Involvement of Na\(^+\)-Ca\(^{2+}\) Exchanger in Intracellular Ca\(^{2+}\) Increase and Neuronal Injury Induced by Polychlorinated Biphenyls in Human Neuroblastoma SH-SY5Y Cells

Simona Magi, Pasqualina Castaldo, Giuseppina Carrieri, Antonella Scorziello, Gianfranco Di Renzo, and Salvatore Amoroso

Department of Neuroscience, Unit of Pharmacology, School of Medicine, University “Politecnica delle Marche,” Ancona, Italy (S.M., P.C., G.C., S.A.); and Unit of Pharmacology, Department of Neuroscience, School of Medicine, University of Naples “Federico II”, Naples, Italy (A.S., G.D.R.)

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ABSTRACT

In SH-SY5Y, a human neuroblastoma cell line, Aroclor 1254 (A1254) induced a dose-dependent (10–50 µg/ml) intracellular calcium concentration ([Ca\(^{2+}\)]\(_i\)) increase. Two rather specific sodium-calcium (Na\(^+\)-Ca\(^{2+}\)) exchanger (NCX) inhibitors, bepridil (10 µM) and KB-R7943 [2-[2-[(4-nitrobenzyloxy)phenyl]-ethyl]isothiourea methanesulfonate] (10 µM), reduced A1254-induced [Ca\(^{2+}\)]\(_i\) increase. A 24-h exposure to 30 µg/ml A1254 caused remarkable SH-SY5Y neuroblastoma cell damage. It is noteworthy that both bepridil and KB-R7943 counteracted A1254-induced neuronal injury. These results indicate that NCX contributes to [Ca\(^{2+}\)]\(_i\) increase and neuronal injury induced by A1254. RT-PCR experiments revealed in SH-SY5Y neuroblastoma cells the expression of NCX1 and NCX3 isoforms. To investigate which isoform was involved in [Ca\(^{2+}\)]\(_i\) increase, we used specific antisense oligodeoxynucleotides (ODNs) to reduce NCX1 or NCX3 protein expression. The results showed that only NCX1 ODN reduced [Ca\(^{2+}\)]\(_i\) increase and neuronal injury induced by A1254. In conclusion, these results indicate that NCX1 may participate to [Ca\(^{2+}\)]\(_i\) increase and neurotoxicity evoked by A1254 in SH-SY5Y neuroblastoma cells.

Polychlorinated biphenyls (PCB) are a class of persistent pollutants present in the environment, and there is increasing evidence that exposure to PCBs can cause neurotoxicity (Hwang et al., 2001; Mariussen et al., 2002; Howard et al., 2003; Sanchez-Alonso et al., 2003, 2004; Kang et al., 2004; Lee and Opanashuk, 2004). A number of potential mechanisms have been proposed to explain PCBs neurotoxicity i.e., alterations in neurotransmitter levels (Bemis and Seegal, 2003; Sanchez-Alonso et al., 2003, 2004; Kang et al., 2004; Lee and Opanashuk, 2004). This antiporter couples the uphill extrusion of Ca\(^{2+}\) to the entrance of Na\(^+\) into the cell (forward mode) and down its electrochemical gradient. However, this mechanism can also operate as a Na\(^+\) efflux-Ca\(^{2+}\) influx pathway, depending not only on membrane potential (Snelling and Nicholls, 1985) but also on the intracellular concentrations of Na\(^+\) and Ca\(^{2+}\) (Amoroso et al., 1997, 2000; Pignataro et al., 2004a,b).

The Na\(^+\)-Ca\(^{2+}\) exchanger (NCX) is a plasmamembrane exchanger mainly involved in the maintenance of cytosolic Ca\(^{2+}\) homeostasis (Sanchez-Armass and Blaustein, 1987). This antiporter couples the uphill extrusion of Ca\(^{2+}\) to the entrance of Na\(^+\) into the cell (forward mode) and down its electrochemical gradient. However, this mechanism can also operate as a Na\(^+\) efflux-Ca\(^{2+}\) influx pathway, depending not only on membrane potential (Snelling and Nicholls, 1985) but also on the intracellular concentrations of Na\(^+\) and Ca\(^{2+}\) (Amoroso et al., 1997, 2000; Pignataro et al., 2004a,b).

In light of the role played by NCX in the maintenance of [Ca\(^{2+}\)]\(_i\), homeostasis, the aim of the present study was to investigate the role played by NCX in PCBs-induced [Ca\(^{2+}\)]\(_i\)
perturbation and neuronal injury. For this purpose, SH-SY5Y cells were exposed to Aroclor 1254 (A1254), a commercial mixture of PCBs, and Fura-2 acetoxyethyl ester-monitored \([\text{Ca}^{2+}]\), and MTT-detected cell injury were evaluated in the presence or in the absence of NCX inhibitors, such as bepridil (Amoroso et al., 2000; Pignataro et al., 2004b), KB-R7943 (Iwamoto and Shigekawa, 1998), and specific NCX antisense oligodeoxynucleotides (ODNs) (Pignataro et al., 2004a).

**Materials and Methods**

**Cell Culture.** The SH-SY5Y cell line (purchased from the American Type Culture Collection, Manassas, VA) was cultured as a monolayer in polystyrene dishes (100 mm diameter) and grown in RPMI 1640 medium (Invitrogen, Carlsbad, CA) containing 10% heat-inactivated fetal bovine serum (Invitrogen), 1% L-glutamine (200 mM) (Invitrogen), 1% sodium pyruvate (100 mM) (Invitrogen), 100 IU/ml penicillin (Invitrogen), and 100 µg/ml streptomycin (Invitrogen). Cells were grown in a humidified incubator at 37°C in a 5% CO₂ atmosphere. The medium was changed every 2 days. Each experiment was performed with cells (passage 40–60) in multiple well dishes.

**Cytosolic Ca²⁺ Concentration Measurements.** Just before the experiment, 2 x 10⁵ SH-SY5Y cells were detached, centrifuged, and then resuspended in 1 ml of a medium whose composition was 140 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 0.5 mM MgCl₂, 5.5 mM glucose, and 10 mM HEPES, pH adjusted to 7.4 with 1 M Tris (standard buffer). The cells were then incubated with 5 µM Fura-2 acetoxyethyl ester (Calbiochem, San Diego, CA) for 1 h at 30°C. After the loading period, the medium was diluted with 2 volumes of the same balanced salt solution, incubated at 37°C, and then washed twice before the experiment was performed (Amoroso et al., 2000). \([\text{Ca}^{2+}]\), values were measured in a 2-ml suspension of SH-SY5Y cells at 37°C in a quartz cuvette equipped with a magnetic stirrer bar. Fura-2 fluorescence was monitored in a PerkinElmer model 55 LS spectrofluorimeter (PerkinElmer Life and Analytical Sciences, Boston, MA). The excitation wavelengths were at 340 and 380 nm (bandpass, 5 nm), and emission was at 509 nm (bandpass, 5 nm). \([\text{Ca}^{2+}]\), values were determined according to the equation of Grynkiewicz et al. (1985).

**Determination of Cell Viability Evaluated as Mitochondrial Activity.** Cell viability evaluated as mitochondrial activity was quantified by measuring dehydrogenase activity retained in the cytosolic fraction of SH-SY5Y cells after death. The mean value of 106 SH-SY5Y cells was detached, centrifuged, and then resuspended in 1 ml of a medium whose composition was 140 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 0.5 mM MgCl₂, 5.5 mM glucose, and 10 mM HEPES, pH adjusted to 7.4 with 1 M Tris (standard buffer). The cells were then incubated with 5 µM Fura-2 acetoxyethyl ester (Calbiochem, San Diego, CA) for 1 h at 30°C. After the loading period, the medium was diluted with 2 volumes of the same balanced salt solution, incubated at 37°C, and then washed twice before the experiment was performed (Amoroso et al., 2000). \([\text{Ca}^{2+}]\), values were measured in a 2-ml suspension of SH-SY5Y cells at 37°C in a quartz cuvette equipped with a magnetic stirrer bar. Fura-2 fluorescence was monitored in a PerkinElmer model 55 LS spectrofluorimeter (PerkinElmer Life and Analytical Sciences, Boston, MA). The excitation wavelengths were at 340 and 380 nm (bandpass, 5 nm), and emission was at 509 nm (bandpass, 5 nm). \([\text{Ca}^{2+}]\), values were determined according to the equation of Grynkiewicz et al. (1985).

**RT-PCR Analysis of mRNA Expression of NCX Isoforms in SH-SY5Y Cells.** Total RNA was extracted from SH-SY5Y cells by using TRIzol reagent (Invitrogen). To avoid contamination with genomic DNA, the extracted RNA was treated with 10 U/µl of RNAase-free DNase I (Stratagene, Heidelberg, Germany) for 1 h at 37°C. The purity and integrity of RNA were checked by denaturing agarose gel electrophoresis. Two micrograms of total RNA were reverse-transcribed in the presence of oligo(dT) using SuperScript III reverse transcriptase (Invitrogen) according to the protocol by the manufacturer. The retrotranscribed cDNAs (2 µl) were then amplified by a MJ Research PTC 2000 Peltier Thermal Cycler (MJ Research, Watertown, MA) by using the primers shown in Table 1, described previously by Quednau et al. (1997) and Papa et al. (2003). Each 50-µl reaction containing 1.25 U of Taq DNA polymerase (Eppendorf-5 Prime, Inc., Boulder, CO) and 10 pmol of each primer was amplified (30 cycles) by the following procedure: 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. The amplification products were visualized on 2% agarose gel electrophoresis, loading approximately half (25 µl) of each reaction volume per lane.

**Oligonucleotide Transfection.** One day after plating, SH-SY5Y cells were transfected with the appropriate phosphorothioate ODNs (Table 1) described previously by Pignataro et al. (2004a) using Lipofectamine 2000 (Invitrogen) according to the protocol by the manufacturer. In brief, oligonucleotides (5 µM) were dissolved in Lipofectamine (DNA/Lipofectamine 2000, 1:1) and then incubated at room temperature for 30 min before being added to the culture medium. Control dishes received Lipofectamine only. To evaluate cell transfection efficiency, ODNs were mixed with a plasmid encoding a green fluorescent protein (GFP) reporter. The GFP-positive cells were counted using a fluorescence microscope.

**TABLE 1**

NCX1, NCX2, NCX3, and GAPDH primers used in RT PCR reactions and chimeric phosphorothioated antisense (AS1, AS3) and sense (S1, S3) ODNs used to reduce the NCX isoforms expression

<table>
<thead>
<tr>
<th>GenBank Accession Number</th>
<th>Sequence (5’ to 3’)</th>
<th>Base Pairs</th>
<th>Method</th>
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<tbody>
<tr>
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<td>cccagctttagggagaccaaacagac</td>
<td>503</td>
<td>RT-PCR</td>
</tr>
<tr>
<td>NCX2 U068141 Sense</td>
<td>cccagcttctggagaccaaacagac</td>
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<td>RT-PCR</td>
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<td>516</td>
<td>RT-PCR</td>
</tr>
<tr>
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<td></td>
<td>Transfection</td>
</tr>
<tr>
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<td></td>
<td>Transfection</td>
</tr>
<tr>
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<td>gcatcccaacaag</td>
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</tr>
<tr>
<td>NCX3 S3</td>
<td>ettttcttctgatgctgct</td>
<td></td>
<td>Transfection</td>
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</table>
Results

Effect of Different Concentrations of A1254 on \([\text{Ca}^{2+}]_i\) in the Presence and Absence of Extracellular \(\text{Ca}^{2+}\) in SH-SY5Y Cells. When SH-SY5Y cells were exposed to different concentrations (10, 30, and 50 \(\mu\text{M}\)) of A1254, a dose-dependent increase of \([\text{Ca}^{2+}]_i\) occurred. This increase started almost immediately and lasted for the whole considered time. The elevation of \([\text{Ca}^{2+}]_i\) induced by A1254 (30 \(\mu\text{M}\)) was completely abolished by the removal of extracellular \(\text{Ca}^{2+}\) (Fig. 1).

Effect of Bepridil and the Isothioureia Derivative KB-R7943, Two Rather Specific Inhibitors of NCX, on the \([\text{Ca}^{2+}]_i\) Elevation Induced by A1254. Bepridil and the isothioureia derivative KB-R7943 added to the incubation medium at the concentration of 10 \(\mu\text{M}\) known to inhibit NCX activity (Iwamoto and Shigekawa, 1998; Amoroso et al., 2000; Annunziato et al., 2004; Pignataro et al., 2004b) did not modify basal \([\text{Ca}^{2+}]_i\), whereas they counteracted \([\text{Ca}^{2+}]_i\) increase induced by 30 \(\mu\text{g/ml}\) A1254 (Fig. 2).

Effect of Bepridil and KB-R7943 on A1254-Induced Neuronal Injury. When SH-SY5Y cells were exposed to 30 \(\mu\text{g/ml}\) A1254 for 24 h, a significant reduction of cell survival, which was evaluated as mitochondrial activity by means of MTT method, occurred. Bepridil and KB-R7943 at the concentration (10 \(\mu\text{M}\)) that had reduced \([\text{Ca}^{2+}]_i\), increases triggered by A1254, were able to prevent neuronal injury evoked by PCB mixture (Fig. 3).

RT-PCR Analysis of SH-SY5Y mRNA Expression of NCX1, NCX2, and NCX3. Regarding the NCX family, three dominant genes coding for three different NCX1 (Nicoll et al., 1990), NCX2 (Li et al., 1994), and NCX3 (Nicoll et al., 1996) proteins have been identified in several mammal tissues, including the central nervous system. To characterize the pattern of the expression of the three NCX isoforms in SH-SY5Y cells, RT-PCR analysis of NCX1, NCX2, and NCX3 mRNA expression was performed. As shown in Fig. 4, only mRNA expression of NCX1 and NCX3 was detected in SH-SY5Y cell line (Fig. 4).

Effect of NCX1 and NCX3 Antisense ODNs on \([\text{Ca}^{2+}]_i\) Elevation 293

![Fig. 1. Effect of different concentrations of A1254 on \([\text{Ca}^{2+}]_i\) in the presence and in the absence of extracellular \(\text{Ca}^{2+}\) ions in SH-SY5Y cells. SH-SY5Y cells were exposed to different concentrations (10, 30, and 50 \(\mu\text{g/ml}\)) of A1254 for 31 min. Each point represents the mean ± S.E. (bars) of four values. In the experiments performed in the absence of extracellular \(\text{Ca}^{2+}\) ions, A1254 was added at the concentration of 30 \(\mu\text{g/ml}\). The arrow indicates the minute at which A1254 was added. Inset shows a typical calibration trace in the presence of A1254 (30 \(\mu\text{g/ml}\)). \([\text{Ca}^{2+}]_i\) values were measured in sample containing 2 × 10⁶ cells/2 ml.](image)

![Fig. 2. Effect of bepridil and the isothiourea derivative KB-R7943 on the \([\text{Ca}^{2+}]_i\) elevation induced by A1254. SH-SY5Y cells were exposed to a standard medium or to A1254 in the presence or in the absence of 10 \(\mu\text{M}\) bepridil or 10 \(\mu\text{M}\) KB-R7943. Each point represents the mean ± S.E. (bars) of 4 values. The arrows indicate the minute at which NCX inhibitors and A1254 were added. \([\text{Ca}^{2+}]_i\) values were measured in a sample containing 2 × 10⁶ cells/2 ml.](image)

![Fig. 3. Effect of bepridil and KB-R7943 on A1254-induced neuronal injury. SH-SY5Y cells were exposed to 30 \(\mu\text{g/ml}\) A1254 for 24 h in the presence or in the absence of 10 \(\mu\text{M}\) bepridil or 10 \(\mu\text{M}\) KB-R7943 and then assessed in their ability to produce formazan. Each column represents the mean ± S.E. (bars) of eight values. F = 14.35 (one-way ANOVA). * denotes statistical significance (p < 0.001) versus all other experimental groups; ** denotes statistical significance (p < 0.05) versus control group.](image)
ODNs, the basal intracellular calcium concentrations were similar to those of untransfected cells. When the effect of NCX1 and NCX3 antisense ODNs on [Ca\textsuperscript{2+}]\textsubscript{i}, increase evoked by A1254 was considered, a reduction (≥60%) of [Ca\textsuperscript{2+}]\textsubscript{i}, elevation induced by the PBC mixture occurred in cells transfected with NCX1 antisense ODN, whereas, in cells transfected with NCX3 antisense ODN, A1254 caused the same increase of [Ca\textsuperscript{2+}]\textsubscript{i}, as that detected in untransfected cells (Fig. 6A). It is noteworthy that in cells transfected with NCX1 ODN antisense, inhibition of [Ca\textsuperscript{2+}]\textsubscript{i}, increase evoked by A1254 was coupled to a marked reduction of neuronal injury (Fig. 6B). In the absence of exogenous Ca\textsuperscript{2+}, whereas, in NCX3 antisense ODN-transfected SH-SY5Y cells, the extent of neuronal injury induced by A1254 was not modified exactly as [Ca\textsuperscript{2+}]\textsubscript{i}, NCX1 and NCX3 sense ODNs did not modify both [Ca\textsuperscript{2+}]\textsubscript{i}, increase and neuronal injury induced by A1254 (data not shown).

Discussion

The results of present study showed that, in SH-SY5Y neuroblastoma cells, the PBC mixture A1254 induces a dose-dependent increase of [Ca\textsuperscript{2+}]\textsubscript{i}, that is dependent upon extracellular Ca\textsuperscript{2+} ions. In fact, the removal of extracellular Ca\textsuperscript{2+}, completely abolished A1254-induced [Ca\textsuperscript{2+}]\textsubscript{i}, increase. These results are in agreement with previous studies performed in cultured cerebellar granule cells by Mundy et al. (1999). Regarding the mechanism by which A1254 induced an influx of Ca\textsuperscript{2+}, it has been suggested that extracellular Ca\textsuperscript{2+} entry occurs via L-type voltage-sensitive calcium channels (VSCC), because A1254-evoked intracellular Ca\textsuperscript{2+} oscillations was abolished by the addition of nifedipine (Inglefield and Shafer, 2000). We now report that NCX exchanger may be involved in Ca\textsuperscript{2+} entry induced by the PBC mixture. In fact, both bepridil, a rather specific inhibitor of NCX (Amoroso et al., 2000; Annunziato et al., 2004b; Pignataro et al., 2004b), and KB-R7943, besides its peculiar NCX-blocking properties, also exerts an inhibitory effect on several other ionic transport mechanisms, such as L-type VSCC and receptor-operated ion channels such as NMDA (Matsuda et al., 2001; Annunziato et al., 2004). Therefore, the possibility exists that these drugs' inhibition of the [Ca\textsuperscript{2+}]\textsubscript{i}, increase induced by A1254 might be due to the blockade of Na\textsuperscript{+} and/or Ca\textsuperscript{2+} channels and/or NMDA receptor activation. On the other hand, this possibility seems to be supported by the results of Inglefield and Shafer (2000) showing that A1254-induced Ca\textsuperscript{2+} oscillations in developing neocortical cells can be blocked by the VSCC antagonist nifedipine, by the voltage-sensitive Na\textsuperscript{+} channel antagonist tetrodotoxin (Almers and Levinson, 1975; Weiser and Wilson, 2002), and by ionotropic glutamate receptor antagonists. To clarify whether NCX is effectively involved in A1254-induced [Ca\textsuperscript{2+}]\textsubscript{i}, increase, the pattern of the expression of the three known NCX isoforms NCX1, NCX2 and NCX3 (Annunziato et al., 2004) was first characterized in SH-SY5Y cells by RT-PCR analy-
sis. Then, the effect of A1254 on [Ca^{2+}], increase was investigated in SH-SY5Y transfected with the antisense NCX1 or NCX3 ODNs, because only these two isoforms were detected. The results of these experiments showed that a significant reduction (≈60%) of [Ca^{2+}] elevation induced by the PCB mixture occurred in cells transfected with antisense NCX1 ODN. These findings demonstrate that the NCX is effectively involved in [Ca^{2+}] elevation induced by PCB mixture, as suggested by results obtained with bepridil and KB-R7943.

In addition, it is noteworthy that, between the two NCX isoforms detected in SH-SY5Y cells, only NCX1 seems to be involved in this phenomenon. In fact, no inhibition of A1254-induced [Ca^{2+}], increase was found in cells transfected with antisense NCX3 ODN. If the inhibition of NCX reduces A1254-induced Ca^{2+} influx, this implies that NCX is operating in the reverse mode (Ca^{2+}-entry/Na^{+} efflux pathway). This mode of operation could be explained by the fact that A1254 induces a membrane depolarization, as suggested by Inglefield and Shafer (2000), with a consequent opening of Na^{+}-sensitive voltage channels. This event leads to an increase of intracellular Na^{+} ion concentration, which in turn may force the NCX to work in the reverse mode (Baker and McNaughton, 1976; DiPolo, 1979; Sanchez-Armass and Blaustein, 1987; Annunziato et al., 2004). This chain of events seems to be supported by the results showing that tetrodotoxin, a well known blocker of fast-activated Na^{+} channels (Almers and Levinson, 1975; Weiser and Wilson, 2002), prevents Ca^{2+} oscillations evoked by A1254 in developing cortical neurons (Inglefield and Shafer, 2000). Thus, the hypothesis that, in SH-SY5Y cells, A1254 induces a membrane depolarization with a consequently intracellular increase of Na^{+} ions, which in turn activates NCX to operate in the reverse mode, seems reasonable.

However, the inhibition of NCX did not fully counteract [Ca^{2+}], elevation induced by the PCB mixture. These results suggest that other mechanisms may be also involved in A1254-elicited [Ca^{2+}], increase, such as extracellular Ca^{2+} influx through L-type VSCC (Inglefield and Shafer, 2000), inhibition of Ca^{2+}-ATPase activity, Ca^{2+} sequestration by mitochondria and microsomes (Kodavanti et al., 1993), and mobilization of intracellular Ca^{2+} stores (Inglefield et al., 2001).

Another finding of the present study that deserves to be discussed is that the inhibition of A1254-elicited [Ca^{2+}], increase mediated by NCX is able to protect SH-SY5Y cells from the injury induced by the exposure to the PCB mixture. In fact, both bepridil and KB-R7943, at the same concentrations that inhibited [Ca^{2+}], increase, caused a significant reduction of cell injury elicited by A1254 and, in cells transfected with antisense NCX1 ODN, the extent of cell damage induced by the PCB mixture was significantly lower than that observed in naive cells. On the other hand, it is well known that an increase of [Ca^{2+}], can trigger intracellular pathways leading to cell death (Kristian and Siesjo, 1998; Kang et al., 2002, 2004). It is noteworthy that cell protection from A1254-elicited injury obtained by the inhibition of NCX was not complete, exactly as the reduction of A1254-elicited [Ca^{2+}], increase. In conclusion, the results of the present study seem to suggest that NCX may participate in [Ca^{2+}], increase induced by A1254 exposure and that its inhibition may contribute to protecting cells from the injury evoked by the PCB mixture.

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References


Address correspondence to: Prof. Salvatore Amoroso, Department of Neuroscience, Unit of Pharmacology, School of Medicine, University “Politecnica delle Marche”, Via Tronto 10/A, 60020 Torrette, Ancona, Italy. E-mail: s.amoroso@univpm.it