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Expression of the Na^+/Ca^{2+} Exchanger Ameliorates Ionomycin-Induced Cell Death

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PC12 cells were stably transfected with cDNA encoding the Na^+/Ca^{2+} exchanger (NCX1.4). A robust Na^+-dependent Ca^{2+} uptake confirmed the functional expression of the protein. When NCX1.4 expressing cells (NO) and vector transfected control cells (VC) were exposed to 0.5–20 μM ionomycin for 6 h, a dose-dependent increase in LDH release was observed. LDH release was significantly reduced in NO when compared with VC. When either VC and NO were treated with 3 μM ionomycin and 1.1 mM EGTA, the increase in LDH release was nearly abolished. However, when VC and NO were treated with ionomycin and then EGTA was added 2 min later, LDH release remained elevated. These data suggest ionomycin-induced cell death was Ca^{2+} dependent and expressing NCX1.4 may have ameliorated cell death by reducing elevated $[Ca^{2+}]_I$.

Key Words: **calcium; ionomycin; ion transport; neurodegeneration; PC12 cells.**

Neurons undergo transient changes in free intracellular Ca^{2+} concentration ($[Ca^{2+}]_I$) as a fundamental element of their intracellular signaling. However, under pathological conditions, pronounced elevation of $[Ca^{2+}]_I$ activates proteases, phospholipases and endonucleases, initiates cytoskeletal degradation, impaired energy production, and ultimately results in cell death (1). Calcium overload has been associated with cell injury and death in many cell types. In neurons, glutamate-induced neurotoxicity is associated with a prolonged increase in $[Ca^{2+}]_I$ and excessive intracellular calcium overload plays a major role in several acute neuronal losses such as ischemia and brain trauma (2, 3). Calcium elevation is considered to be the final pathway for neuronal degenerative disease such as Alzheimer's disease (4, 5).

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As the maintenance of intracellular Ca^{2+} homeostasis is necessary for survival, neurons use several mechanisms to reduce elevated $[Ca^{2+}]_I$. Two processes located at the plasma membrane mediate Ca^{2+} extrusion from cells: the Ca^{2+}-ATPase (6) and the Na^+/Ca^{2+} exchanger (7). The Na^+/Ca^{2+} exchanger is a membrane protein expressed in virtually all excitable cells and highly expressed in brain (8). Three isoforms, NCX1 (9), NCX2 (10), and NCX3 (11) of the Na^+/Ca^{2+} exchanger have been identified. The Na^+/Ca^{2+} exchanger mediates the movement of 3 Na^+ in exchange for 1 Ca^{2+}. Importantly, the exchanger is reversible and can mediate both Ca^{2+} efflux and influx.

The Na^+/Ca^{2+} exchanger could contribute to either increasing or decreasing $[Ca^{2+}]_I$ depending on the membrane potential and ion gradients for Na^+ and Ca^{2+}. The exact role of the Na^+/Ca^{2+} exchanger in neuronal Ca^{2+} homeostasis has been difficult to delineate, primarily due to the lack of specific inhibitors. Experiments have relied on Na^+-dependent or amiloride inhibitable events to define phenomena mediated by the exchanger (12, 13). Recently, 2-[2-[4-(4 nitrobenzyloxy) phenyl] ethyl] isothiourea methanesulfonate (KB-R7943) thought to selectively inhibit the Na^+/Ca^{2+} exchanger, was shown to protect CA1 neurons in rat hippocampal slices against hypoxic/hypoglycemic injury (14). However, KB-R7943 is known to block Na^+ channels, Ca^{2+} channels and certain K^+ channels (15). Another approach is to use antisense oligonucleotides to reduce the expression of the exchanger (16). It was found that Na^+/Ca^{2+} exchange appeared to mediate Ca^{2+} entry in response to membrane depolarization and was responsible for up to 70% of the Ca^{2+} removal from the cytoplasm upon membrane repolarization in rat pancreatic β-cells. Overexpression approach has also been used. Transgenic male mice overexpressing the cardiac Na^+/Ca^{2+} exchanger showed an increased susceptibility to ischemia/reperfusion injury in the heart (17).

The various findings point to a very important role played by Na^+/Ca^{2+} exchange in neuronal Ca^{2+} ho-

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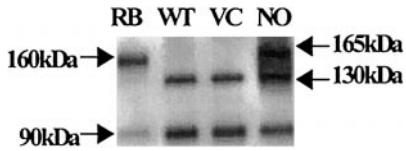

FIG. 1. Western blot of NCX1 protein expression in rat brain, wild-type, empty vector transfected and NCX1.4 expressing PC12 cells. RB showed two NCX1 specific antibody labeled proteins with MW of 90 and 160 kDa. WT and VC showed the same 90 and 130 kDa bands. NO showed an additional band about 165 kDa confirming a novel membrane protein expression by transfection of NCX1.4.

meostasis and processes of Ca^{2+}-dependent neurodegeneration. The present study was designed to help define the role of Na^+/Ca^{2+} exchange in mechanisms of neuronal death caused by intracellular calcium overload. We stably transfected PC12 cells with a Na^+/Ca^{2+} exchanger NCX1.4 isoform. Our results showed that expression of NCX1.4 in PC12 cells could ameliorate cell death induced by ionomycin.

MATERIALS AND METHODS

Expression of NCX1.4 isoform in PC12 cells. Rat pheochromocytoma (PC12) cells (ATCC No. CRL1721) were maintained at 37°C in DMEM (Sigma) with 10% heat-inactivated horse serum (Sigma) and 5% heat-inactivated fetal bovine serum (Sigma). Plasmid DNA containing a dog NCX1.4 construct was obtained from Dr. Debora Nicoll (Department of Physiology and Medicine, UCLA). This NCX1.4 isoform was generated from a dog cardiac NCX1.1 cDNA (GenBank No. M57523) by replacing the alternative-splicing region with a partial clone obtained from the dog brain. The brain isoform contained AD exons whereas the cardiac isoform contained exons ACDEF in the alternative splicing region. The NCX1.4 cDNA was subcloned into a CMV promoter- containing pcDNA3.1(−) (Invitrogen) using *Bam*HI and *Hind*III sites to generate the final pS7 construct. PC12 cells were transfected with pS7, or pcDNA3.1(−) as a negative control, using standard LipofectAMINE transfection protocol (Life Technologies). Cell lines that stably expressed the Neo gene were selected with 0.6 mg/ml G418 for 6 weeks. Clones that stably expressed the NCX1.4 were screened and identified by Northern and Western blot.

Western analysis of NCX1.4 transfected PC12 cell line. The primary antibodies were generated as previously described (18). Plasma membrane fractions from PC12 cells were prepared by suspending cells in 0.32 mM sucrose and 10 mM HEPES pH 7.4 with protease inhibitors (soybean trypsin inhibitors, 50 μg/ml; pepstatin, 0.7 μg/ml; and leupeptin, 0.5 μg/ml). The cells were homogenized and centrifuged at 5000*g* for 5 min to pellet nuclei and mitochondria. The supernatant was then centrifuged at 125,000*g* for 45 min to sediment the plasma membrane fraction and the protein concentration was determined by Lowry. Fifty micrograms of the membrane fraction was separated in a 9% SDS–PAGE gel. The resolved proteins were transferred to a PVDF membrane and immunoblotted with primary antibodies. A Western-Light Plus Chemiluminescent Detect System (TROPIX) was used for immunodetection.

Assay of the Na^+/Ca^{2+} exchange activity. Na^+/Ca^{2+} exchange activity was assayed as described previously with slight modification (19). Cells were Na^+ loaded with buffer containing 132 mM NaCl, 1 mM ouabain, 25 μM nystatin, 2 mM $MgCl_2$, and 10 mM HEPES pH 7.4 on ice for 10 min, rinsed with the same buffer but without nystatin, and divided as 2×10^5 cells/tube. One group was incubated in Na^+-containing buffer, while the other group was treated with Na^+-free buffer. The Na^+-containing buffer contained 10 mM HEPES, 137 mM NaCl, 0.1 mM EGTA, 0.55 mM $CaCO_3$, 1 mM ouabain, and 0.83 μCi/ml $^{45}Ca^{2+}$(ICN); while the Na^+-free buffer contained the same components except 137 mM choline was substituted for 137 mM NaCl. The free calcium concentration in the above solutions was 257 μM verified using calcium sensitive electrode (20). The time course of Na^+/Ca^{2+} exchange was measured by incubating the cells at 37°C for 20 s, 45 s, 1, 2, and 5 min with each buffer and was terminated by addition of $LaCl_3$ at a final concentration of 1 mM. Cells were washed with $LaCl_3$ three times and transferred to scintillation vials containing 3 ml of ScintiSafe LCS-cocktail solution. $^{45}Ca^{2+}$ was counted using a Packard 1900TR Liquid Scintillation Analyzer (Packard Instrument Company).

Ionomycin induced cell death. Ionomycin (Calbiochem) was dissolved in dimethylsulfoxide (DMSO) as 1 mM stock and diluted into culture medium (DMEM, 5% FBS, 10% HS) at final concentrations from 0.5–20 μM. 2×10^5 cells was used for each treatment. LDH release was measured after 6 h using published methods (21).

RESULTS AND DISCUSSION

Characterization of a PC12 Cell Line Expressing NCX1.4

In Western blots (Fig. 1) of wild-type (WT) or pcDNA3.1(−) vector transfected cells (VC), the banding pattern differed from that seen in adult rat brain (RB). The NCX1.4 expressing PC12 cells (NO) showed an additional labeled protein band at 165 kDa. Since this band could be detected by the anti-NCX antibodies, is expressed in NO but not WT or VC, and is similar in size to the major NCX protein expressed in RB, this 165 kDa protein band is very likely the product of the transfected NCX1.4 gene. The slight difference in size between this 165 kDa band and the 160 kDa band in the RB sample may be due to the difference in species of the gene (dog versus rat) and differential protein modification in RB and PC12 cells. The deduced amino acid sequence of NCX1.4 predicts a protein of 120 kDa but the expressed protein is most often seen in Western blots as a protein of 160 kDa with various lower MW species due to differential pro-

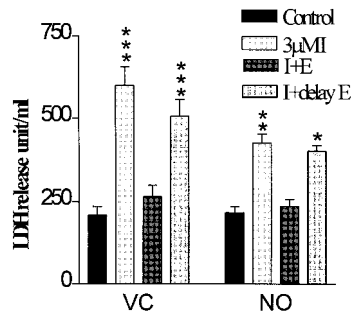

FIG. 2. Time course of Na^+-dependent Ca^{2+} uptake in VC and NO. Na^+-dependent Ca^{2+} uptake of both VC (■) and NO (▲) was determined by subtracting the values obtained in Na^+-containing buffer from the Na^+-free choline buffer. Each point represents the mean value ± SEM ($n = 6$). Two-way-ANOVA showed a significant difference of Ca^{2+} uptake with time ($P < 0.05$) and Ca^{2+} uptake of NO was significantly different when compared to VC ($P < 0.001$).

cessing, partial proteolysis, glycosylation, and incomplete reduction of the protein (22, 23).

The kinetics of Ca^{2+} transport by NO was determined and compared to VC. The time course of the Na^+-dependent Ca^{2+} uptake showed a rapid initial uptake reaching a maximum in about 2 min (Fig. 2). The maximum Ca^{2+} uptake measured in WT was negligible (0.07 nM/10^5 cells/2 min). VC showed a slightly higher Ca^{2+} uptake (0.16 nM/10^5 cells/2 min), but NO had a Ca^{2+} uptake as high as 0.70 nM/10^5 cells/2 min. Although the WT did not exhibit significant Na^+-dependent Ca^{2+} uptake, they were capable of robust Ca^{2+} uptake when stably transfected with NCX1.4. In a sense, WT PC12 could be considered equivalent to an NCX1 "knock-down." Expressing the NCX1.4 cDNA in a PC12 cell background provided an excellent model system to determine Ca^{2+} homeostasis mechanisms mediated by the Na^+/Ca^{2+} exchanger. The Ca^{2+} dependency of the Na^+-dependent Ca^{2+} uptake was determined by varying the Ca^{2+} concentration from 1 to 100 μM and measuring at 2 min. Data obtained from NO were fit to a rectangular hyperbola using computer assisted non-linear regression analysis (Graph Pad, San Diego, CA). An apparent V_{max} of 0.5 nmol/10^5 cells/2 min and a K_m of 35 μM were obtained (data not shown). To further confirm that the Ca^{2+} uptake was mediated by Na^+/Ca^{2+} exchange, inhibitors of the exchanger were tested. KB-R7943 (Kanebo, LTD, Japan) was tested and showed a dose-dependent inhibition of Na^+-dependent Ca^{2+} uptake in NO with half maximum inhibition at 30 μM (data not shown).

Expression of NCX1.4 Reduced Cell Death Induced by Ionomycin

We hypothesized that NO should be more efficient at clearing an intracellular Ca^{2+} load and therefore should be resistant to ionomycin-induced cell death. We found that increasing ionomycin concentrations induced a dose-dependent cell death in both NO and VC and the LDH release of NO was significantly less when compared to VC ($P < 0.001$). To answer whether NCX1.4 expression influenced cellular vulnerability in a non-specific way, or whether LDH release from NO was non specifically reduced, VC and NO were treated with H_2O_2 (0.1–10 mM) similar to ionomycin treatment and LDH release was determined 1 h later. In contrast to the data obtained with the ionomycin, expression of NCX1.4 afforded no additional resistance to H_2O_2-induced cytotoxicity (data not shown).

In order to test the hypothesis that extracellular calcium was mediating the ionomycin-induced cell death, equal molar concentration of EGTA/Ca^{2+} were added during ionomycin exposure (Fig. 3). The LDH release of the 3 μM ionomycin treated cells was significantly higher than control for either VC or NO. In addition, the LDH release from VC was significantly higher than NO when both were treated with 3 μM ionomycin. When both NO and VC were treated with 3 μM ionomycin and 1.1 mM EGTA, LDH release was similar to control cells. These results indicate that the influx of extracellular calcium induced by ionomycin was necessary to cause cell death. Next, it was determined whether the initial influx of Ca^{2+} was sufficient to initiate the delayed cell death. Another group of cells were treated with 3 μM ionomycin for 2 min and then 1.1 mM EGTA was added for the remaining 6 h of incubation. Both NO and VC showed increased LDH release similar to when no EGTA was used (Fig. 3). The difference was significant when compared to control with both VC ($P < 0.001$) and NO ($P < 0.05$). These data suggest that PC12 cells expressing NCX1.4 acquired an active and efficient mechanism of Ca^{2+} extrusion that at least partially counteracts ionomycin-induced rises in $[Ca^{2+}]_I$. From these data it can be inferred that the Na^+/Ca^{2+} exchanger plays an important role in cellular Ca^{2+} homoeostasis when cells are exposed to rapid and large increases in $[Ca^{2+}]_I$.

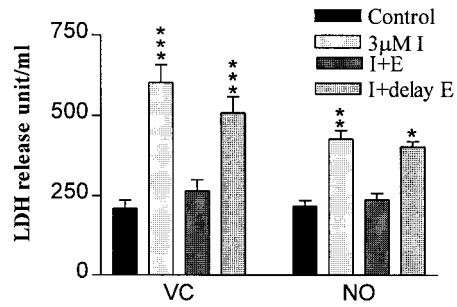

FIG. 3. Ionomycin-induced cytotoxicity was calcium dependent. VC and NO were treated with 10 μM DMSO (control), 3 μM ionomycin (I), 3 μM ionomycin + 1.1 mM EGTA (I + E) and 3 μM ionomycin with 1.1 mM EGTA added 2 min later (I + delay E). Control with only culture medium and control with 1.1 mM EGTA were also tested and showed no difference compared to control with 10 μM DMSO. The results are the mean ± SEM of duplicates in three independent experiments ($n = 6$). ANOVA and Tukey's multiple comparison tests show that there were significant differences between control and 3 μM ionomycin treatment, and between control and I + delay E treatment, in both VC and NO. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

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