depressed the increase in $[Ca^{2+}]_i$ induced by the activation of NCX reverse mode in C6 glioma cells (Fig. 8 and Table. 1), suggesting showed that CD decreased NCX activity by disruption of caveolae.

In the interaction of NCX other factor, such as nitric oxide (NO) can be incorporated (Teubl et al., 1999), endogenous NO is synthesized from the amino acid L-arginine by three isoforms of the enzyme NO synthase (NOS; inducible NOS (iNOS), neuronal NOS (nNOS), endothelial NOS (eNOS), (Moncada et al., 1991). In endothelial cells, eNOS co-purified with caveolin-1 and NCX 1 in sucrose density gradients (Teubl et al., 1999). Caveolin-1 may direct interact with eNOS and inhibits eNOS activity in endothelial cells (Ju et al., 1997). In skeletal muscles, caveolin-3 binds and regulates nNOS (Garcia-Cardenas et al., 1997; Venema et al., 1997). I will investigate the effects of caveolins AS-oligos on NCX and NOS activity in C6 glioma cell. Recently, many researchers took advantage of NO donor drugs, S-nitroso-N-acetyl-penicillamine (SNAP), sodium nitroprusside (SNP), PAPA-NONOate and so on, in NO studies (Megson et al., 2000). I will experiment effects of NO-donors on the interaction between NCX and caveolins.

To investigate the modulation of NOS activity by caveolin, to detect the
intracellular NO concentration changes by the absence of caveolin in the membrane, 4’, 5’-diaminofluorescein diacetate (DAF-2DA), NO-sensing fluorescence dye, will be employed.

On conclusion, in the present study, I identified the expression of caveolins and NCX, and investigated the interaction between NCX 1 protein and caveolin proteins in cultured C6 glioma cells. Our data represented that C6 glioma cells are expressed caveolin-1 and -2, but not caveolin-3. Also, NCX 1 co-precipitated with caveolin-1 and -2. These results suggest that caveolin-1 and/or caveolin-2 also associates with NCX 1 in the absence of caveolin-3. It is possible that all types of caveolin proteins might function as signaling factor of NCX 1. Cultured C6 glioma cells can be useful as a model system to study the role of signal transduction events in glial cells and the brain.
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