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Ba^{2+}-induced chromaffin cell death: cytoprotection by Ca^{2+} channel antagonists

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Abstract

Exposure of bovine adrenal medullary chromaffin cells to Ba^{2+} ions (in the absence of Ca^{2+} ions) caused their death, measured as lactate dehydrogenase (LDH) release. The concentration of Ba^{2+} required to damage the cells by about 65% ranged between 1 and 10 mM (no Ca^{2+} added); the required exposure time was rather brief (15 min–4 h). The simultaneous presence of Ca^{2+}, Mg^{2+} or Zn^{2+} together with Ba^{2+} (2 mM, 4 h) afforded cyprotection (60–80%). Individual selective blockers of Ca^{2+} channel subtypes afforded no protection. However, combined nifedipine (3 μM) plus ω-conotoxin MVIIC (3 μM) offered full protection. Substantial protection was also seen with the "wide-spectrum" Ca^{2+} channel blockers penfluridol (0.3 μM), lubeluzole (3 μM), dotarizine (3 μM), flunarizine (3 μM), and mibefradil (3 μM). This protection was due to blockade of Ba^{2+} entry through Ca^{2+} channels because dotarizine (10 μM) inhibited the increase in cytosolic $[Ba^{2+}]$ seen in fura-2-loaded chromaffin cells. Once Ba^{2+} accumulated in the cytosol, it was not extruded by the Na^+/Ca^{2+} exchanger, as shown by the prolonged and sustained elevation of the fura-2 signal. This contrasts with the fast dissipation of the fura-2 signal generated by $[Ca^{2+}]_i$ elevation. Thus, Ba^{2+} overload can cause cell death by mechanisms similar to those reported for Ca^{2+} overload and might be used as a novel and convenient tool to search for new cytoprotective compounds.

Keywords: Ba^{2+}; Cell death; Cytotoxicity; Cytoprotection; Chromaffin cell; Ca^{2+} channel antagonist

1. Introduction

The idea that Ca^{2+} overload leads to neuronal cell death has become a dogma (Choi, 1995; Choi et al., 1988; Siesjö, 1981). Ba^{2+} ions are often used as surrogates for Ca^{2+} ions in multiple Ca^{2+}-dependent biological mechanisms. However, the behaviour of Ba^{2+} differs considerably from that of Ca^{2+}, as the following examples illustrate. For instance, Ba^{2+} permeates better than Ca^{2+} the voltage-dependent Ca^{2+} channels and causes less inactivation of such channels (Hagiwara and Byerly, 1981). This leads to an enhanced current through Ca^{2+} channels (Albillos et al., 1994) and to a greater divalent cation entry into chromaffin cells, when Ba^{2+} is used instead of Ca^{2+} (Artalejo et al., 1987). In addition, Ba^{2+} exhibits a poor affinity for the Ca^{2+} binding sites on the plasmalemmal Ca^{2+} ATPase and Na^+/Ca^{2+} exchanger (Schilling et al., 1989; Wagner-Mann et al., 1992). These properties of Ba^{2+} make this cation likely to enter the cell more easily than Ca^{2+}, and to leave the cell with more difficulty. Thus, it may be that chronic exposure to Ba^{2+} may lead to its accumulation inside the cells more readily than Ca^{2+}. Hence, the use of Ba^{2+} can become a useful tool to cause the death of cells endowed with Ca^{2+} channels, and to study the cytoprotective properties of new drugs. We tested this hypothesis in the study presented here.

2. Materials and methods

2.1. Materials and solutions

Dulbecco's modified Eagle's medium (DMEM) and foetal calf serum were obtained from Gibco, Madrid, Spain; lubeluzole, flunarizine and penfluridol were from Janssen, Beerse, Belgium; dotarizine, was from Laboratorios Ferrer, Barcelona, Spain; nifedipine and furnidipine

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were from Alter, Madrid, Spain; mibefradil from Roche Laboratories; ω-conotoxin GVIA was from Bachem Feinchemikalien (Switzerland); ω-conotoxin MVIIC and ω-agatoxin IVA were from Peptide Institute (Osaka, Japan). Thapsigargin, ryanodine, caffeine and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) were from Sigma, Spain.

Concentrated solutions of drugs were prepared in water (mibefradil, ω-conotoxin GVIA, ω-conotoxin MVIIC, ω-agatoxin IVA) or dimethylsulfoxide (dotarizine, nifedipine, nimodipine, furnidipine, lubeluzole, penfluridol thapsigargin and ryanodine). Appropriate dilutions were then made in Krebs–HEPES. Caffeine was dissolved directly in Krebs–HEPES. Dimethylsulfoxide (DMSO), at the final concentration used (less than 0.1%), had no effect on any of the parameters tested. The assay kit for measuring the activity of lactate dehydrogenase (LDH) was purchased from Boehringer Mannheim. Barium was used as the salt $BaCl_2 \cdot 2H_20$.

2.2. Preparation of bovine chromaffin cell cultures

Bovine adrenal medullary chromaffin cells were isolated as previously described (Livett, 1984) with some modifications (Moro et al., 1990). To reduce the number of endothelial cells in the culture, which could alter the LDH measurements, the cells were pre-plated for 30 min and proliferation inhibitors (cytosine arabinoside, L-leucine methyl esther and fluorodeoxyuridine) were added to the culture in the DMEM medium. For cell death studies, cells were plated at a density of 5×10^5 on plastic culture wells (24-well Costar plates) coated with 0.01 mg ml^{-1} of poly-L-lysine, containing 1 ml DMEM supplemented with 5% foetal calf serum, 10 μM cytosine arabinoside, 10 μM fluorodeoxyuridine, 50 IU ml^{-1} penicillin and 50 μg ml^{-1} streptomycin. Cultures were maintained for 2–3 days at 37°C in a water-saturated atmosphere with 5% CO_2. After 24 h, the medium was replaced by 1-ml serum-free fresh medium and subsequently changed every 2 days. Trypan blue exclusion yielded cell viability values greater than 95%. Cells were normally used after days 2–3, to avoid excessive growth of endothelial cells.

2.3. Monitoring cell viability and cell death after exposure of cells to Ba^{2+} and drugs

Cells cultured for 2–3 days were washed first with 1 ml of Krebs–HEPES solution (in mM): NaCl, 144; KCl, 5.9; $MgCl_2$, 1.2; sodium HEPES, 10; glucose, 11, pH 7.4; thereafter, different protocols were used. For concentration–response curves of Ba^{2+}, the cells were exposed to 1 ml of Krebs–HEPES/0 Ca^{2+} solution containing different concentrations of Ba^{2+} (0.1 to 10 mM) for 24 h; after this time period, the medium was removed for measurement of extracellular LDH (LDH_e) and the cells were lysed with water to measure the intracellular content of LDH (LDH_i). For the time-dependent curves, 2 mM Ba^{2+} was added to the cells for a given time (5 min to 24 h) and after each time period, samples were collected to measure extracellular and intracellular LDH. To explore the actions of other divalent cations or different drugs in the presence of Ba^{2+}, divalent cations were incubated first for 30 min; and then the same cation or drug to be tested was added to the cells in the presence of 2 mM Ba^{2+} for another 30-min (this medium was kept for LDH_e measurement). Finally, the cation or drug, without Ba^{2+} now, was added again and the cells were kept in the incubator for 4-h. Intracellular and extracellular LDH were measured at the end of the 4-h period and also after the 30-min exposure to Ba^{2+}. Therefore, extracellular LDH was the sum of LDH_e after the 30-min exposure to Ba^{2+} plus LDH_e at the end of the 4-h incubation period, right at the end of the experiment.

2.4. LDH assay

Extracellular and intracellular LDH activity was spectrophotometrically measured by following tetrazolium reduction at an absorbance wavelength of 492 nm (Boehringer Mannheim kit). Total LDH activity was defined as the sum of intracellular and extracellular LDH activity. Released LDH was defined as the percentage of extracellular compared to total LDH activity.

2.5. Measurement of Trypan blue exclusion

Trypan blue exclusion was estimated in cultures plated at a lower density (100,000 cells/well). The number of cells stained and unstained was determined by counting 100 cells per well. Dead cells stained with Trypan blue were counted by two separate investigators.

2.6. Measurement of changes of the $[Ca^{2+}]_i$ and $[Ba^{2+}]_i$ in fura-2-loaded chromaffin cells

For these experiments, cells were plated on 1-cm diameter glass coverslips, at a density of 50,000 cells per coverslip. Cells were loaded with fura-2 by incubating them with fura-2/acetoxymethyl-esther (fura-2/AM) (4 μM) for 30 min at 37°C in Krebs–HEPES solution. The loading with the fluorescent dye was terminated by washing the coverslip to which the cells were attached several times with Krebs–HEPES. Then, cells were kept at room temperature for 15 to 30 min. The fluorescence of fura-2 in single cells was measured with the photomultiplier-based system described by Neher (1989), which produces a spatially averaged measure of $[Ca^{2+}]_i$. Fura-2 was excited with light alternating between 360 and 390 nm, using a Nikon 40 × fluorite objective. Emitted light was transmitted through a 425-nm dichroic mirror and 500–545 nm barrier filter before being detected by the photomultiplier. $[Ca^{2+}]_i$ was calculated from the ratios of the light emitted when the dye was excited by the two alternating excitation

wavelengths (Grynkiewicz et al., 1985). Since these conditions were used to measure the changes in $[Ca^{2+}]_i$ induced by Ba^{2+} in the absence of Ca^{2+}, and the Ca_i^{2+} software was not calibrated for Ba^{2+}, changes in fluorescence reflecting Ba_e^{2+} entry and a concomitant increase in $[Ba^{2+}]_i$ were expressed as apparent changes in Ca_i^{2+} concentrations, $[Ca^{2+}]_{app}$ (Von Rüden et al., 1993).

2.7. Statistical analysis

Data are expressed as means ± S.E.M. The statistical significance of differences between means was determined by an analysis of variance (ANOVA). If significant differences were found, an appropriate multiple comparison Fisher Paired least significant differences (PLSD) test was done. In some cases, the Student's *t*-test was used (see figure legends). Differences were considered significant at the level of $P < 0.05$ and $P < 0.01$.

3. Results

3.1. Cell death caused by Ba^{2+}

After a given treatment, cell death was measured as a function of LDH released into the extracellular medium. Before the experiment started, cells were washed once with Krebs–HEPES solution containing 2 mM Ca^{2+}; thereafter, this solution was withdrawn and Krebs–HEPES solution containing no Ca^{2+} and various concentrations of Ba^{2+} (0.1 to 10 mM) was added to the different wells. Extracellular LDH was measured 24 h after Ba^{2+} addition. Under these experimental conditions, Ba^{2+} induced cell damage. However, the concentration–response curve was very steep; the threshold concentration of Ba^{2+} to cause cell damage was 0.5 mM and the maximum effect was observed at 1 mM. Basal LDH release (24 h incubation of cells in Krebs–HEPES containing 2 mM Ca^{2+}) was less than 10%; maximum LDH release with 1–10 mM Ba^{2+} was above 60% (Fig. 1a).

To test the time-dependence of the Ba^{2+}-induced damage, cells were pre-incubated with 2 mM Ba^{2+} for different time periods ranging from 5 min to 24 h (Fig. 1b); Ba^{2+} was then removed and samples were immediately collected to measure LDH release. Cell death increased from 6 ± 0.7% in control conditions to 53 ± 5 and 69 ± 5.4% after a 30-min and 4-h incubation in 2 mM Ba^{2+}, respectively; maximum damage was achieved after 4 h and no further increase in the damage was observed even after a 24-h incubation with Ba^{2+}. Therefore, all subsequent studies to test the cytotoxic effects of Ba^{2+} were done by challenging the cells with 2 mM Ba^{2+} for 30 min, followed by a 4-h incubation at 37°C in normal Krebs–HEPES solution.

In order to corroborate the results of LDH released into the extracellular media in the presence of Ba^{2+}, we also

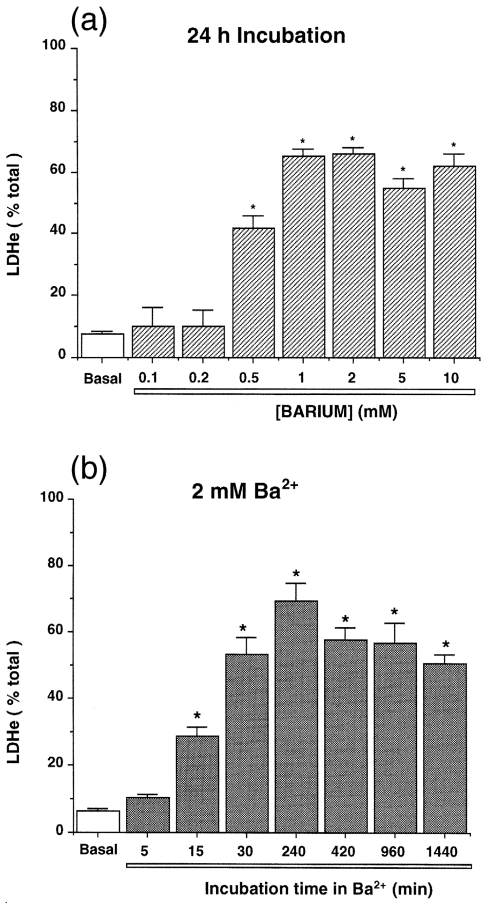

Fig. 1. Concentration and time dependence of the cytotoxic effects of Ba^{2+}. Panel A shows the cytotoxic effect of increasing concentrations of Ba^{2+}, measured as LDH release (ordinate), in cells exposed for 24 h to the cation (0.1 to 10 mM), in the absence of Ca^{2+}. Data correspond to the means ± S.E.M. for 4–24 wells for two to six different batches of cells. Panel B shows the cytotoxic effect of Ba^{2+} as a function of the time of cell exposure (5 min to 24 h); LDH was measured immediately after each period of cell exposure to Ba^{2+}. Data correspond to means ± S.E.M. for nine wells; three different batches of cells were used. $^{*}P < 0.05$ with respect to basal LDH release (ANOVA).

counted Trypan blue stained cells when treated with two different concentrations of Ba^{2+} (0.5 and 2 mM) and at different time intervals (15, 30 and 60 min). Fig. 2a shows that Ba^{2+} increased the number of stained cells from 11.8 ± 2.4% in control cells to 30.8 ± 4% in cells incubated with 2 mM Ba^{2+} for 60-min; cells were counted 4-h later. We also observed a significant concentration-dependent increase (Fig. 2b) in the percentage of dead cells; stained cells increased from 11 ± 2% when incubated in 2 mM Ca^{2+} and 0 Ba^{2+} (Basal) to 23 ± 2% and 35 ± 3% when incubated in the presence of 0.5 and 2 mM Ba^{2+} in 0 Ca^{2+} for 4 h. Although cell damage is clearly observed with this method, the results are underestimated with respect to the LDH results (Fig. 1a and b). A possible explanation for these results is that when the cells are completely lysed by Ba^{2+} they cannot be quantified with Trypan blue because their membranes are destroyed and

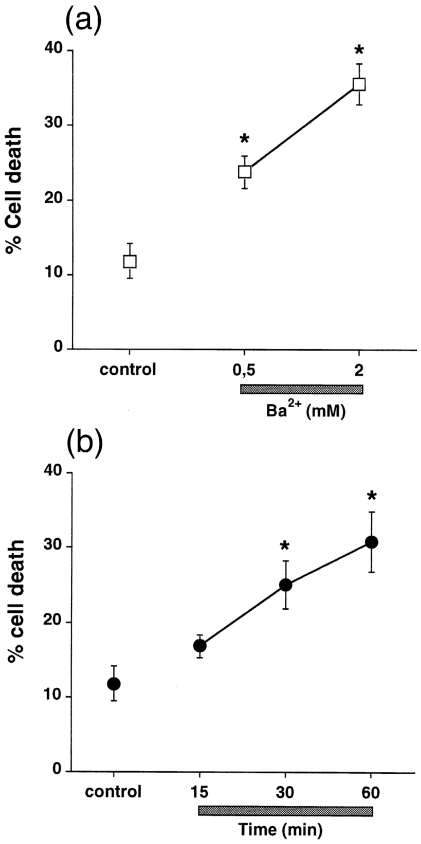

Fig. 2. Cell death expressed as percentage of cells stained with Trypan blue. (a) Represents the time dependence of the damage induced by 2 mM Ba^{2+} and (b) shows cell death after a 4-h incubation with 0.5 and 2 mM Ba^{2+}. The data correspond to the means ± S.E.M. of duplicates performed with three different batches of cells. $^{*}P < 0.005$ with respect to basal stained cells (Student's *t*-test).

the cell is no longer attached to the bottom of the well; in the case of the LDH measurements, when the cell is destroyed its content is totally released into the medium and can be measured biochemically.

The possibility exists that the extracellular LDH measured may to some extent indicate the presence of cells that are not necessarily dead, but merely detached due to removal of Ca^{2+}. This was unlikely since after a 24-h incubation of the cells in a 0 Ca^{2+} Krebs–HEPES solution containing 0.1 or 0.2 mM Ba^{2+}, the LDH released into the medium was around 10%, a figure similar to that found in cells incubated in Krebs–HEPES solution containing 2 mM Ca^{2+} (Fig. 1a). The following additional experiments demonstrated that LDH activity measured in 0 Ca^{2+}/Ba^{2+}-containing media was due to cell death.

In one experiment, cells were incubated for 24-h in a 2-mM Ba^{2+} (0 Ca^{2+}) solution. Then, the media were collected and divided into two aliquots. One aliquot was centrifuged at 1000 rpm for 10 min, to precipitate possible detached intact cells. LDH was assayed in the supernatant, as well as in the aliquot that was not centrifuged. LDH found in the centrifuged aliquot was $56 \pm 2.3\%$ ($n = 4$ wells) and that in the non-centrifuged aliquot was $52 \pm 2.1\%$ ($n = 4$ wells). This finding suggests that LDH from intact living cells was not present in the 0 Ca^{2+}/Ba^{2+}-containing media.

When observed under a phase-contrast microscope, Ba^{2+}-treated cells (2 mM for 24 h) were attached to the coverslip, at a density similar to the cells incubated in 2 mM Ca^{2+}. Cells incubated in 2 mM Ca^{2+} were round, tended to group in clusters and exhibited a strong birefringency (Fig. 3a). In contrast, cells incubated in the presence of Ba^{2+} exhibited poor birefringence and had a granular necrotic appearance (Fig. 3b).

3.2. Divalent cations other than Ba^{2+} protect against the damaging effects of Ba^{2+}

Other divalent cations like Ca^{2+}, Mg^{2+} and Zn^{2+} were tested for their ability to cause cell death, and for their capacity to protect against the cell damage caused by Ba^{2+}. In the latter case, the cells were exposed first to the protecting divalent cation for 30 min, then the cation together with 2 mM Ba^{2+} was added to the cells, and after the 30-min pulse with Ba^{2+}, this medium was washed out

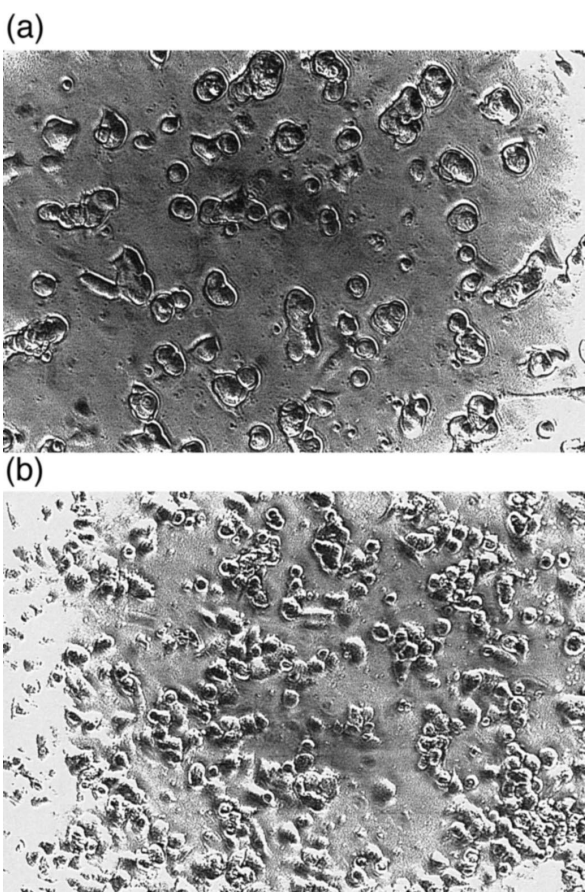

Fig. 3. Phase-contrast photomicrographs of chromaffin cells incubated for 24 h in Krebs–HEPES solution containing 2 mM Ca^{2+} (panel A) or in 0 Ca^{2+} solution containing 2 mM Ba^{2+} (panel B).

and new Krebs–HEPES with the protecting divalent cation was added to the cells for 3.5 h. Extracellular LDH released into the medium was measured after the 4-h incubation period, together with the intracellular content of LDH.

Ca^{2+} ions were very efficient and potent in protecting against the damaging effects of Ba^{2+}. Ca^{2+} ions per se (0.125 to 20 mM) did not damage the cells (not shown), but they afforded cytoprotection against the Ba^{2+}-induced damage (Fig. 4a). The maximum protection was achieved at 10 mM Ca^{2+}. Mg^{2+} (2 to 20 mM) and Zn^{2+} (0.1 to 2 mM) did not increase LDH release by themselves after a 4-h incubation period (not shown). Mg^{2+} caused a mild protection, which reached significant levels at 20 mM (Fig. 4b). Zn^{2+} was equally protective at all the concentrations used, 0.1 to 2 mM (Fig. 4c).

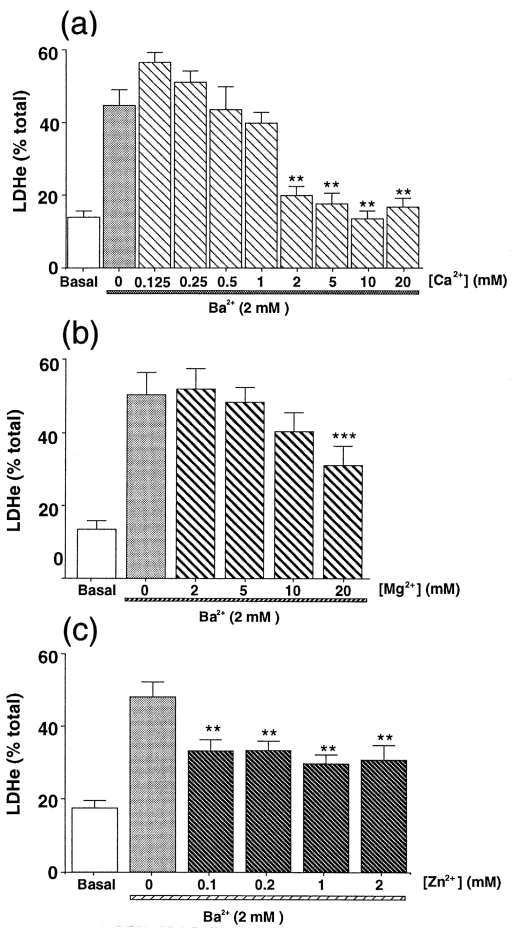

Fig. 4. Protection by divalent cations against the cytotoxic effects of Ba^{2+}. Cells were incubated first for 30 min in the presence of the concentrations of each divalent cation (Ca^{2+}, Mg^{2+} or Zn^{2+}) shown in the abscissa of panels A, B and C; then, still in the presence of the cation, cells were incubated for another 30 min with 2 mM Ba^{2+}. Ba^{2+} was removed and cells were incubated for a further 4-h period in a Krebs–HEPES solution containing the respective concentration of each divalent cation. Data are means ± S.E.M. for 9–12 wells in the case of Ca^{2+} (panel A), 12 wells in the case of Mg^{2+} (panel B), and 16 wells in the case of Zn^{2+} (panel C). In each case, the experiments were performed with cells from three to four different batches. $^{**}P < 0.01$ with respect to Ba^{2+} alone (ANOVA).

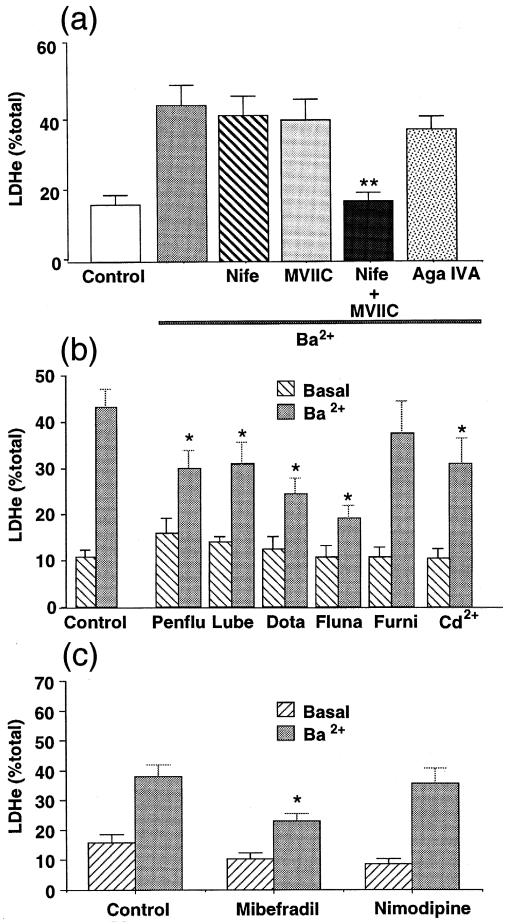

Fig. 5. Protection by selective Ca^{2+} channel blockers and by "wide spectrum" Ca^{2+} channel blockers against Ba^{2+}-induced cell damage. Panel A shows the effects of 3 μM nifedipine (Nife, an L-type calcium channel blocker), 2 μM ω-agatoxin IVA (Aga IVA; a P/Q-type Ca^{2+} channel blocker), and 2 μM ω-conotoxin MVIIC (MVIIC; an N/P/Q-type Ca^{2+} channel blocker) and their combination, on LDH release induced by 2 mM Ba^{2+}. Cells were exposed to Ba^{2+} for 30 min; LDH was estimated 4 h thereafter. Data are means ± S.E.M. for 12 wells using three different batches of cells. $^{**}P < 0.01$ with respect to Ba^{2+} alone. Panels B and C show the cytoprotection afforded by the "wide spectrum" Ca^{2+} channel blockers penfluridol (Penflu, 0.3 μM), lubeluzole (Lube, 3 μM), dotarizine (Dota, 3 μM), flunarizine (Fluna, 3 μM), Cd^{2+} (200 μM), and mibefradil (3 μM), as well as by the two 1,4-dihydropyridine derivatives furnidipine (Furni, 3 μM) and nimodipine (Nimo, 3 μM) against the cytotoxic effects of Ba^{2+} (2 mM). Protecting drugs were in contact with the cells for 30 min before exposure to Ba^{2+} and until the end of the experiment. Data are means ± S.E.M. for 9–12 wells; three different batches of cells were used. $^{*}P < 0.05$ with respect to Ba^{2+} alone (ANOVA).

3.3. Protection by Ca^{2+} channel blockers against Ba^{2+}-induced cell death

To explore if Ba^{2+} entered through voltage-dependent Ca^{2+} channels, we used various Ca^{2+} channel blockers. Since bovine chromaffin cells express L, N and P/Q

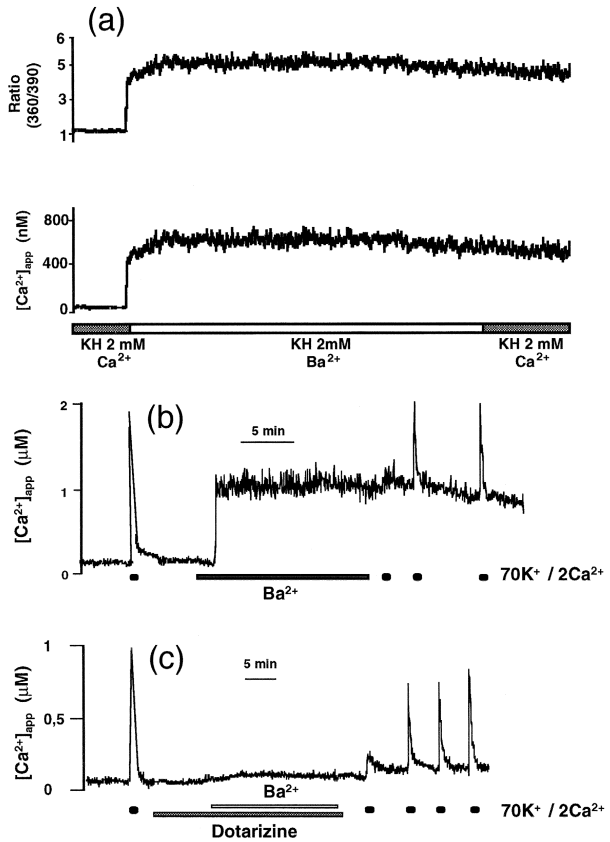

Fig. 6. Panel A shows Ba^{2+} entry, expressed as the ratio of fluorescence at 360/390 and as changes of the apparent cytosolic Ca^{2+} concentration, $[Ca^{2+}]_{app}$., in a fura-2-loaded cell. In panel B, a single fura-2-loaded cell was initially bathed in Krebs–HEPES solution containing 2 mM Ca^{2+}. Once the fluorescence signal was stable, the cell was stimulated for 10 s with 70 mM K^+ to see the $[Ca^{2+}]_i$ rise (dots at the bottom). Then, the Krebs–HEPES solution containing Ca^{2+} was switched to another one containing 2 mM Ba^{2+} but no Ca^{2+}, as indicated by the horizontal bar underneath. The superfusion of Ba^{2+} gave a fast increase in $[Ca^{2+}]_{app}$. In panel C another cell is shown, treated according to the same protocol as above, but superfused with 10 μM dotarizine for 10 min before and during Ba^{2+} addition. These results were reproduced in seven cells with Ba^{2+} alone and in three cells with dotarizine.

subtypes of Ca^{2+} channels (Albillos et al., 1996), we first used selective drugs and toxins to block the different subtypes of voltage-dependent Ca^{2+} channels, alone or in combination. As shown in Fig. 5a, the dihydropyridine nifedipine, an L-type Ca^{2+} channel blocker, had no cytoprotective effects; neither ω-conotoxin MVIIC nor ω-agatoxin IVA, which block non-L-type Ca^{2+} channels, afforded any protection when used alone. However, when all voltage-dependent Ca^{2+} channels were blocked with the combination of nifedipine plus ω-conotoxin MVIIC, full protection against Ba^{2+}-induced lesion was observed. Other dihydropyridines like nimodipine (Fig. 5c) and furnidipine (Fig. 5b) also did not provide protection.

As the combination of L- and non-L-type Ca^{2+} channel blockers afforded total protection, we decided to use several "wide-spectrum" Ca^{2+} channel antagonists (Villarroya et al., 1997; Cano-Abad et al., 1998) like penfluridol (0.3 μM), lubeluzole (3 μM), dotarizine (3 μM), flunarizine (3 μM), and Cd^{2+} (200 μM). A clear cytoprotection against Ba^{2+}-induced damage was observed with all these blockers (Fig. 5b). Mibefradil, shown to be a blocker of various subtypes of Ca^{2+} channels (Bezprozvanny and Tsien, 1995), also afforded protection (Fig. 5c). None of these drugs were cytotoxic per se at the concentrations used.

3.4. Ba^{2+} entry and its prolonged accumulation in the cytosol of fura-2-loaded chromaffin cells

In a previous study, a cytosolic Ba^{2+} signal could be detected in single fura-2-loaded bovine chromaffin cells (Von Rüden et al., 1993). This signal was expressed as the apparent cytosolic Ca^{2+} concentration, $[Ca^{2+}]_{app}$, which was calculated from the ratio 360/390 (Fig. 6a). The rate of Ba^{2+} entry to the cell interior and the dissipation of the $[Ba^{2+}]_i$ signal were estimated using this technique.

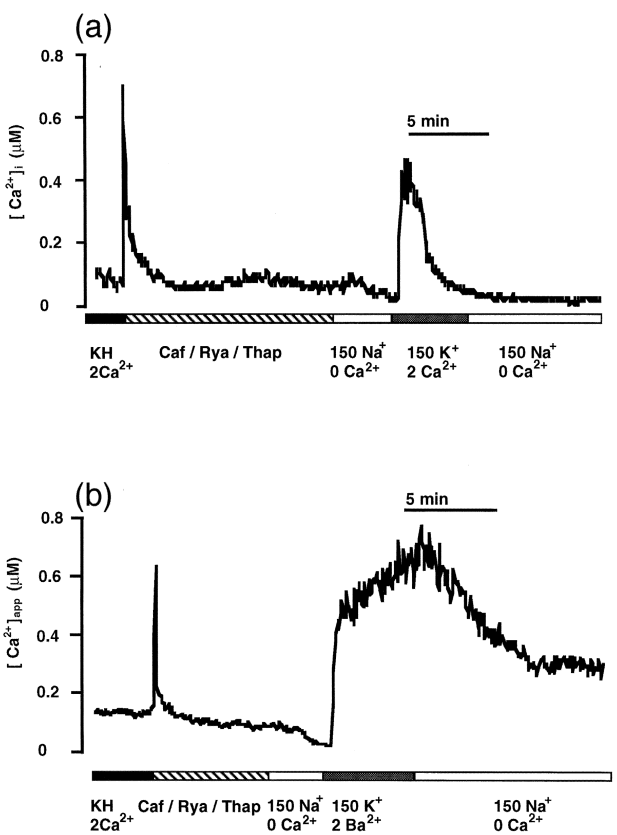

Fig. 7. Dissipation of the $[Ca^{2+}]_i$ signal (panel A) and $[Ba^{2+}]_i$ signal (panel B) after a high K^+ challenge given to fura-2-loaded single chromaffin cells. Initially, cells were superfused with Krebs–HEPES solution containing 2 mM Ca^{2+} (KH 2 Ca^{2+}). Then, cells were superfused with a Krebs–HEPES solution (2 mM Ca^{2+}) containing 10 μM ryanodine (Rya), 10 mM caffeine (Caf) and 1 μM thapsigargin (Thap) for the time period indicated in the horizontal bars. In the high K^+ solution (150 K^+/2 Ca^{2+}, 150 K^+/2 Ba^{2+}), all NaCl was replaced by KCl. The cells were sequentially superfused with each solution for the time periods indicated by the horizontal bars at the bottom of each trace.

In the fura-2-loaded cell shown in Fig. 6b, the initial resting $[Ca^{2+}]_i$ was around 0.1 μM. A challenge of $70K^+/2\ Ca^{2+}$ for 10 s produced a transient elevation of the $[Ca^{2+}]_i$, to 1.9 μM. After the $[Ca^{2+}]_i$ returned to basal levels, extracellular Ca^{2+} was replaced by 2 mM Ba^{2+}. The fluorescent signal rose rapidly and adopted an oscillatory pattern well above the basal levels. This pattern lasted the 15-min time exposure to Ba^{2+} and the signal remained

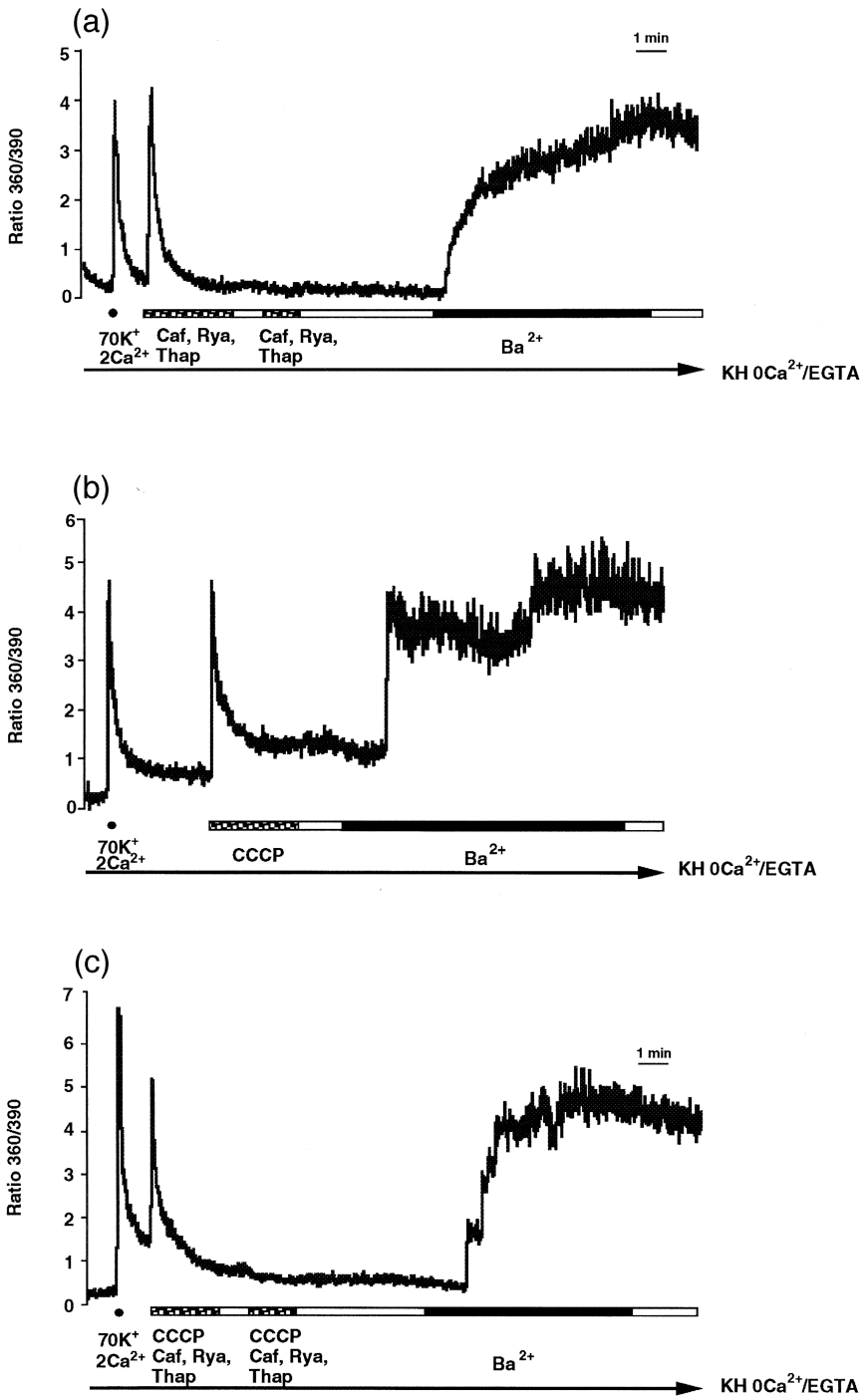

Fig. 8. Ba^{2+} entry, expressed as the ratio of fluorescence at 360/390 in fura-2-loaded cells after depletion of internal Ca^{2+} stores (endoplasmic reticulum and mitochondria). The cells were continuously bathed in Krebs–HEPES solution containing 0 Ca^{2+} and 0.2 mM EGTA. The cells were stimulated with a brief 5-s pulse of 70 mM K^+ and 2 mM Ca^{2+} to test the functionality of the cell, and thereafter the cells were superfused with the combination of 10 μM ryanodine (Rya), 10 mM caffeine (Caf) and 1 μM thapsigargin (Thap) to deplete the Ca^{2+} stores of the endoplasmic reticulum. Once the Ca^{2+} signal was at the basal level, Ba^{2+} was superfused to see its entry into the cell (a). In (b), the same experiment was carried out depleting Ca^{2+} from the mitochondria using CCCP 2μM (b) and depleting Ca^{2+} from the endoplasmic reticulum and mitochondria using a combination of 10 μM ryanodine (Rya), 10 mM caffeine (Caf), 1 μM thapsigargin (Thap) and 2 μM CCCP (c).

elevated after removal of Ba^{2+}. It is interesting that the application of $70K^+/2\ Ca^{2+}$ pulses produced a transient elevation of $[Ca^{2+}]_i$ before the Ba^{2+}-induced signal started to decline, suggesting that the cell had repolarised before Ba^{2+} was washed out from the cell cytosol.

The results shown in Fig. 6c were obtained with a fura-2-loaded cell that gave a sharp initial $[Ca^{2+}]_i$ increase (around 1 μM) upon a 10-s challenge with a $70K^+/2Ca^{2+}$ solution. Dotarizine (10 μM) prevented the increase of $[Ca^{2+}]_{app}$ induced by 2 mM Ba^{2+}. Immediately after the washout of dotarizine and Ba^{2+}, the response to $70K^+/2Ca^{2+}$ was abolished. This response recovered gradually thereafter, indicating washout of dotarizine.

One possible mechanism for the prolonged elevation of Ba^{2+} in the cytosol is its poor affinity for the Na^+/Ca^{2+} exchanger of the plasma membrane. This was tested in an experiment, the results of which are shown in Fig. 7. The idea of this experiment was to deplete and knockout the intracellular stores of Ca^{2+}, in such a manner that when the cytosolic Ca^{2+} (or Ba^{2+}) concentrations increased, Ca^{2+} (or Ba^{2+}) ions could only serve as substrates for the plasmalemmal transport systems. In panel A, a fura-2-loaded chromaffin cell was initially superfused with Krebs–HEPES solution (2 mM Ca^{2+}). Once the basal $[Ca^{2+}]_i$ stabilised at around 0.1 μM, the cell was superfused with Krebs–HEPES containing 10 μM ryanodine, 10 mM caffeine and 1 μM thapsigargin. This led to a transient rise of $[Ca^{2+}]_i$ due to the release of Ca^{2+} from intracellular stores and to its subsequent extrusion. Under these conditions, the ryanodine receptor channel and the smooth endoplasmic reticulum Ca^{2+} pump were irreversibly blocked. Then, the cell was superfused with a nominal 0 Ca^{2+} solution ($150\ Na^+/0\ Ca^{2+}$), which caused a gradual decline of the basal $[Ca^{2+}]_i$. To increase the $[Ca^{2+}]_i$, the cell was subsequently challenged with a solution containing 150 mM K^+ (without Na^+) and 2 mM Ca^{2+} ($150\ K^+/2\ Ca^{2+}$ solution). Finally, the cell was superfused again with the $150\ Na^+/0\ Ca^{2+}$ solution in order to activate the Na^+/Ca^{2+} exchanger. Observe that $[Ca^{2+}]_i$ declined quickly to basal levels.

Fig. 7b shows the results of a similar experiment, but the cell was challenged with $150\ K^+/2\ Ba^{2+}$ in order to increase $[Ba^{2+}]_i$, measured here as $[Ca^{2+}]_{app}$. The reintroduction of Na^+ caused a gradual slow decline of the $[Ba^{2+}]_i$. In contrast to $[Ca^{2+}]_i$, the $[Ba^{2+}]_i$ did not reach basal levels and remained elevated at a plateau until the end of the experiment.

3.5. Ba^{2+}-induced cell death is mediated by Ba^{2+} entry and not by Ba^{2+}-induced Ca^{2+} release from intracellular Ca^{2+} stores

To demonstrate that the increase in the fluorescence ratio in fura-2-loaded cells was due to Ba^{2+} entry and not to Ba^{2+}-induced Ca^{2+} release from intracellular stores, we conducted experiments with 0 Ca^{2+} EGTA (0.2 mM) and depleted the endoplasmic reticulum (ER) Ca^{2+} store with 10 μM ryanodine, 10 mM caffeine and 1 μM thapsigargin, alone or in combination with the mitochondrial uncoupler CCCP (2 μM). As shown in Fig. 8a, once Ca^{2+} from the ER was depleted, the addition of Ba^{2+} in 0 Ca^{2+}/EGTA still increased the fluorescence signal. A similar pattern was observed when CCCP was added (Fig. 8b). CCCP caused an elevation of $[Ca^{2+}]i$, probably due to Ca^{2+} release from mitochondria and ER (Montero et al., 2000; Alonso et al., 1999). Therefore, these results indicate that Ba^{2+} entry into the cell causes cell death. The damage induced by Ba^{2+} alone, expressed as LDH release, was not significantly increased when Ba^{2+} was incubated in the presence of either thapsigargin + ryanodine + caffeine alone, or in combination with CCCP (data not shown).

4. Discussion

The experiments described here suggest that Ba^{2+} ions may become an excellent tool to overload chromaffin cells with the cation, to cause cell death and hence to study the basic mechanisms of this process, as well as to search for new compounds with potential neuroprotecting properties. The overloading of cells with Ba^{2+} might imply the following sequence of events: (i) blockade by Ba^{2+} of K^+ channels; (ii) cell depolarisation induced by Ba^{2+}; (iii) the subsequent opening of voltage-dependent Ca^{2+} channels; (iv) Ba^{2+} entry into the cell; (v) quick elevation of the $[Ba^{2+}]$ in the cytosol; and (vi) poor buffering of Ba^{2+} by the Ca^{2+} buffering systems, with its sustained elevation in the cytosol.

Bovine chromaffin cells express Ca^{2+}-dependent K^+ channels (Marty, 1981; Marty and Neher, 1985; Artalejo et al., 1993) and voltage-dependent K^+ channels (Sala and Soria, 1991). Thus, as in other cell systems (Eaton and Brodwick, 1980; Armstrong et al., 1982), Ba^{2+} might block the various subtypes of K^+ channels expressed by bovine chromaffin cells. This could ultimately be the mechanism through which Ba^{2+} causes chromaffin cell depolarisation and the opening of voltage-dependent Ca^{2+} channels.

Under our experimental conditions, Ba^{2+} induced concentration- and time-dependent damage (Figs. 1 and 2) that reached maximum values around 60% Therefore, there was a 40% resistant population that did not become damaged even if we increased the external concentration of Ba^{2+} up to 10 mM or if the cells were incubated for 24 h in 2 mM Ba^{2+}. A possible explanation could be that in our bovine adrenal medulla cell preparations there are adrenaline-containing cells (around 80%), noradrenaline-containing cells (around 20%) and endothelial cells. In order to diminish as much as possible the number of endothelial cells, we pre-plated the cells for 30 min and performed experiments within 48 h. In spite of this, endothelial cells still grew. Since we have three different

subpopulations of cells in our cultures, it could be that one of these subpopulations is resistant to the damage caused by Ba^{2+}, and that is why we did not observe more that 60% cell death.

An interesting issue that emerges from these results relates to the use of Ba^{2+} to induce neurotransmitter release (Heldman et al., 1989; Forsberg and Pollard, 1988; Von Rüden et al., 1993; Pzywara et al., 1993). The fact that Ba^{2+} can cause cell death after 15–20 min exposure to 2 mM Ba^{2+} (Fig. 1b) should be considered when making an interpretation of the results of exocytosis, since under these circumstances secretion may also be due to disrupture of the plasma membrane.

It is well established that Ba^{2+} exhibits a considerably greater capability than Ca^{2+} to permeate the voltage-dependent Ca^{2+} channels (Hagiwara and Byerly, 1981). Thus, in a previous study we observed that 10 mM Ba^{2+} doubled the inward Ca^{2+} channel current generated by 10 mM Ca^{2+} in cat chromaffin cells (Albillos et al., 1994). The ready access of Ba^{2+} to the cell interior sharply contrasts with its poor ability to be extruded out of the cell, or to enter the intracellular Ca^{2+} stores. Once the cation gains the cytosol, the intracellular concentration of Ba^{2+}, measured as $[Ca^{2+}]_{app}$ in fura-2-loaded cells, remained elevated for a long time (Fig. 6a). This contrasts with the quick dissipation of a cytosolic Ca^{2+} transient generated after a K^+ depolarising stimulus (Ca_i^{2+} spikes in Fig. 6). This is due to the poor affinity of Ba^{2+} for the Ca^{2+}-binding sites on intracellular and plasmalemmal Ca^{2+} transport systems, i.e. Ca^{2+} ATPases and Na^+/Ca^{2+} exchanger (Schilling et al., 1989; Wagner-Mann et al., 1992). We corroborated that this is so in an experiment performed with fura-2-loaded chromaffin cells, with the intracellular Ca^{2+} stores of which were depleted. External Na^+ exchanged with cytosolic Ba^{2+} very slowly (Fig. 7b), as compared with the exchange with cytosolic Ca^{2+} (Fig. 7a).

The possibility exists that Ba^{2+} causes cell damage indirectly through the release of Ca^{2+} from intracellular stores. However, this is unlikely for two reasons: (1) we have previously shown that Ba^{2+} does not mobilise Ca^{2+} from intracellular stores (Von Rüden et al., 1993); and (2) incubation of chromaffin cells in 0 Ca^{2+} solution caused a drastic depletion of intracellular Ca^{2+} stores, as recently shown in our laboratory with aequorin, which is targeted to the endoplasmic reticulum of bovine chromaffin cells (Alonso et al., 1999). In this work, we performed experiments with cells bathed in 0 Ca^{2+}/EGTA, and after depleting Ca^{2+} stores with thapsigargin, caffeine and ryanodine, we observed that Ba^{2+} caused an increase in fluorescence (Fig. 8a), indicating Ba^{2+} entry. A similar picture was observed when we used the mitochondrial uncoupler, CCCP (Fig. 8b). These results, together with the measurement of LDH (data not shown), indicate that Ba^{2+} itself causes cell death since depletion of internal Ca^{2+} stores did not modify the damage induced by Ba^{2+} alone. We also observed that depletion of internal stores is not a sufficient signal to cause cell death, measured as LDH release, although it is known to cause cell apoptosis in other cell systems (Baffy et al., 1993; Lam et al., 1994; He et al. 1997).

Depolarisation itself seems not to be associated with cell death because Ca^{2+} ions, which caused a clear cytoprotection against Ba^{2+} cytotoxicity (Fig. 3a), did not prevent the Ba^{2+}-induced cell depolarisation (not shown). The observation that Ca^{2+} and the inorganic Ca^{2+} channel blockers Mg^{2+}, Zn^{2+} and Cd^{2+} caused cell protection (Figs. 4 and 5) supports the view that the primary event in causing cell death is Ba^{2+} entry through non-inactivating Ca^{2+} channels and its sustained accumulation in the cytosol, where it remains unbuffered. It is interesting that sustained depolarisation of chromaffin cells with high K^+ solutions containing Ca^{2+} is incapable of producing cell death over a 24-h period (Maroto et al., 1994). This can be easily explained if we consider that, like neurones (Hagiwara and Byerly, 1981), depolarised chromaffin cells inactivate their Ca^{2+} channels in a voltage- and Ca_i^{2+}-dependent manner (Michelena et al., 1993). This inactivation might constitute the basis for a physiological mechanism whose function might be the protection of excitable cells against Ca^{2+} accumulation produced by excessive cell firing of action potentials. This mechanism seems to be circumvented by Ba^{2+}, which does not inactivate Ca^{2+} channels (Hagiwara and Byerly, 1981).

An interesting question relates to the subtypes of Ca^{2+} channels used by Ba^{2+} to enter the cytosol and to cause cell death. Bovine chromaffin cells express L, N and P/Q subtypes of high voltage-activated Ca^{2+} channels, and all of them are highly permeable to Ba^{2+} (Albillos et al., 1993, 1996). Partial blockade of Ca^{2+} entry through L channels (nifedipine), P/Q channels (ω-agatoxin IVA) or N/P/Q channels (ω-conotoxin MVIIC) did not afford protection; however, a considerable protection against Ba^{2+} cytotoxicity was produced by blockade of all channels with combined nifedipine plus ω-conotoxin MVIIC (Fig. 5a), thus suggesting that any subtype of Ca^{2+} channel serves as a pathway for Ba^{2+} to gain access to the cell interior to cause cell death.

In looking for non-toxin/non-peptide compounds to block Ba^{2+} entry and to provide cell protection, we used the so-called "wide-spectrum Ca^{2+} antagonists' such as penfluridol, lubeluzole, dotarizine, and flunarizine as well as mibefradil, a novel wide-spectrum Ca^{2+} antagonist. All of these compounds afforded significant cytoprotection against Ba^{2+}-induced cytotoxicity (Fig. 5b and c). This is in line with their known ability to block Ca^{2+} and Ba^{2+} entry through L- as well as non-L-subtypes of Ca^{2+} channels (Villarroya et al., 1995, 1997; Hernández-Guijo et al., 1997; Bezprozvanny and Tsien, 1995), and with the blockade by dotarizine of the increase in $[Ca^{2+}]_{app}$ (Fig. 6c). Thus, the wide-spectrum Ca^{2+} antagonist dotarizine affords cytoprotection by blocking Ba^{2+} entry through Ca^{2+} channels.

In conclusion, we have demonstrated in this study that, like Ca^{2+}, the overloading of chromaffin cells with Ba^{2+} causes their death. In previous studies, we used pharmacological agents such as veratridine to induce Ca^{2+} cell load. Veratridine causes an oscillatory pattern of cell depolarisation and $[Ca^{2+}]_i$ increments (López et al., 1995) that ultimately leads to Ca^{2+} overloading and cell death (Maroto et al., 1994, 1996). In the case of Ba^{2+}, we do not need to use depolarising agent such as veratridine, since Ba^{2+} itself causes cell depolarisation. Ba^{2+} is a more simple and reliable tool than Ca^{2+} to overload, and pulses of few minutes are enough to trigger the cascade of events causing cell death. It will be interesting to know whether Ba^{2+} also damages other cells such as cerebellar granular neurones that express L, N and P/Q-types of Ca^{2+} channels (Randall and Tsien, 1995) in approximately similar proportions to bovine chromaffin cells (Albillos et al., 1996). And it will be even more interesting to know whether the pharmacological blockade of one or various of those channels suffices to cause neuronal protection, or whether blockade of all channels is needed, as seems to be the case for chromaffin cells. It is becoming widely accepted that acute ischemic brain injury is a complex etiopathogenic process. Neuronal protection might therefore require the combined use of drugs with different targets and mechanisms of action. The experiments reported here suggest that this is true, since several of the voltage-dependent neuronal Ca^{2+} channels must be blocked to prevent Ba^{2+} overloading and cell death.

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