Na/Ca Exchanger Overexpression Induces Endoplasmic Reticulum–Related Apoptosis and Caspase-12 Activation in Insulin-Releasing BRIN-BD11 Cells

Oscar Diaz-Horta, Adama Kamagate, André Herchuelz, and Françoise Van Eylen

Ca^{2+} may trigger programmed cell death (apoptosis) and regulate death-specific enzymes. Therefore, the development of strategies to control Ca^{2+} homeostasis may represent a potential approach to prevent or enhance cell apoptosis. To test this hypothesis, the plasma membrane Na/Ca exchanger (NCX1.7 isoform) was stably overexpressed in insulin-secreting tumoral cells. NCX1.7 overexpression increased apoptosis induced by endoplasmic reticulum (ER) Ca^{2+}-ATPase inhibitors, but not by agents increasing intracellular calcium concentration ($[Ca^{2+}]_i$), through the opening of plasma membrane Ca^{2+}-channels. NCX1.7 overexpression reduced the rise in $[Ca^{2+}]_i$ induced by all agents, depleted ER Ca^{2+} stores, sensitized the cells to Ca^{2+}-independent proapoptotic signaling pathways, and reduced cell proliferation by ~40%. ER Ca^{2+} stores depletion was accompanied by the activation of the ER-specific caspase (caspase-12), and the activation was enhanced by ER Ca^{2+}-ATPase inhibitors. Hence, Na/Ca exchanger overexpression, by depleting ER Ca^{2+} stores, triggers the activation of caspase-12 and increases apoptotic cell death. By increasing apoptosis and decreasing cell proliferation, overexpression of Na/Ca exchanger may represent a new potential approach in cancer gene therapy. On the other hand, our results open the way to the development of new strategies to control cellular Ca^{2+} homeostasis that could, on the contrary, prevent the process of apoptosis that mediates, in part, β-cell autoimmune destruction in type 1 diabetes. *Diabetes* 51: 1815–1824, 2002

Cytosolic Ca^{2+} regulates a large number of cellular processes; its concentration is finely regulated, in turn, by various channels, pumps, and exchangers. Although each Ca^{2+} transporter has been the object of abundant research, the interplay between these transporters and their relative contribution to Ca^{2+} equilibrium in the different cellular compartments remain largely unknown (1).

From the Laboratory of Pharmacology, Brussels University School of Medicine, Brussels, Belgium.

Address correspondence and reprint requests to André Herchuelz, Laboratoire de Pharmacodynamie et de Thérapeutique, Université Libre de Bruxelles, Faculté de Médecine, Route de Lennik, 808-Bâtiment GE, B-1070 Bruxelles, Belgium. E-mail: herchu@ulb.ac.be.

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$[Ca^{2+}]_i$ intracellular calcium concentration; CPA, cyclopiazonic acid; ER, endoplasmic reticulum; MTT, 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide; PMCA, plasma membrane Ca^{2+}-ATPase; SERCA, sarco(endo) plasmic reticulum Ca^{2+}-ATPase.

Cytosolic Ca^{2+} has also been implicated as a proapoptotic agent involved in triggering apoptosis and regulating death-specific enzymes (2). Apoptosis, also known as programmed cell death, plays a major role in the normal development of most organs (3). However, too much or too little apoptosis is also implicated in a large number of clinical disorders, including ischemia, cancers, type 1 diabetes, neurodegenerative disorders such as Alzheimer's disease, and viral infections, e.g., HIV-1 (4). The family of cysteine proteases, known as caspases, are critical mediators of apoptosis (5) that can be triggered by three distinct pathways: oligomerization of death receptors located on the plasma membrane, mitochondrial damage, and the recently discovered endoplasmic reticulum (ER) pathway (6,7). In the latter triggering mechanism, "ER stress," including disruption of ER Ca^{2+} homeostasis or accumulation of excess protein in the ER, causes the activation of caspase-12 (8) that triggers the process of apoptosis (7). Therefore, the development of strategies to control intracellular Ca^{2+} homeostasis may represent a potential approach to prevent or enhance programmed cell death. This could be accomplished by overexpressing the Na/Ca exchanger, an ubiquitous mechanism for Ca^{2+} extrusion from excitable and nonexcitable cells (9).

In the present study, we examined the effect of Na/Ca exchanger overexpression on Ca^{2+}-induced apoptosis in insulin-producing cells. Indeed, Ca^{2+} plays a major role in the process of insulin release from the pancreatic β-cell (10), and in type 1 diabetes (insulin dependent), the autoimmune destruction of pancreatic β-cells appears to be mediated by apoptosis (11), a phenomenon that could involve Ca^{2+}. Thus, serum from newly diagnosed type 1 diabetic subjects was shown to activate Ca^{2+} inflow in mouse β-cells through voltage-sensitive Ca^{2+}-channels and to promote β-cell destruction by apoptosis (12). Likewise, chronic cytokine treatment (interleukin-1β plus interferon-γ) induces a low voltage–activated Ca^{2+} current in mouse islet cells, a phenomenon associated with a sustained increase in basal intracellular calcium concentration ($[Ca^{2+}]_i$) and apoptotic cell death (13). Glucose itself, the main physiological stimulus of insulin release, when used at a high concentration (17 or 27 mmol/l), was observed to trigger apoptosis in both mouse and rat β-cells, a process that again was Ca^{2+}-dependent (14).

The pancreatic β-cell is equipped with two systems allowing Ca^{2+} extrusion, the plasma membrane Ca^{2+}-ATPase (PMCA) and the Na/Ca exchanger (15,16). The latter system couples the electrogenic transport of 3 Na^+

ions for 1 Ca^{2+} ion (9). In rat β-cells, like in cardiac myocytes, Na/Ca exchange is the major mechanism for Ca^{2+} extrusion (17,18) and participates in the control of $[Ca^{2+}]_i$ and of insulin release (18,19).

The Na/Ca exchanger (NCX) was cloned 10 years ago, and three mammalian isoforms have been identified: NCX1, -2, and -3. Several splice variants of NCX1 and -3 have been described, each exhibiting a specific tissue distribution (reviewed in 20). Rat pancreatic islet cells, purified β-cells, and the insulinoma cell line (RINm5F cells) express two NCX1 splice variants (NCX1.3 and -1.7).

In the current study, apoptosis rates were determined in an insulin-secreting cell line (BRIN-BD11) overexpressing the human NCX1.7 exchanger isoform (21). BRIN-BD11 cells, produced by electrofusion of normal rat pancreatic β-cells and RINm5F cells (22), were chosen instead of RINm5F cells because they show a fully functional glucose responsiveness and do not suffer from the inherent defect recorded with parental RINm5F cells (22). Our data reveal that overexpression of the exchanger depletes ER Ca^{2+} stores, a phenomenon that leads to ER stress, activation of caspase-12, and increase in Ca^{2+}-mediated apoptosis. Overexpression of the exchanger also reduced cell proliferation and sensitized the cells to non–Ca^{2+}-dependent apoptotic signals.

RESEARCH DESIGN AND METHODS

Cell culture and stable transfection. BRIN-BD11 cells, produced by electrofusion of normal rat pancreatic β-cells and RINm5F cells (22), were cultured in complete RPMI medium containing 11.1 mmol/l glucose supplemented with 10% FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO_2/95% air at 37°C. Cells were plated at 3×10^5 cells/ml on a 35-mm plastic Petri dish and incubated for 24 h before transfection. BRIN-BD11 cells were then stably transfected with the mammalian expression vector pcDNA3(-) containing a 2.9-kb insert encoding for the human pancreatic β-cell NCX1.7 by using LipofectAMINE PLUS reagent (Life Technologies). BRIN-BD11 cells were also transfected with the pcDNA3.1(-) vector carrying the neomycin resistance gene but lacking the NCX1.7 cDNA. Positive clones were selected through resistance against G418 (250 μg/ml) (Life Technologies) and verified for NCX1.7 overexpression by RT-PCR, Western blot analysis, immunofluorescence, and Na/Ca exchange activity (21).

Except otherwise stated, all experiments were carried out in complete RPMI medium in a humidified atmosphere of 5% CO_2/95% air at 37°C. Likewise, all viability/cytotoxic/apoptotic assays were carried out over 48 h, except nuclear chromatin staining and caspase-12 activation assay, which were carried out over 24 h.

Cell viability/cytotoxicity assay. Control cells and cells overexpressing the Na/Ca exchanger were seeded at 1.5×10^5 cells/ml, cultured overnight in flat-bottom 96-well plates, and treated with a variety of agents known to induce apoptosis and/or to affect $[Ca^{2+}]_i$. Cell viability/cytotoxicity was determined by the 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) assay as previously described (23). This assay is based on the ability of viable cells, but not death cells, to reduce MTT, with the reaction generating a dark blue insoluble formazan product. Briefly, after exposure of the cells to the different agents for 48 h, 10 μl MTT in PBS (5 mg/ml) were added to 100 μl medium in each well and further incubated for 4 h. A total of 100 μl of 40 mmol/l HCl/isopropanol was then added to stop MTT reduction and to dissolve the dye. After a 10-min incubation at 37°C, colorimetric measures were made at 540 nm with an ELISA (enzyme-linked immunosorbent assay) plate reader (Labsystems Oy, Helsinki, Finland). Results are expressed as percentage of absorbances relative to controls (defined as 100%) after subtraction of non–cell-derived background.

Nuclear chromatin staining. Cells were cultured overnight on circular cover glasses at 2.5×10^5 cells/ml in complete RPMI medium and treated with the previously mentioned agents for 24 h. The incubation time was 24 instead of 48 h because apoptotic cells usually detach from the culture plates and hence escape detection. After washing with PBS, cells were incubated in PBS containing Hoechst 33342 (20 μg/ml) for 10 min at 37°C. The dye was then removed, and apoptotic cells were identified by the presence of condensed nuclei, which were stained blue and visualized using a fluorescent microscope (UV excitation at 340–380 nm) (14).

Quantification of DNA fragmentation. Cells (2.5×10^5 cells/ml) were washed with Hanks' balanced salt solution, detached using nonenzymatic cell dissociation buffer, and collected by centrifugation. Fragmented DNA was isolated and quantified as previously described (24), with some modifications. A total of 300 μl lysis buffer (0.5% Triton X-100, 20 mmol/l EDTA, and 5 mmol/l Tris-HCl; pH 7.4) was added to the cells. After 20 min incubation at 4°C, cells were centrifuged at 15,000*g* for 30 min. Pellets were resuspended in 300 μl lysis buffer containing 100 μg Proteinase K. Sixty micrograms of Proteinase K was also added to the supernatants. Samples were incubated overnight at 37°C with gentle shaking, and DNA was extracted with phenol-chloroform. Each DNA fraction was incubated in TNE buffer (10 mmol/l Tris-HCl, 200 mmol/l NaCl, and 1 mmol/l EDTA; pH 7.4) in the presence of Hoechst 33258 at 0.5 μg/ml. Fluorescence of fragmented (supernatant) and nonfragmented DNA (pellets), respectively, was measured with a spectrofluorometer (excitation at 356 nm and emission at 492 nm). Results were expressed as percent of fragmented DNA.

Gel electrophoresis of low molecular weight DNA. DNA extraction was performed as described above. After ethanol precipitation, fragmented DNA was incubated in 30 μl TER buffer (10 mmol/l Tris-HCl, 1 mmol/l EDTA, and 2 μg/ml RNAse A; pH 8) for 1 h at 37°C with gentle shaking. Samples were run on 1.2% agarose gels. DNA was visualized by ethidium bromide staining (24).

Cytosolic Ca^{2+} concentration measurements. Cells previously plated on round glass coverslips were incubated with fura-2 acetoxymethyl ester (final concentration 2 μmol/l) during 60 min at 37°C in Krebs-Ringer bicarbonate–buffered solution supplemented with glucose (2.8 mmol/l). The coverslips were then transferred to a tissue chamber mounted on an inverted fluorescence microscope (Diaphot TDM; Nikon, Tokyo) for epifluorescence. Fura-2 fluorescence of single cells was measured by dual-excitation fluorimetry using a camera-based image analysis system (Magical; Applied Imaging, Sunderland, U.K.). The excitation and emission wavelengths were set at 340/380 and 510 nm, respectively, and a pair of ratioable images (at the excitations of 340 and 380 nm, 30-ms interval) were taken every 2.5 s. $[Ca^{2+}]_i$ was calculated from the ratios of the 340- and 380-nm signals as previously described (18).

Measurements of the Ca^{2+} concentration in the ER. Furaptra loading, permeabilization, and measurements of intracellular Ca^{2+} stores were performed as previously described (25), with some modifications. Briefly, cells were loaded with furaptra acetoxymethyl ester (4 μmol/l) and 0.05% (wt/vol) Pluronic F-127 in Krebs-Ringer bicarbonate–buffered solution supplemented with glucose (2.8 mmol/l) for 60 min at 37°C. After rinsing, round glass coverslips with the attached cells were then transferred to a tissue chamber mounted on an inverted fluorescence microscope. Permeabilization of the plasma membrane was carried out with 4 μmol/l digitonin in intracellular medium containing 140 mmol/l KCl, 10 mmol/l Na_2ATP, and 10 mmol/l HEPES, pH 7.0, adjusted with KOH. Free Ca^{2+} was buffered at 200 nmol/l with 2 mmol/l EGTA and the appropriate amount of Ca^{2+} using the Max Chelator program (Stanford University). Furaptra fluorescence of single loaded cells was measured by use of dual-excitation microfluorimetry with a SPEX photometric system (Optilas, Alphen aan den Rijn, Holland). The excitation wavelengths (340 and 380 nm) were alternated at the frequency of 1 Hz. The emission wavelength was set at 510 nm. After the sudden drop of fluorescence, the detergent was removed and intracellular buffer was added. Thapsigargin (1 μmol/l) was used to estimate the content of free Ca^{2+} in the ER.

Western blot analysis. Approximately 3×10^6 cells were collected and lysed in lysis buffer (NP40 1%, TRIS 10 mmol/l, NaCl 200 mmol/l, EDTA 5 mmol/l, glycerol 10%, and protease inhibitors; pH = 7). Lysates containing 25 μg protein were run on 12% acrylamide gels, transferred onto nitrocellulose, and analyzed (21) using a 1/1,000 dilution of a polyclonal antibody raised against caspase-12 (kindly supplied by P. Vandenabeele, Department of Molecular Biology, University of Ghent, Ghent, Belgium). Because caspase-12 activation is an early process, cells were exposed to apoptotic agents for 24 h.

Caspase activity detection using fluorescence microscopy. Caspase activity was measured using the Caspatag fluorescein Caspase (VAD) activity kit (Intergen, Oxford, U.K.) and fluorescence microscopy. The cells were plated on sterile glass coverslips for 24 h and further incubated for 48 h in the absence or presence of 1 μmol/l thapsigargin. Cells were then incubated for 1 h at 37°C under 5% CO_2 with the fluorescent caspase inhibitor FAM-VAD-FMK. The medium was then removed and cells washed twice with wash buffer. The coverslips were mounted on a slide using a drop of wash buffer and observed under a fluorescence microscope (Olympus AX70 System Microscope; Omnilabo, Aartselaar, Belgium) to view green fluorescence of caspase-positive cells (excitation at 490 nm and emission at 520 nm.).

Cell proliferation assay. Cells were seeded at 1.5×10^5 cells/ml and cultured for 24 h in flat-bottom 96-well plates in the absence of FCS. Cells

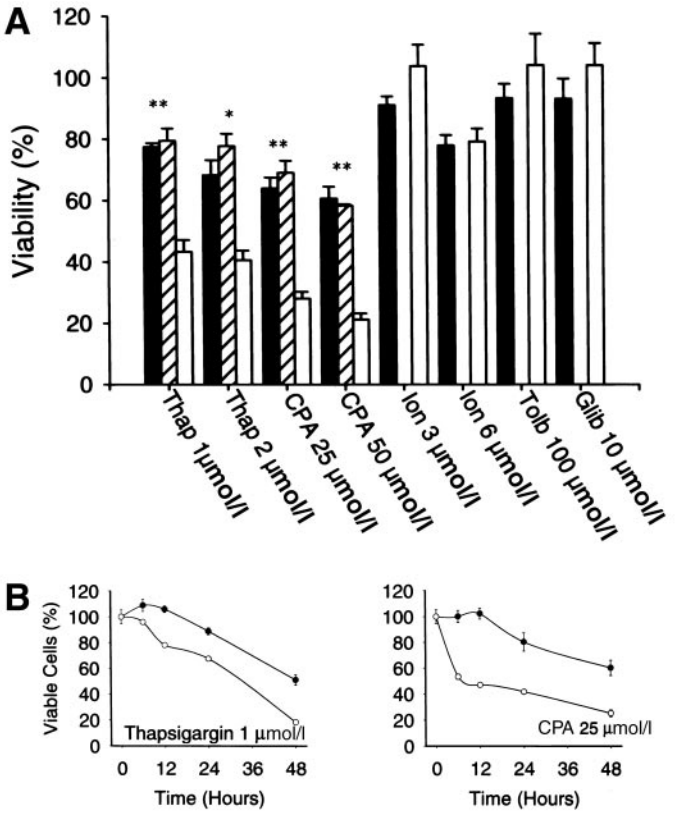

FIG. 1. *A*: Effect of NCX1.7 overexpression on cell viability in the presence of thapsigargin (Thap; 1 and 2 µmol/l), CPA (25 and 50 µmol/l), ionomycin (Ion; 3 and 6 µmol/l), tolbutamide (Tolb; 100 µmol/l), and glibenclamide (Glib; 10 µmol/l). The MTT assay was used to measure cell survival. ■, nontransfected cells; ▨, vector-transfected cells only; □, NCX1.7-transfected cells. Data are expressed as means ± SE from at least four individual experiments, each comprising at least four replicates. *$P < 0.005$; **$P < 0.001$. *B*: Time course of the effect of thapsigargin and CPA on cell viability. Data are expressed as means ± SE from at least four individual experiments, each comprising four replicates.

were then incubated with 0, 3, 6, and 10% of FCS during 48 h to measure serum-induced proliferation using the MTT assay as described above and by cell counting using trypan blue and a Bürcher cell. All data are expressed as means ± SE. Statistical significance of differences between data were assessed by using ANOVA and Tukey's post test.

RESULTS

Most of the experiments were carried out using a clone of BRIN-BD11 cells expressing high levels of Na/Ca exchange activity (clone 8), characterized in a previous work (21). However, similar results were obtained with another clone overexpressing NCX1.7 (clone 4, see below). In addition, five clones transfected with the expression vector lacking the NCX1.7 construct were generated. Overexpression was assessed at the mRNA and protein level, and appropriate targeting to the plasma membrane was assessed by microfluorescence and increase in Na/Ca exchange activity. In response to extracellular Na^+ removal (stimulation of reversal Na/Ca exchange), overexpressing cells showed a four- to fivefold larger increase in $[Ca^{2+}]_i$ compared with control cells (21), indicating a four to five times higher Na/Ca exchange activity.

The agents used to increase $[Ca^{2+}]_i$ comprised glucose and the hypoglycemic sulfonylureas tolbutamide and glibenclamide, the sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) inhibitors thapsigargin and cyclopiazonic acid (CPA), and the ionophore ionomycin. Glucose and the hypoglycemic sulfonylureas increase $[Ca^{2+}]_i$ by closing ATP-dependent K^+ channels, which depolarize the plasma membrane, open voltage-sensitive Ca^{2+} channels, and hence increase Ca^{2+} inflow (26). SERCA inhibitors block the ER Ca^{2+}-ATPases and increase $[Ca^{2+}]_i$ by releasing Ca^{2+} from the ER (27). Ionomycin acts as a Ca^{2+} ionophore, namely by permeabilizing the plasma membrane and the intracellular membranes to Ca^{2+}, and hence increases both Ca^{2+} inflow from the outside and the release of the ion from intracellular stores.

The MTT assay was used to measure cell viability. Figure 1*A* illustrates the effect of various agents increasing $[Ca^{2+}]_i$ on cell viability. In control cells, SERCA inhibitors (thapsigargin and CPA) decreased cell viability ($P < 0.001$), whereas the hypoglycemic sulfonylureas gliben-

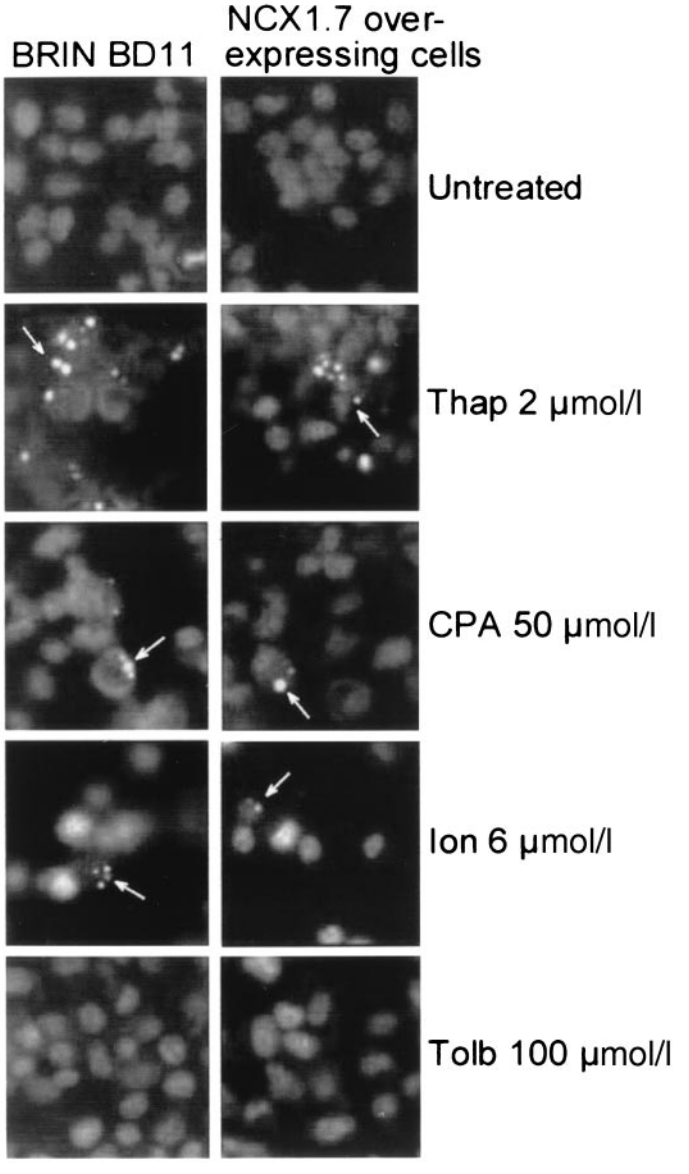

FIG. 2. Fluorescent microscopic analysis of nontransfected (left panels) and NCX1.7-overexpressing BRIN-BD11 cells (right panels) stained with Hoechst 33342. The nuclei of cells exposed to 2 µmol/l thapsigargin, 50 µmol/l CPA, and 6 µmol/l ionomycin showed condensation and chromatin fragmentation (arrows). Normal nuclear morphology of untreated and tolbutamide-treated cells (100 µmol/l) is also shown.

clamide and tolbutamide did not. The ionophore ionomycin decreased cell viability but only at the highest concentration used (6 μmol/l, $P < 0.001$). In cells overexpressing the Na/Ca exchanger, the effect of SERCA inhibitors was increased ($P < 0.001$), whereas that of ionomycin (3 and 6 μmol/l) and the hypoglycemic inhibitors was not. Similar data were obtained using another independent clone overexpressing NCX1.7 (data not shown). In cells transfected with the expression vector lacking the NCX1.7 construct, the effect of thapsigargin or CPA on cell viability was not different from that observed in nontransfected cells ($P > 0.05$) (Fig. 1*A* hatched columns). Figure 1*B* illustrates the time course of the effect of thapsigargin and CPA on cell viability. In overexpressing cells, there was a shift to the left in the time dependence of the two inhibitors inducing apoptosis, confirming that overexpression sensitizes the cells to apoptotic cell death. For instance, with thapsigargin, a significant decrease in cell viability was observed after 12 and 24 h in overexpressing and control cells, respectively ($P < 0.001$). With CPA, a significant decrease in cell viability was observed after 6 and 24 h in overexpressing and control cells, respectively ($P < 0.001$).

To evaluate to what extent the reduction in cell viability was due to apoptosis, nuclear DNA staining with the fluorescent probe HOE 33342 was examined (Fig. 2). Whereas control cells showed a normal nuclear cell morphology, cells treated with the SERCA inhibitors or ionomycin (6 μmol/l) showed chromatin condensation and fragmentation characteristic of apoptosis, whether in control or overexpressing cells. Cells treated with glucose (2.8, 11.1, and 28 mmol/l, data not shown) or glibenclamide (0.1 and 0.25 mmol/l, data not shown) and tolbutamide showed a normal morphology (Fig. 3). No quantification of the process of apoptosis was tempted using this method because apoptotic cells usually detach from culture plates and hence escape detection.

To quantify apoptosis, the amount of fragmented DNA was measured using HOE 33258 (Fig. 3*B*). A result similar to that obtained with the MTT test was observed. Thus, SERCA inhibitors and ionomycin (6 μmol/l) increased DNA fragmentation in control cells, a phenomenon that was enhanced in Na/Ca exchanger–overexpressing cells ($P < 0.005$ or 0.001), except in the case of ionomycin. The percentage of fragmented DNA in glibenclamide- and tolbutamide-treated cells did not differ from that of untreated control or overexpressing cells (Fig. 3*B*).

One of the hallmarks of apoptosis is nuclear DNA fragmentation into oligonucleosomal fragments, which can be visualized as a DNA ladder by agarose gel electrophoresis. In contrast, DNA electrophoresis of necrotic cells yields a "smear" pattern due to random DNA degradation. Using this method, we observed that thapsigargin and CPA cause cell death by apoptosis rather than by necrosis in both control and Na/Ca exchanger–overexpressing cells (Fig. 3*A*). Fragmented DNA was not visible in untreated cells and cells treated with sulfonylureas (100 μmol/l tolbutamide and 10 μmol/l glibenclamide) or ionomycin (3 μmol/l). At 6 μmol/l, ionomycin induced DNA fragmentation, but only in Na/Ca exchanger–overexpressing cells.

To examine the mechanism by which Na/Ca exchanger

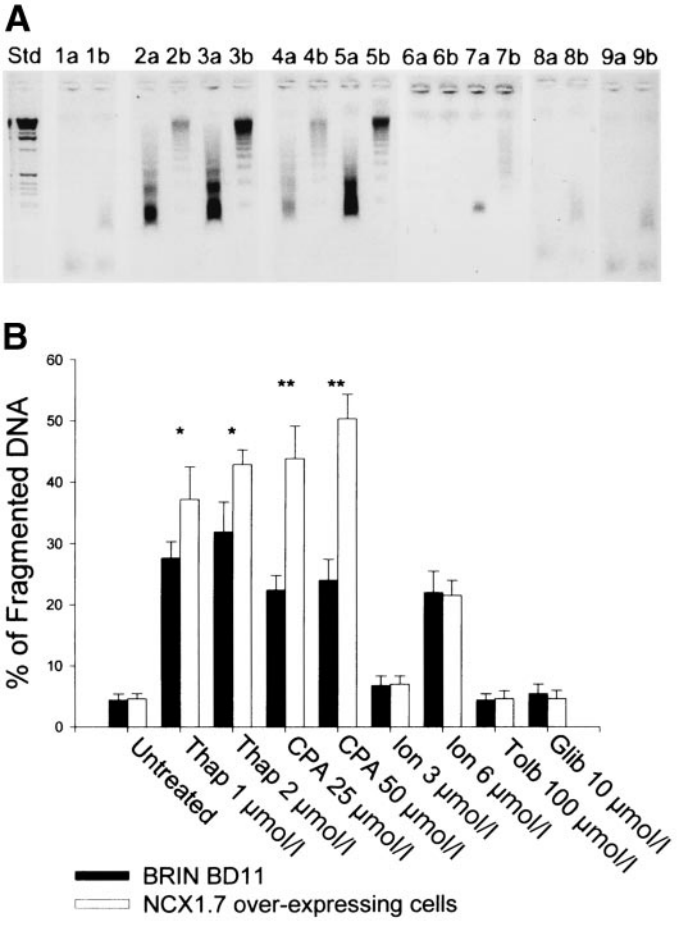

FIG. 3. *A*: Gel electrophoresis of low molecular weight DNA in nontransfected (*a*) and NCX1.7-overexpressing BRIN-BD11 cells (*b*) in untreated cells (*lanes 1a* and *b*) or after exposure to 1 (*lanes 2a* and *b*) and 2 μmol/l thapsigargin (*lanes 3a* and *b*), 25 (*lanes 4a* and *b*) and 50 μmol/l CPA (*lanes 5a* and *b*), 3 (*lanes 6a* and *b*) and 6 μmol/l ionomycin (*lanes 7a* and *b*), 100 μmol/l tolbutamide (*lanes 8a* and *b*), and 10 μmol/l glibenclamide (*lanes 9a* and *b*). No evidence of DNA fragmentation was found in untreated cells or in cells exposed to 3 μmol/l ionomycin, 100 μmol/l tolbutamide, or 10 μmol/l glibenclamide. Low molecular weight DNA was extracted, run on 1.2% agarose gels, and visualized by ethidium bromide staining. Molecular mass standards are indicated on the left (Std). *B*: Quantification of DNA fragmentation in control and NCX1.7-overexpressing cells in the presence or absence of thapsigargin (Thap; 1 and 2 μmol/l), CPA (CPA; 25 and 50 μmol/l), ionomycin (Ion; 3 and 6 μmol/l), tolbutamide (Tolb; 100 μmol/l), and glibenclamide (Glib; 10 μmol/l). Fragmented and nonfragmented DNA was extracted, isolated, and quantified spectrofluorometrically after incubation with Hoechst 33258. Data are expressed as percentage of fragmented DNA of the total and given as means ± SE from three individual experiments, each comprising four replicates. *$P < 0.005$; **$P < 0.001$.

overexpression favors apoptosis, the effect of SERCA inhibitors and ionomycin on $[Ca^{2+}]_i$ was evaluated (Fig. 4). In control cells, thapsigargin and CPA induced a major but transient increase in $[Ca^{2+}]_i$, a phenomenon that was reduced by ~60 and 40%, respectively, in Na/Ca exchanger–overexpressing cells ($P < 0.0001$). On the contrary, in cells transfected with the expression vector lacking the NCX1.7 construct, the effect of thapsigargin and CPA on $[Ca^{2+}]_i$ was slightly larger, although not significantly so, than that observed in control nontransfected cells ($P > 0.1$ and 0.075, respectively) (Fig. 4). In control cells, ionomycin induced an even more marked increase in $[Ca^{2+}]_i$; the increase was also more sustained than that induced by SERCA inhibitors. In Na/Ca exchanger–overexpressing

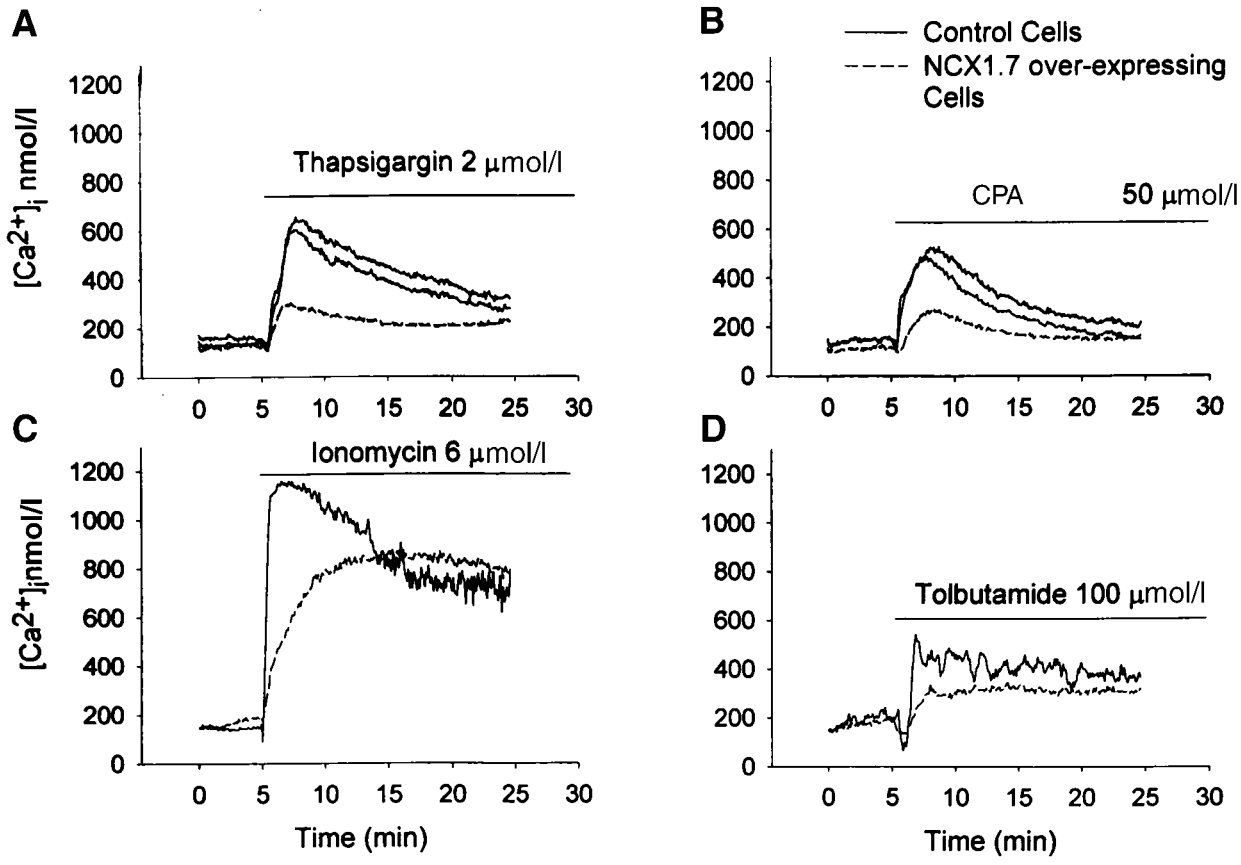

FIG. 4. Effect of NCX1.7 overexpression on $[Ca^{2+}]_i$ in nontransfected and NCX1.7-overexpressing BRIN-BD11 cells exposed to 2 μmol/l thapsigargin (*A*), 50 μmol/l CPA (*B*), 6 μmol/l ionomycin (*C*), and 100 μmol/l tolbutamide (*D*). *A* and *B*: Effect of the expression of the vector only, lacking the NCX1.7 construct, on thapsigargin- and CPA-induced changes in $[Ca^{2+}]_i$, are presented as the two upper curves. The period of exposure to the drugs is indicated by a bar above the curves. The curves shown are the mean of 64–85 individual traces.

cells, ionomycin also increased $[Ca^{2+}]_i$, but the increase was more sluggish. However, at steady state, the rise in $[Ca^{2+}]_i$ was of the same magnitude in control and overexpressing cells (Fig. 4, lower left panel). Tolbutamide also increased $[Ca^{2+}]_i$; the pattern of rise was similar to that of ionomycin, except that it was of a lower magnitude. Thus, in overexpressing cells, the increase was more progressive but of similar magnitude at the steady state compared with control cells ($P > 0.05$) (Fig. 4*D*). Taken as a whole, these data show that overexpression of the Na/Ca exchanger reduces the rise in $[Ca^{2+}]_i$ induced by all agents. The fact that the rise induced by thapsigargin and CPA was reduced indicates that Na/Ca exchanger overexpression provokes a depletion of ER Ca^{2+} stores.

To objectify such a depletion, the low-affinity Ca^{2+} indicator furaptra was used to monitor free Ca^{2+} in the ER of individual BRIN cells after controlled permeabilization of the plasma membrane, as previously described (25). Figure 5*A* and *B* shows the process of permeabilization. After recording the fluorescence obtained by excitation at 340 and 380 nm, cells were permeabilized in intracellular medium containing 4 μmol/l digitonin. After the sudden drop in fluorescence caused by the loss of cytoplasmic furaptra, the detergent was immediately removed while the measurement of the fluorescence at both wavelengths was continued. The loss of cytoplasmic furaptra was associated with an inversion of the 340/380-nm fluorescence excitation ratio, indicating that the remaining indicator was exposed to higher concentrations of free Ca^{2+} prevailing in the ER. In a previous study (25), it was shown that thapsigargin released ER Ca^{2+} pool, which was sensed by furaptra under the present condition. In control cells, thapsigargin induced a major drop in furaptra 340/380-nm fluorescence excitation ratio (Fig. 5*C*); the effect of thapsigargin was reduced by ~70% in Na/Ca exchanger–overexpressing cells ($P < 0.01$) (Fig. 5*D*). This confirms that the Ca^{2+} content of the ER was markedly reduced in cells overexpressing the Na/Ca exchanger.

The family of cysteine proteases (caspases) are critical mediators of programmed cell death. Recently, caspase-12 was found to be predominantly associated with the ER and to be activated by "ER stress," including disruption of ER Ca^{2+} homeostasis, as induced by SERCA inhibitors (7). To further determine the mechanism by which Na/Ca exchanger overexpression increases apoptosis, we examined the activation of caspase-12, as indicated by cleavage of procaspase-12. Procaspase migrates as a 60-kDa protein, and activation of caspase-12 leads to two lower molecular weight proteins (including caspase-12) of ~46 and 36 kDa (Fig. 6) (28). Figure 6 shows that Na/Ca exchanger overexpression induced the cleavage of procaspase-12, even in untreated cells (compare *lanes a* and *b*). The cleavage was increased by thapsigargin and ionomycin (24 h exposure) to a greater extent in overexpressing than in control cells. Density scanning of caspase bands showed that the appearance of caspase-12 (36 kDa) was accompanied by a decrease in the amount of procaspase-12 (60 kDa) (Fig. 6,

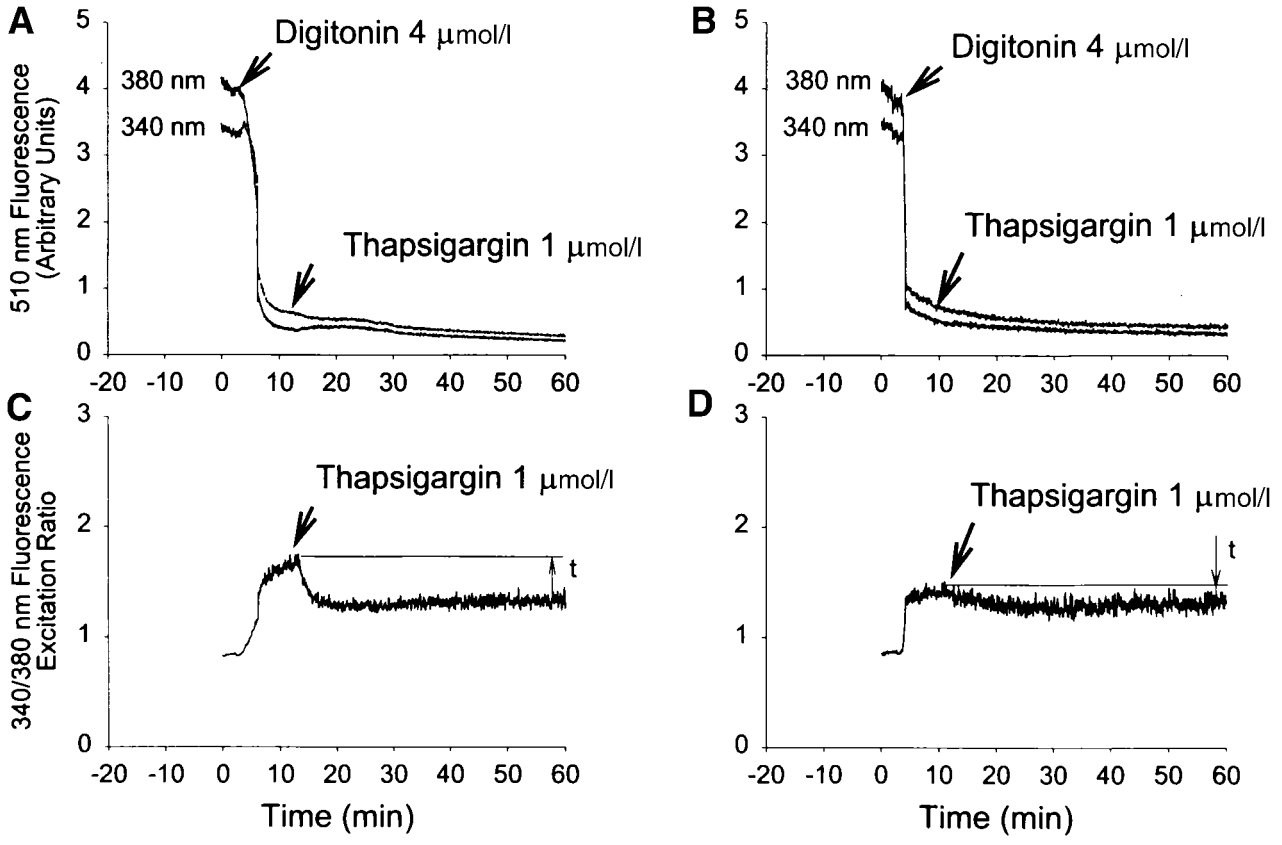

FIG. 5. ***A*** **and** ***B*****: Controlled permeabilization of individual control (*A*) and NCX1.7-overexpressing cells (*B*). The furaptra 340- and 380-nm excitation fluorescence signals were recorded continuously at 510 nm during exposure to a medium containing 3 mmol/l ATP and 200 nmol/l Ca^{2+}. Digitonin (4 μmol/l) was added as indicated by the first arrow and immediately removed after the sudden drop in 340- and 380-nm excitation fluorescence. After stabilization of the fluorescence signals, 1 μmol/l thapsigargin was added (second arrow). *C* and *D*: Furaptra 340- and 380-nm fluorescence excitation ratio of the same cells. Addition of digitonin was associated with an increase in fluorescence excitation ratio, indicating that the remaining indicator was exposed to higher concentrations of free Ca^{2+} prevailing in the ER. The curves shown are representative of six independent experiments. The *t* indicates the time at which the thapsigargin-sensitive ER Ca^{2+} fraction was measured.**

compare *lanes a* and *b* in treated and untreated cells), confirming the activation of caspase-12.

The presence of activated caspases was also examined using FAM-VAD-FMK, a carboxyfluorescein (FAM) derivative of benzyloxycarbonyl valylalanyl aspartic acid fluoromethyl ketone (zVAD-FMK), which is a potent and nonselective inhibitor of caspase activity. The inhibitor is cell permeable, noncytotoxic, and binds irreversibly to active caspases, with positive cells distinguished from negative cells by fluorescence microscopy (29). Figure 7 shows FAM-VAD-FMK fluorescence in untreated (Fig. 7*A*, *C*, and *E*) and thapsigargin-treated cells (Fig. 7*B*, *D*, and *F*). Whereas, in the absence of thapsigargin treatment, a discrete fluorescence was observed in some nontransfected and vector-transfected cells, a more accentuated fluorescence was seen in NCX1.7-overexpressing cells.

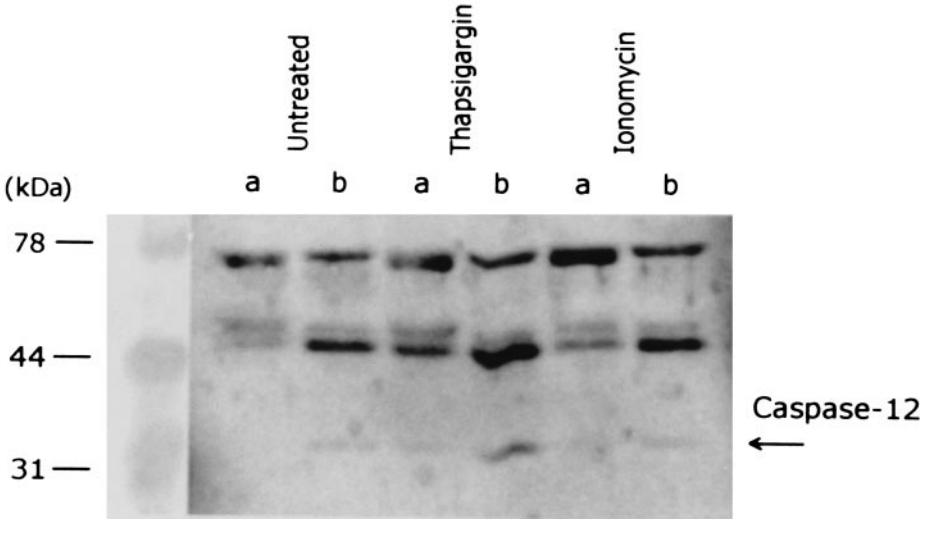

FIG. 6. Effect of NCX1.7 overexpression on caspase-12 activation. Western blot analysis of non transfected cells (*lane a*) and NCX1.7-overexpressing cells (*lane b*) in the absence or the presence of thapsigargin (2 μmol/l) and ionomycin (6 μmol/l). The lanes were loaded with 25 μg protein. The positions and sizes of the molecular mass standards are indicated on the left and expressed in kDa. The position of caspase-12 is shown on the right.

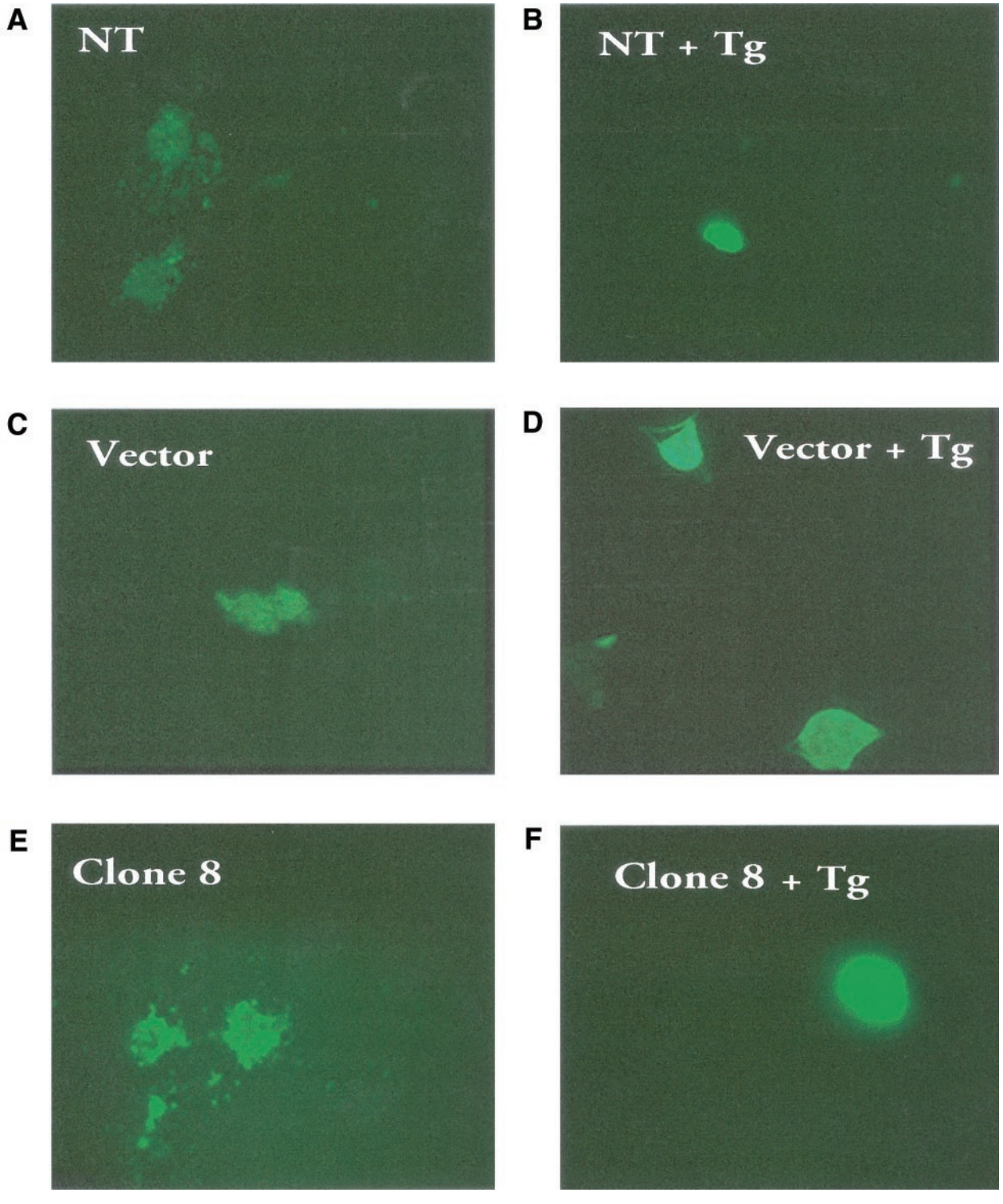

FIG. 7. Detection of activated caspases using FAM-VAD-FMK. Fluorescence microscopy analysis of nontransfected (NT) (*A* and *B*), vector-transfected (*C* and *D*), and NCX1.7-overexpressing cells (*E* and *F*) treated (*B*, *D*, and *F*) or not treated (*A*, *C*, and *E*) with thapsigargin (Tg; 1 μmol/l) for 48 h. After incubation with the SERCA inhibitor, the cells were exposed to FAM-VAD-FMK for 1 h at 37°c under 5% CO_2 and examined using fluorescence microscopy.

Thapsigargin markedly increased the fluorescence of both control and transfected cells, with NCX1.7-transfected cells displaying a higher fluorescence labeling than non-transfected cells. This confirms the higher caspase activity in NCX1.7-overexpressing cells, whether in the absence or presence of thapsigargin.

Because caspase-12 activation was increased in overexpressing cells, even when they were untreated and namely in a condition not associated with increased apoptosis, we examined whether such caspase-12 activation may nevertheless sensitize the cell to apoptosis. Figure 8 shows that this was indeed the case. Thus, overexpressing cells showed a higher rate of apoptosis even when triggered by non–Ca^{2+}-dependent signaling pathways. Figure 8 shows

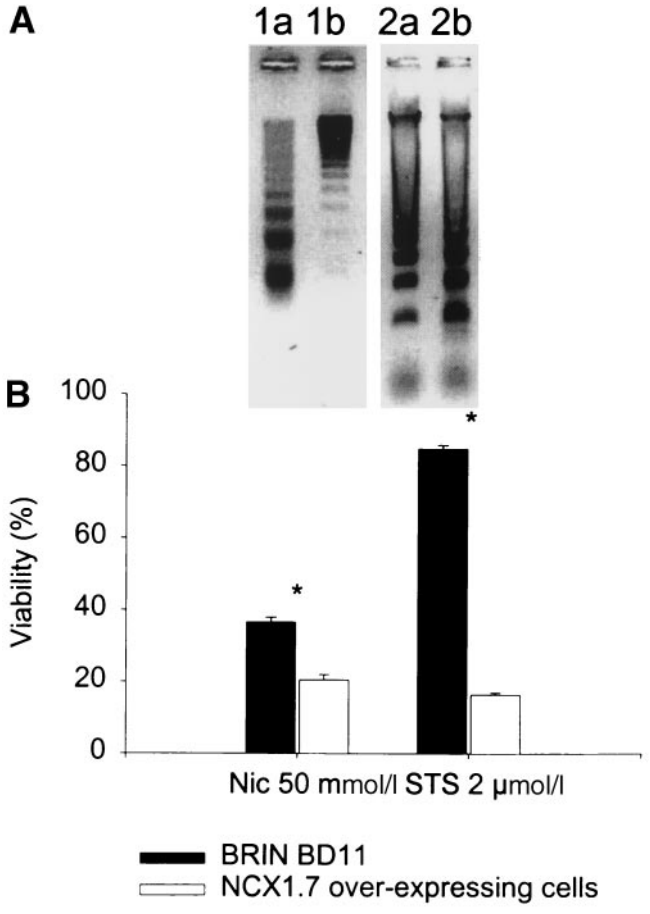

FIG. 8. *A*: Gel electrophoresis of low molecular weight DNA in non-transfected (*a*) and NCX1.7-overexpressing BRIN-BD11 cells (*b*) after exposure to 50 mmol/l nicotinamide (*lanes 1a* and *b*) and 2 μmol/l stauroporine (*lanes 2a* and *b*). Same presentation as in Fig. 3*A*. *B*: Effect of NCX1.7 overexpression on cell viability in the presence of nicotinamide (Nic; 50 mmol/l) and stauroporine (STS; 2 μmol/l). Same presentation as in Fig. 1.

that nicotinamide, the inhibitor of the DNA repair enzyme poly(ADP-ribose) polymerase (30) and stauroporine, the broad spectrum protein kinase inhibitor (31), induced a higher rate of apoptosis in overexpressing than in control cells, as determined by the MTT test and DNA laddering. Although the second agent acts through Ca^{2+}, the first does not. In nicotinamide-treated cells, the percentage of DNA fragmentation was 42 ± 3% in overexpressing cells compared with 28 ± 3% in control cells ($P < 0.01$).

On harvesting the cells, it was apparent that the growth of NCX1.7-overexpressing cells was lower than that of control cells. To evaluate such a difference, the effect of serum on cell proliferation, over a period of 48 h, was measured using the MTT test and cell counting. Figure 9*A* shows that at serum concentrations of 3–10%, cell proliferation was reduced by ~40% in overexpressing compared with control cells ($P < 0.001$). Such a reduction in proliferation was also observed in another independent clone overexpressing NCX1.7 (data not shown). Cell counting (Fig. 9*B*) revealed a similar reduction in proliferation (−35 to 43%, $P < 0.05$).

DISCUSSION

In the present study, the Na/Ca exchanger was stably overexpressed in an insulin-secreting cell line, specifically to examine the extent to which such overexpression may modulate Ca^{2+} equilibrium within the cell and modulate Ca^{2+}-induced cytotoxicity and/or apoptosis.

The major observation was that Na/Ca exchanger overexpression led to a reduction in the rise in $[Ca^{2+}]_i$ induced by agents stimulating Ca^{2+} entry into the cell, to a depletion of the ER Ca^{2+} stores, and to an increase in cell death, whether mediated by Ca^{2+}-dependent or -independent pathways. Cell death appeared to be due to apoptosis as assessed by nuclear DNA staining, DNA laddering, and the quantification of fragmented DNA.

The view that Na/Ca exchanger overexpression would lead to a reduction in the rise in $[Ca^{2+}]_i$ induced by agents stimulating Ca^{2+} entry into the cell was best objectified in the case of tolbutamide. Ionomycin also increases Ca^{2+} inflow, but may also release Ca^{2+} from intracellular stores. Interestingly, the initial phase of the increase in $[Ca^{2+}]_i$ induced by ionomycin was reduced in overexpressing cells, whereas the late phase was not. The initial phase most probably reflects Ca^{2+} entry into the cell from the outside instead of Ca^{2+} release from intracellular stores. Therefore, the data obtained with ionomycin (reduction of the first phase) are consistent with those obtained with the hypoglycemic sulfonylureas. In a previous study, we also showed that the rise in Ca^{2+} induced by glucose was slightly reduced in Na/Ca exchanger–overexpressing cells compared with control cells (21).

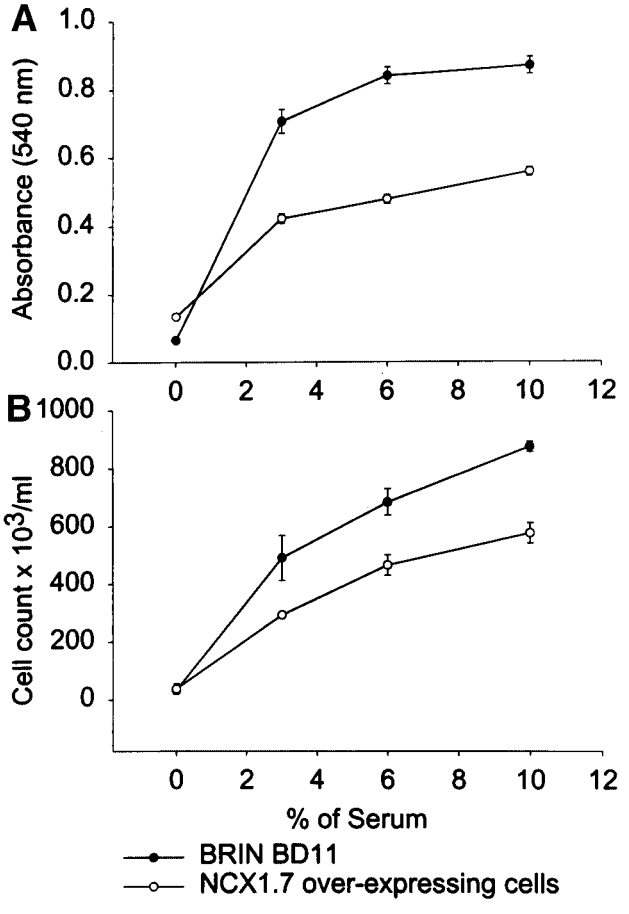

FIG. 9. Effect of NCX1.7 overexpression on serum-induced proliferation. Cells (1.5×10^4/well) were incubated in the presence of various concentrations of serum. After 48 h incubation, cell proliferation was analyzed using the MTT test (*A*) or cell counting (*B*).

The view that Na/Ca exchanger overexpression led to a depletion of the ER Ca^{2+} stores is based on the observation that SERCA inhibitors, thapsigargin and CPA, induced a smaller increase in $[Ca^{2+}]_i$ in Na/Ca exchanger–overexpressing than in control cells, indicating that the amount of Ca^{2+} releasable from the ER was reduced in the former cells. The depletion could be directly objectified by the measurement of the ER Ca^{2+} concentration using furaptra. Both the reduction in $[Ca^{2+}]_i$ and the depletion of ER Ca^{2+} stores can best be understood as the result of an increase in Ca^{2+} extrusion from the cell. This is consistent with our recent demonstration that Na/Ca exchange contributes to ~70% of the extrusion of Ca^{2+} from rat pancreatic β-cells (18). In addition, control cells transfected with the vector alone (without the NCX1.7 construct) did not show any difference in thapsigargin- or CPA-induced $[Ca^{2+}]_i$ changes compared with nontransfected cells (Fig. 4*A* and *B*). Also in agreement with such a view, overexpression of an isoform of the PMCA (2wb) led to similar results, including a reduction in the increase in $[Ca^{2+}]_i$, a depletion in ER Ca^{2+} stores, and an increased rate of apoptosis (unpublished data). In a previous study, the overexpression of a PMCA in CHO cells also led to a decrease in ER Ca^{2+} levels (1).

Taken as a whole, these observations indicate that the increased rate of apoptosis observed in overexpressing cells was due to ER Ca^{2+} depletion. Indeed, apoptosis was increased despite a reduction in the rise in $[Ca^{2+}]_i$, whether SERCA inhibitors or ionomycin were used. Another indication that apoptosis induced by SERCA inhibitors and ionomycin resulted from ER Ca^{2+} depletion rather than from $[Ca^{2+}]_i$ elevation is that the hypoglycemic sulfonylureas, which do not provoke ER Ca^{2+} depletion, did not induce apoptosis, either in control or overexpressing cells, despite the fact that they increased $[Ca^{2+}]_i$ to a similar extent than SERCA inhibitors. Likewise, ionomycin, while provoking a higher increase in $[Ca^{2+}]_i$ than in SERCA inhibitors, induced less apoptosis than the latter. Last, the increased rate of apoptosis was accompanied by the activation of caspase-12. Caspase-12, which is localized in the ER, is specifically activated by ER stress, including disruption of ER Ca^{2+} homeostasis, but not by membrane- or mitochondrial-targeted apoptotic signals (7). Incidentally, our data do not exclude that Na/Ca exchanger overexpression may also lead to a disruption in mitochondrial Ca^{2+} homeostasis that may also result in apoptosis. Indeed, there are dynamic interactions between ER and mitochondrial Ca^{2+} stores (32).

Na/Ca exchanger overexpression markedly increased the proapoptotic action of SERCA inhibitors compared with that of ionomycin. This probably is a result of the double action of ionomycin, which permeates both the plasma membrane and the intracellular membranes to Ca^{2+}. By doing so, ionomycin induces a lower release of Ca^{2+} from the ER because the permeation of the plasma membrane to Ca^{2+} increases $[Ca^{2+}]_i$, a phenomenon that may counteract ER Ca^{2+} depletion.

In the current study, one interesting observation was that Na/Ca exchanger overexpression induced caspase-12 activation without increasing the rate of apoptosis. Caspase-12 activation nevertheless sensitized overexpressing cells to apoptotic agents regardless of whether the involved signaling pathway was Ca^{2+} dependent or independent. This is compatible with the view that caspase-12 is an initiator rather an executor caspase (28).

In a previous study, glucose and hypoglycemic sulfonylureas were shown to induce apoptosis in mouse and rat pancreatic β-cells (14). Such a phenomenon was not observed in the current study using BRIN-BD11 cells. Although the present study provides no explanation for such a difference, one possibility is that native β-cells, because they are nontumoral and obtained from islet disruption (e.g., in Ca^{2+}-free medium), are more prone to undergo apoptosis than untreated tumoral cells.

The decrease in cell proliferation appeared to result from the same cause (reduction in ER Ca^{2+} stores due to increase in Ca^{2+} outflow) because it could be observed in two clones overexpressing NCX1.7 (current study) and in three clones overexpressing PMCA2wb (unpublished data). This reduction in cell proliferation was not unexpected because Ca^{2+} is essential for cell growth and survival, although its effects are so widespread that its exact mechanism(s) remain(s) to be completely elucidated (33).

In conclusion, we have shown that Na/Ca exchanger overexpression, by depleting ER Ca^{2+} stores, triggers the activation of caspase-12 and increases apoptotic cell death by Ca^{2+}-dependent and -independent pathways. Overexpression of the exchanger also led to a decrease in cell proliferation. Because excessive cell proliferation and decreased ability to undergo apoptosis are two hallmarks of cancer or malignancies (4), overexpression of Na/Ca exchanger in cancer cells by gene therapy may represent a new potential approach in cancer therapy. On the other hand, our results also clear a path for the development of new strategies to control cellular Ca^{2+} homeostasis that could, on the contrary, prevent the process of apoptosis that mediates, in part, β-cell autoimmune destruction in type 1 diabetes. Indeed, if it is possible to increase apoptosis by overexpressing the Na/Ca exchanger, then it should be possible to reduce it, e.g., by transfecting a Na/Ca exchanger antisense oligonucleotide or by overexpressing a SERCA that may lead to an increase in ER Ca^{2+} stores.

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