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INVOLVEMENT OF Na^+-Ca^{2+} EXCHANGER IN INTRACELLULAR Ca^{2+} INCREASE AND NEURONAL INJURY INDUCED BY POLYCHLORINATED BIPHENYLS IN HUMAN NEUROBLASTOMA SH-SY5Y CELLS

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Na^+-Ca^{2+}-exchanger and PCBs Ca^{2+} increase.

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Abbreviations used: A1254, Aroclor 1254; AS1, NCX1 antisense; AS3, NCX3 antisense; KB-R7943, 2-[2-[4-(4-nitrobenzyloxy) phenyl] ethyl] isothiourea methanesulphonate; $[Ca^{2+}]_i$, intracellular calcium concentration; DMSO, dimethylsulphoxide; Fura-2AM, fura-2 acetoxymethyl ester; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IP3, inositol 1,4,5-triphosphate; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide; NCX, sodium calcium exchanger; NMDA, N-methyl-D-aspartate; ODNs, oligodeoxynucleotides; PBS, phosphate buffered saline; PCBs, polychlorinated biphenyls; VSCCs, voltage-sensitive Ca^{2+} channels.

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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 exchanger (NCX) inhibitors like bepridil (10 μM) and 2-[2-[4-(4-nitrobenzyloxy) phenyl] ethyl] isothiourea methanesulphonate (KB-R7943) (10 μM), reduced A1254-induced $[Ca^{2+}]_i$ increase. 24 hours exposure to 30 μg/ml A1254 caused a remarkable SH-SY5Y neuroblastoma cell damage. Interestingly, 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, expression of NCX1 and NCX3 isoforms. In order to investigate which isoform was involved in $[Ca^{2+}]_i$ increase and neuronal damage induced by A1254, 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.

Introduction

Polychlorinated biphenyls (PCBs) 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, 1999), perturbation in intracellular second messenger systems (Kodavanti and Tilson, 1997; Yang and Kodavanti, 2001) and elevation of intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) (Sharma et al., 2000). This last effect has been attributed to a Ca^{2+} influx (Mundy et al., 1999; Inglefield and Shafer, 2000), or to the inhibition of Ca^{2+}-ATPase activity at the synaptic level and Ca^{2+} sequestration by mitochondria and microsomes (Kodavanti et al., 1993). In addition it has been reported that PCBs can also perturb Ca^{2+} homeostasis by acting on ryanodine (Wong and Pessah, 1996; Mundy et al., 1999) and inositol 1,4,5-triphosphate (IP3) receptor-sensitive Ca^{2+} release (Inglefield et al., 2001).

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, 2004b).

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-2AM monitored $[Ca^{2+}]_i$ and 3-[4,5-

dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) detected cell injury, were evaluated in the presence or in the absence of NCX inhibitors like bepridil (Amoroso et al., 2000; Pignataro et al., 2004a), KB-R7943 (Iwamoto and Shigekawa, 1998) and specific NCX antisense oligodeoxynucleotides (ODNs) (Pignataro et al., 2004b).

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Materials and Methods

Cell culture

The SH-SY5Y cell line (purchased from the American Type Colture Collection) was cultured as monolayer in polystyrene dishes (100 mm diameter) and grown in RPMI 1640 medium (Gibco, Grand Island, NY USA) containing 10% heat inactivated fetal bovine serum (Gibco, Grand Island, NY USA), 1% of 200 mM L-glutamine (Gibco Grand Island, NY USA), 1% of sodium pyruvate 100 mM (Gibco Grand Island, NY USA), 100 IU/ml penicillin (Gibco, Grand Island, NY USA), and 100 μg/ml streptomycin (Gibco, Grand Island, NY USA). Cells were grown in a humidified incubator at 37°C in a 5% CO_2 atmosphere. The medium was changed every 2 days. Each experiment was performed with cells (passage 40-60) in multiple well flow dishes.

Cytosolic Ca^{2+} concentration measurements

Just before the experiment, $2x10^6$ SH-SY5Y cells were detached, then centrifuged and resuspended in 1 ml of a medium whose composition was in mM: NaCl 140, KCl 5, $CaCl_2$ 1, $MgCl_2$ 0.5, glucose 5.5, Hepes 10; pH was adjusted to 7.4 with 1 M Tris (standard buffer). The cells were then incubated with 5 μM Fura-2AM (Calbiochem, San Diego, CA, USA) for 1 hour 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). $[Ca^{2+}]_i$ 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 Perkin Elmer model 55 LS spectrophotofluorimeter. The excitation wavelengths were at 340 and 380 nm (bandpass 5 nm) with emission at 509 nm (bandpass 5 nm). $[Ca^{2+}]_i$ 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 cultured cells, using the MTT (Sigma-Aldrich, Milan, Italy) assay (Mossman, 1983; Amoroso et al., 1999). The assay is based on the ability of living cells to convert dissolved MTT into insoluble formazan. Therefore, the amount of formazan produced is proportional to the number of living cells. Briefly SH-SY5Y cells ($3x10^5$) were incubated in 2 ml MTT solution (0.5 mg/ml) for 1 hour in a humified 5% CO_2 incubator at 37°C. Thereafter the medium was removed and cells were washed twice with PBS. Then, 1 ml of dimethylsulphoxide (DMSO) was added to the cells to solubilize the formazan. The absorbance was read at 540 nm in a plate reader (Victor^2 1420 Multilabel Counter, Perkin Elmer). Data are expressed as amount of formazan produced. In control cultures the same volume of A1254 vehicle was added. The osmotic pressure, measured by Autostat Osmometer 6030 (Arkay, KDK corporation, Japan) in media containing standard buffer or A1254 (30 μg/ml) or its vehicle, was identical (302 mOsm). When experiments with oligos were performed, A1254 was added 5 hours after the transfection.

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, Milan, Italy). To avoid contamination with genomic DNA, the extracted RNA was treated with 10U/μl of RNAase-free DNAase I (Stratagene, Milan, Italy) for 1 hour at 37°C. The purity and integrity of RNA was checked by denaturing agarose gel electrophoresis. Two micrograms of total RNA were reverse-transcribed in the presence of oligo(dt), by using SuperScript III reverse transcriptase (Invitrogen, Milan, Italy), according to the manufacturer protocol. The retrotranscribed cDNAs (2 μl) were then amplified in a MJ Research PTC 2000 Peltier Thermal Cycler (CELBIO, Milan, Italy) by using the primers shown in Table 1, previously described by Quednau et al., (1997) and Papa et al., (2003). Each 50 μl reaction

containing 1.25 U of Taq DNA Polymerase (Eppendorf, Milan, Italy) 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 agarose (2%) gel electrophoresis, loading approximately half (25 µl) of each reaction volume per lane.

Oligos Transfection

One day after plating, SH-SY5Y cells were transfected with the appropriate phosphorothioate ODNs (Table 1) previously described by Pignataro et al., (2004b), by using lipofectamine 2000 according to the manufacturer protocol (Invitrogen, Milan, Italy). Briefly, oligos (5 µM) were dissolved in lipofectamine (DNA/lipofectamine 2000 1:1), and then incubated at room temperature for 30 min before the addition to the culture medium. Control dishes received lipofectamine only. In order to evaluate cell transfection efficiency, ODNs were mixed with a plasmid encoding the enhanced green fluorescent protein as marker (Castaldo et al., 2004). Efficiency transfection was ≅ 90% (data not shown).

Drugs and Chemicals

All the chemicals were of analytical grade and were purchased from Sigma (Milan, Italy). A1254 solution in isooctane (Lot Number M-841B, Analyte Lot NT01022, stock solution 1000 ±5 µg/ml) was purchased from Ultra Scientific, North Kingstown, RI; bepridil was obtained from Sigma Italy, KB-R7943 was obtained from Tocris UK. Bepridil was dissolved in a mixture of acetone and water (stock solution 10 mM), whereas KB-R7943 was dissolved in a mixture of DMSO and water (stock solution 10 mM).

Statistics

Data were analyzed by one-way ANOVA followed by Dunnett's test.

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Results

Effect of different concentrations of A1254 on $[Ca^{2+}]_i$ in the presence and in the absence of extracellular Ca^{2+} in SH-SY5Y cells.

When SH-SY5Y cells were exposed to different concentrations (10, 30 and 50 μg/ml) of A1254, a dose-dependent increase of $[Ca^{2+}]_i$ occurred. This increase started almost immediately and lasted for the whole considered time. The elevation of $[Ca^{2+}]_i$ induced by A1254 (30 μg/ml) was completely abolished by removal of extracellular Ca^{2+} (Fig.1).

Effect of bepridil and the isothiourea derivative KB-R7943, two rather specific inhibitors of NCX, on the $[Ca^{2+}]_i$ elevation induced by A1254.

Bepridil and the isothiourea derivative KB-R7943, added to the incubation medium at the concentration of 10 μM, known to inhibit NCX activity (Iwamoto and Shigekawa, 1998; Amoroso et al., 2000; Annunziato et al., 2004; Pignataro et al., 2004a), did not modify basal $[Ca^{2+}]_i$, whereas they counteracted $[Ca^{2+}]_i$ increase induced by A1254 (30 μg/ml) (Fig.2).

Effect of bepridil and KB-R7943 on A1254-induced neuronal injury.

When SH-SY5Y cells were exposed to A1254 (30 μg/ml) for 24 hours, a significant reduction of cell survival, evaluated as mitochondrial activity by means of MTT method, occurred. Bepridil and KB-R7943, at the concentration (10 μM) which had reduced $[Ca^{2+}]_i$, rises 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 central nervous system. In order 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).

RT-PCR analysis of NCX1 and NCX3 mRNA expression in SH-SY5Y transfected with the respective sense and antisense ODNs.

As shown in Fig 5, the transfection with NCX1 and NCX3 antisenses caused a marked reduction (≅ 80%) of NCX1 and NCX3 mRNA expression, whereas the transfection with the NCX1 and NCX3 senses failed to change mRNA levels of the two isoforms.

Effect of NCX1 and NCX3 antisense ODNs on $[Ca^{2+}]_i$ increase and neuronal injury elicited by A1254.

In SH-SY5Y cells transfected with NCX1 or NCX3 antisense ODNs the basal $[Ca^{2+}]_i$ were similar to those of untransfected cells. When the effect of NCX1 and NCX3 antisense ODNs on $[Ca^{2+}]_i$ increase evoked by A1254 was considered, a reduction (≅ 60%) of $[Ca^{2+}]_i$ elevation induced by the PCB mixture occurred in cells transfected with NCX1 antisense ODN, whereas in cells transfected with NCX3 antisense ODN, A1254 caused the same increase of $[Ca^{2+}]_i$ as that detected in untransfected cells (Fig. 6A). Interestingly, in cells transfected with NCX1 ODN antisense, inhibition of $[Ca^{2+}]_i$ increase evoked by A1254 was coupled to a marked reduction of neuronal injury (Fig. 6B), while in NCX3 antisense ODN transfected SH-SY5Y cells, the extent of neuronal injury induced by A1254 was not modified exactly as $[Ca^{2+}]_{i.}$. NCX1 and NCX3 sense ODNs did not modify both $[Ca^{2+}]_i$ increase and neuronal injury induced by A1254 (data not shown).

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Discussion

The results of present study showed that in SH-SY5Y neuroblastoma cells the PCB mixture A1254 induces a dose-dependent increase of $[Ca^{2+}]_i$ that is dependent upon extracellular Ca^{2+} ions. In fact the removal of extracellular Ca^{2+} completely abolished A1254-induced $[Ca^{2+}]_i$ 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^{2+}, it has been suggested that extracellular Ca^{2+} entry occurs via L-type voltage sensitive calcium channels (VSCCs), since A1254-evoked intracellular Ca^{2+} oscillations was abolished by addition of nifedipine (Inglefield and Shafer, 2000). We now report that NCX exchanger may be involved in Ca^{2+} entry induced by the PBC mixture. In fact both bepridil, a rather specific inhibitor of NCX (Amoroso et al., 2000; Annunziato et al., 2004; Pignataro et al., 2004a) and KB-R7943, which inhibits NCX only when it is operating in the reverse mode (Iwamoto and Shigekawa, 1998), significantly counteracted $[Ca^{2+}]_i$ increase induced by A1254. However, bepridil has been reported also to block Ca^{2+} (Kaczorowski et al., 1989) and Na^+ channels (Kaczorowski et al., 1989), and KB-R7943, besides its peculiar NCX blocking properties, also exerts an inhibitory effect on several other ionic transport mechanisms, such L-type VSCCs and receptor operated ion channels, like NMDA (Matsuda et al., 2001; Annunziato et al., 2004). Therefore the possibility exists that the inhibition produced by these drugs of $[Ca^{2+}]_i$ increase induced by A1254, might be due to the blockade of Na^+ and/or Ca^{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^{2+} oscillations in developing neocortical cells can be blocked by the VSCC antagonist nifedipine, by the voltage sensitive Na^+ channel antagonist, tetrodotoxin (Almers and Levinson, 1975; Weiser and Wilson, 2002) and by ionotropic glutamate receptor antagonists. In order to clarify whether NCX is effectively involved in

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A1254-induced $[Ca^{2+}]_i$ increase, firstly the pattern of the expression of the three known NCX isoforms NCX1, NCX2 and NCX3 (Annunziato et al., 2004) was characterized in SH-SY5Y cells by RT-PCR analysis. Then, the effect of A1254 on $[Ca^{2+}]_i$ increase was investigated in SH-SY5Y transfected with the antisense NCX1 or NCX3 ODNs, since only these two isoforms were detected. The results of these experiments showed that, a significant reduction (≅ 60%) of $[Ca^{2+}]$i elevation induced by the PCB mixture occurred in cells transfected with antisense NCX1 ODN. These findings demonstrate that the NCX is effectively involved in $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ 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 consequent intracellular increase of Na^+ ions which in turn activates NCX to operate in the reverse mode, seems reasonable.

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However, the inhibition of NCX did not fully counteract $[Ca^{2+}]_i$ elevation induced by the PCB mixture. These results suggest that other mechanisms may be also involved in A1254-elicited $[Ca^{2+}]_i$ increase, like extracellular Ca^{2+} influx through L-type VSCCs (Inglefield and Shafer, 2000); inhibition of Ca^{2+}-ATPase activity and 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 paper that deserves to be discussed is that the inhibition of A1254-elicited $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ increase, caused a significant reduction of cell injury elicited by A1254 and in cells transfected with antisense NCX1 ODNs, the extent of cell damage induced by the PCB mixture was significantly lower than that observed in naïve cells. On the other hand it is well known that an increase of $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ increase.

In conclusion, the results of the present paper seem to suggest that NCX may participate to $[Ca^{2+}]_i$ increase induced by A1254 exposure and that its inhibition may contribute to protect cells from the injury evoked by the PCB mixture.

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Footnotes:

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Legends for figures

Fig. 1. Effect of different concentrations of A1254 on $[Ca^{2+}]_i$ in the presence and in the absence of extracellular Ca^{2+} ions in SH-SY5Y cells.

SH-SY5Y cells were exposed to different concentrations (10, 30 and 50 μg/ml) of A1254, for 31 min. Each point represents the mean ± SE (bars) of 4 values. In the experiments performed in the absence of extracellular Ca^{2+} ions, A1254 was added at the concentration of 30 μg/ml. The arrow indicates the minute at which A1254 was added. Inset shows a typical calibration trace in the presence of A1254 (30 μg/ml). $[Ca^{2+}]_i$ values were measured in sample containing $2x10^6$ cells/2 ml.

Fig. 2. Effect of bepridil and the isothiourea derivative KB-R7943 on the $[Ca^{2+}]_i$ 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 μM bepridil or 10 μM KB-R7943. Each point represents the mean ± SE (bars) of 4 values. The arrows indicate the minute at which NCX inhibitors and A1254 were added. $[Ca^{2+}]_i$ values were measured in sample containing $2x10^6$ cells/2 ml.

Fig. 3. Effect of bepridil and KB-R7943 on A1254-induced neuronal injury. SH-SY5Y cells were exposed to A1254 (30 μg/ml) for 24 hrs in the presence or in the absence of 10 μM bepridil or 10 μM KB-R7943 and then assessed in their ability to produce formazan. Each column represents the mean ± SE (bars) of 8 values.

F= 14.35 (one-way ANOVA)

*denotes statistical significance ($p<0.001$) vs all other experimental groups

**denotes statistical significance ($p<0.05$) vs control group

Fig. 4. RT-PCR analysis of SH-SY5Y mRNA expression of NCX1, NCX2 and NCX3 isoforms. In order 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. Each lane shows PCR product obtained by using NCX1, NCX2 and NCX3 isoform primers.

Fig. 5. RT-PCR analysis of NCX1 and NCX3 mRNA expression in SH-SY5Y cells transfected with respective sense and antisense ODNs. Upper part of the figure shows RT-PCR of NCX1 and NCX3 products in SH-SY5Y transfected with the respective sense and antisense ODNs. Normalization of results was ensured by running parallel RT-PCR with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers. Bottom part of the figure indicates optical density values representing the Mean ± SE of three independent experiments.

Fig. 6. Effect of antisense NCX1 or NCX3 ODNs transfection on $[Ca^{2+}]_i$ increase and neuronal injury elicited by A1254.

A. SH-SY5Y naïve and antisense NCX1 or NCX3 ODNs transfected cells were exposed to A1254 (30 μg/ml). Each point represents the mean ± SE (bars) of 4 values. The arrow indicates the minute at which A1254 was added. $[Ca^{2+}]_i$ values were measured in sample containing $2x10^6$ cell/2 ml.

B. SH-SY5Y naïve and antisense NCX1 or NCX3 ODNs transfected cells were exposed to A1254 (30 μg/ml) and assessed in their ability to reduce MTT. Each column represents mean ± SE (bars) of 8 values .

F = 14.38 (one-way ANOVA)

*$p<0.001$ vs control and AS1+A1254 groups.

** $p<0.05$ vs control group; $p<0.001$ vs A1254 group.

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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. Bold indicates phosphorothioated bases.

GenBank accession number	**Sequence (5' to 3')**	**Base pairs (bp)**	**Using**
NCX1 X68191	sense: cccaagcttaatggagagaccaccaagac reverse: cgcggatccttggaagctggtctgtctcc	503	RT-PCR
NCX2 U08141	sense: cccaagcttgcgtgtgggcgatgctca reverse: cgcggatccgacctcgaggcgacagttc	528	RT-PCR
NCX3 U53420	sense: cccaagcttctggaagaggggatgaccc reverse: cgcggatccgtttagggtgttcacccaata	516	RT-PCR
GAPDH	sense: ccatggagaaggctgggg reverse: caaagttgtcatggatgacc	195	RT-PCR
NCX1	AS1: **tgag**acttccaat**tgtt**		Transfection
NCX1	S1: **aaca**attggaagt**ctca**		Transfection
NCX3	AS3: **gcca**tacaca**agag**		Transfection
NCX3	S3: **ctct**tgtgta**tggc**		Transfection

Fig. 1

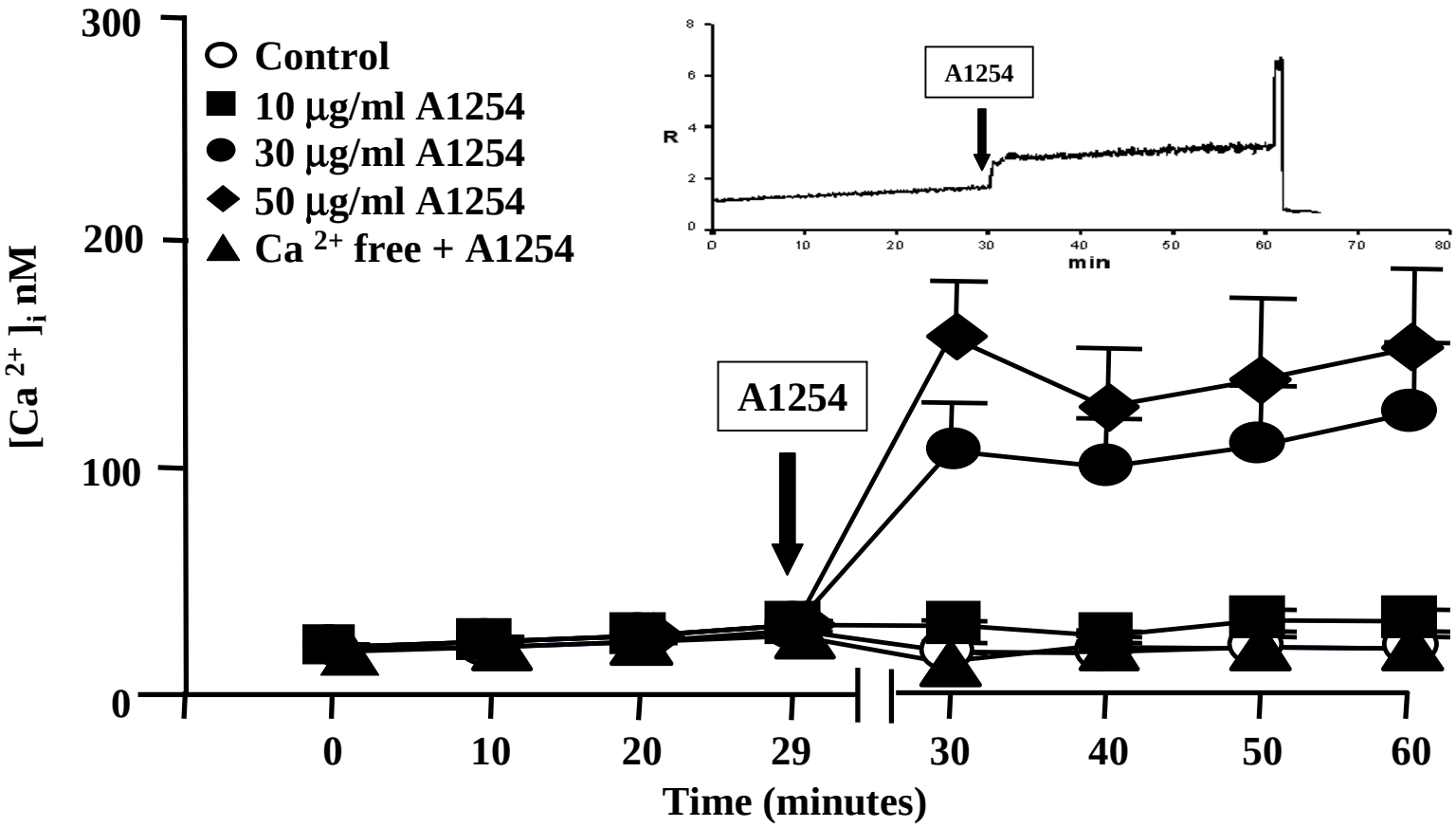

Fig. 2

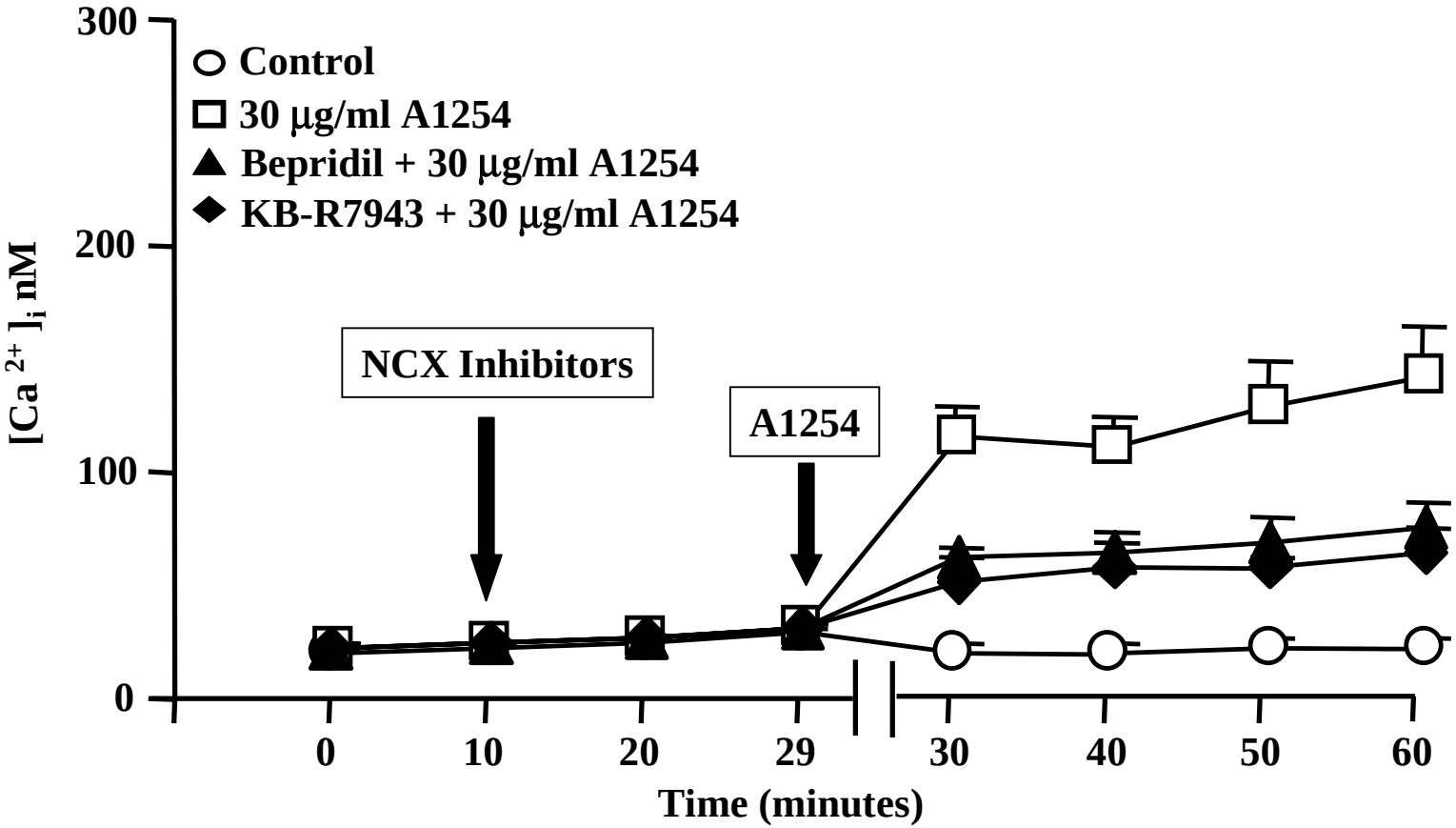

Fig. 3

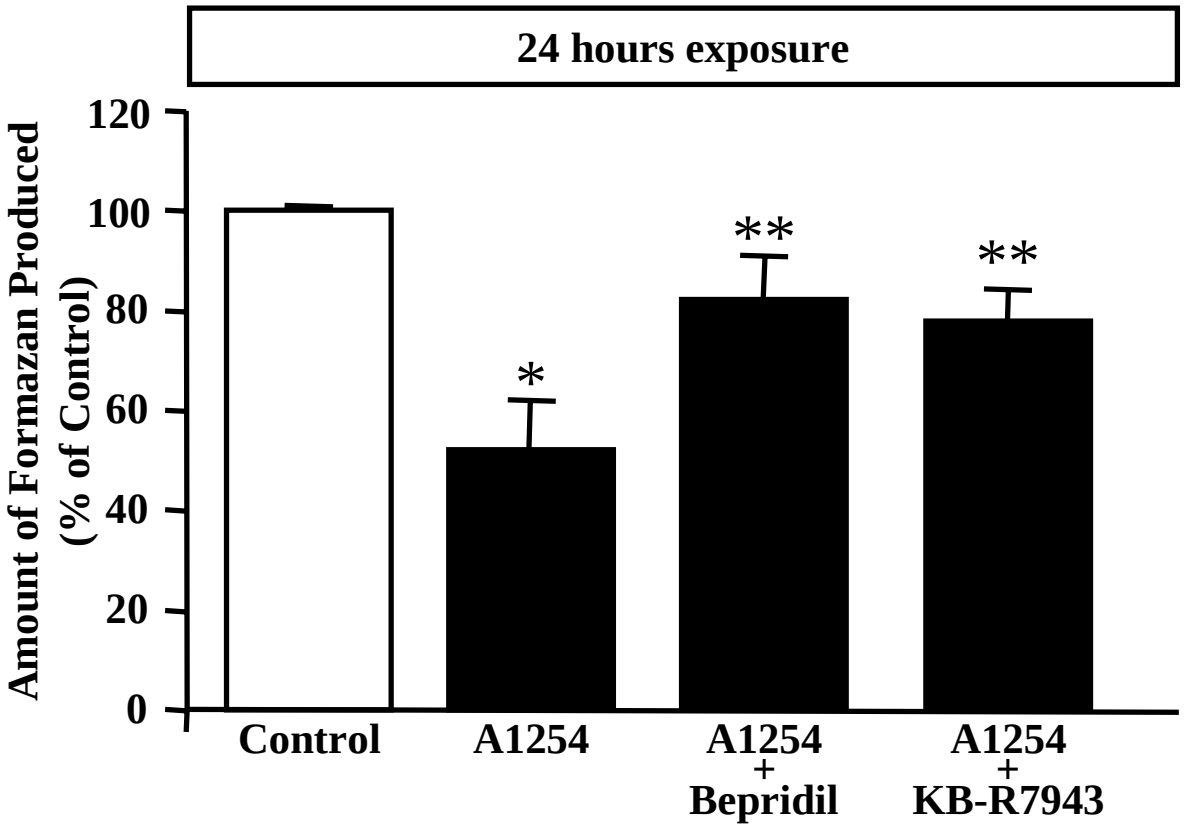

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Fig. 4

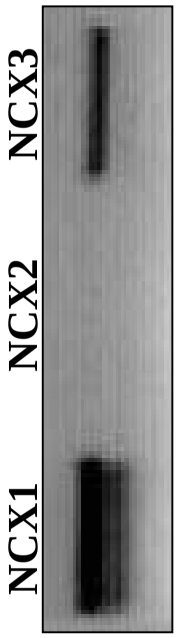

Fig. 5

Fig. 6A

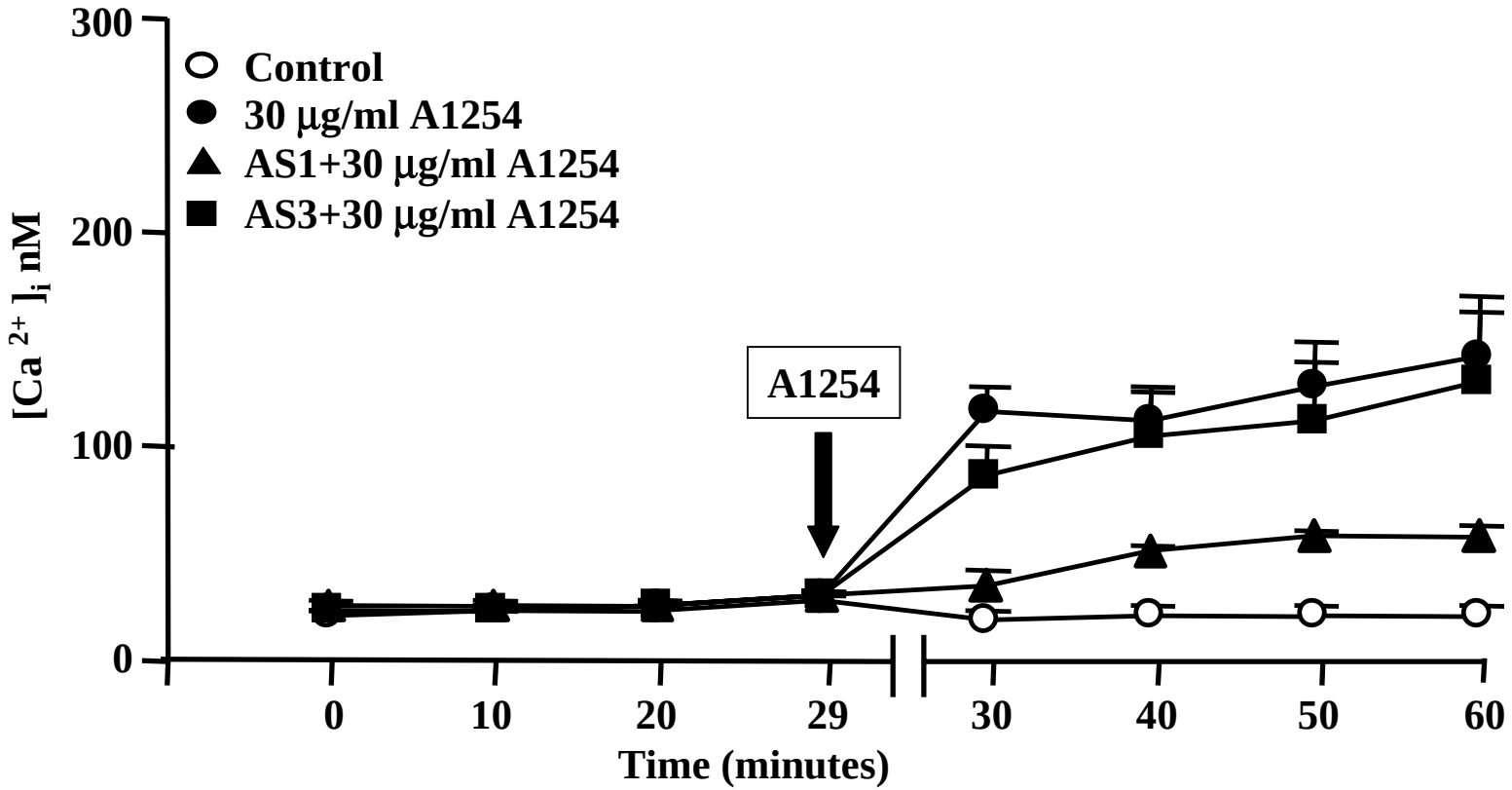

Fig. 6B