0022-3565/09/3303-844–854$20.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
Copyright © 2009 by The American Society for Pharmacology and Experimental Therapeutics
JPET 330:844–854, 2009

Vol. 330, No. 3
154765/3502870
Printed in U.S.A.

Mitochondrial Na^+/Ca^{2+}-Exchanger Blocker CGP37157 Protects against Chromaffin Cell Death Elicited by Veratridine

Santos M. Nicolau, Antonio M. G. de Diego, Lorena Cortés, Javier Egea, José C. González, Marta Mosquera, Manuela G. López, Jesús Miguel Hernández-Guijo, and Antonio G. García

Instituto Teófilo Hernando (S.M.N., A.M.G.D., L.C., J.E., J.C.G., M.M., M.G.L., J.M.H.-G., A.G.G.), Servicio de Farmacología Clínica, Hospital Universitario de la Princesa (A.G.G.), and Departamento de Farmacología y Terapéutica, Facultad de Medicina, Universidad Autónoma de Madrid (S.M.N., A.M.G.D., L.C., J.E., J.C.G., M.M., M.G.L., J.M.H.-G., A.G.G.), Madrid, Spain

Received April 6, 2009; accepted June 8, 2009

ABSTRACT

Mitochondrial calcium (Ca^{2+}) dyshomeostasis constitutes a critical step in the metabolic crossroads leading to cell death. Therefore, we have studied here whether 7-chloro-5-(2-chlorophenyl)-1,5-dihydro-4,1-benzothiazepin-2(3*H*)-one (CGP37157; CGP), a blocker of the mitochondrial Na^+/Ca^{2+}-exchanger (mNCX), protects against veratridine-elicited chromaffin cell death, a model suitable to study cell death associated with Ca^{2+} overload. Veratridine produced a concentration-dependent cell death, measured as lactate dehydrogenase released into the medium after a 24-h incubation period. CGP rescued cells from veratridine-elicited death in a concentration-dependent manner; its EC_{50} was approximately 10 μM, and 20 to 30 μM caused near 100% cytoprotection. If preincubated for 30 min and washed out for 3 min before adding veratridine, CGP still afforded significant cytoprotection. At 30 μM, CGP blocked the veratridine-elicited free radical production, mitochondrial depolarization, and cytochrome *c* release. At this concentration, CGP also inhibited the Na^+ and Ca^{2+} currents by 50 to 60% and the veratridine-elicited oscillations of cytosolic Ca^{2+}. This drastic cytoprotective effect of CGP could be explained in part through its regulatory actions on the mNCX.

In general, it is accepted that a dysregulation of the mechanism that fine tunes the transient or more sustained levels of the cytosolic Ca^{2+} concentrations ($[Ca^{2+}]_c$), leads to excitotoxic neuronal death (Schanne et al., 1979) and to neurodegeneration (Mattson, 2007). However, Ca^{2+} may behave as both a cell survival supporter and a cell death inducer. For instance, cell depolarization and subsequent Ca^{2+} entry into the cytosol helps to sustain the survival of cerebellar granule cells (Gallo et al., 1987) and bovine chromaffin cells (Orozco et al., 2006). However, chronic elevation of $[Ca^{2+}]_c$ by ionophores induces apoptosis (Martikainen et al., 1991). The opposite is also true, i.e., Ca^{2+} antagonists that reduce $[Ca^{2+}]_c$ also cause neuronal death (Koh and Cotman, 1992) and chromaffin cell death (Novalbos et al., 1999). These apparent contradictory findings may be explained in the frame of the hypothesis suggesting that the $[Ca^{2+}]_c$ changes occurring during cell activation must move within a critical set point; beyond this point a cytoprotective signal might turn into a cytotoxic one (Koike et al., 1989). In this context, the suggestion of Nicholls (1985) and White and Reynolds (1995) that Ca^{2+} accumulation into mitochondria could play a neuropro-

This work was supported by the "Consolider Program Ingenio-2010," Ministerio de Ciencia e Innovación, Spain [Grant SAF2006-03589] (to A.G.G.); Instituto de Salud Carlos III, Ministerio de Ciencia e Innovación, Spain [Grant RETICS-RD06/0026] (to A.G.G. and M.G.L.); Comunidad Autónoma de Madrid, Spain [Grant S-SAL-0275-2006] (to A.G.G.); Mútua Madrileña, Madrid, Spain (to A.G.G. and M.G.L.); Agencia Lain Entralgo, Madrid, Spain [Grant NDG07/9] (to A.G.G.); FIS-Instituto de Salud Carlos III [Grant PI080227] (to J.M.H.-G.); Ministerio de Ciencia e Innovación, Spain [Grant SAF2006-0854] (to M.G.L.); a fellowship from Faculdade de Medicina-Universidade Agostinho Neto, government of Angola, to S.M.N.; and a fellowship from Ministério de Ciencia e Innovación, Spain (to J.C.G.).

Article, publication date, and citation information can be found at http://jpet.aspetjournals.org.

doi:10.1124/jpet.109.154765.

ABBREVIATIONS: mNCX, mitochondrial Na^+/Ca^{2+}-exchanger; DMSO, dimethyl sulfoxide; FPL64176, FPL, 2,5-dimethyl-4-[2-(phenylmethyl)benzoyl]-1*H*-pyrrole-3-carboxylic acid methyl ester; 30 K^+/FPL, 30 mM K^+/0.3 μM FPL; MTT formazan, 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan, thiazolyl blue formazan; CGP37157, 7-chloro-5-(2-chlorophenyl)-1,5-dihydro-4,1-benzothiazepin-2(3*H*)-one; TTX, tetrodotoxin citrate, octahydro-12-(hydroxymethyl)-2-imino-5,9:7,10a-dimethan *o*-10aH-[1,3] dioxocino[6,5-d]pyrimidine-4,7,10,11,12-pentol; DMEM, Dulbecco's modified Eagle's medium; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide; Olig, oligomycin; Rot, rotenone; CM-H_2DCFDA, 5-(and 6-) chloromethyl-2′,7′-dichlorodihydro-fluorescein-diacetate acetyl ester; Ver, veratridine; LDH, lactate dehydrogenase; ROS, reactive oxygen species; HRP, horseradish peroxidase.

tective role by removing Ca^{2+} from the cytoplasm fits well in the hypothesis. Conversely, by slowing down the rate of Ca^{2+} exit into the cytosol through the mNCX, the brisk $[Ca^{2+}]_c$ changes could be mitigated and protect cells from a cytotoxic insult.

In this study we have analyzed such hypothesis by using the mNCX blocker CGP as a pharmacological tool to slow down Ca^{2+} efflux from Ca^{2+}-loaded mitochondria into the cytosol (Montero et al., 2000). We have used bovine adrenal medulla chromaffin cells, a paraneuronal cell type that as neurons, possess Na^+ and Ca^{2+} channels as well as K^+ channels. Furthermore, as stated above, mitochondrial Ca^{2+} fluxes including the use of CGP have been thoroughly studied in these cells (Garcia et al., 2006). However, to explore the mNCX function we needed a model of cell death elicited by Na^+ and Ca^{2+} overload; hence, we resorted to veratridine that induces cell death by causing Na^+ and Ca^{2+} overload (Maroto et al., 1994, 1996; Novalbos et al., 1999), and augments superoxide production (Jordán et al., 2000). We have found that CGP affords drastic protection against chromaffin cell death elicited by veratridine. Such protection is linked to the preservation of mitochondrial function in veratridine-treated cells.

Materials and Methods

Reagents. Amphotericin B, cadmium, dimethyl sulfoxide (DMSO), FPL64176, 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan, thiazolyl blue formazan (MTT formazan), oligomycin, rotenone, veratridine, the salts to make the saline solutions, and the lactate dehydrogenase (LDH) kit were obtained from Sigma (Madrid, Spain). CGP37157 (CGP) and tetrodotoxin citrate (TTX) were purchased from Tocris (Biogen Científica, Spain). Dulbecco′s modified Eagle′s medium (DMEM), fetal calf serum, and penicillin/streptomycin were purchased from Invitrogen (Madrid, Spain). Fluo-4AM, JC-1, and 5- (and 6-) chloromethyl-2′,7′-dichlorodihydro-fluorescein-diacetate acetyl ester (CM-H_2DCFDA), pluronic acid were purchased from Invitrogen Molecular Probes (Madrid, Spain). Cytochrome *c* Elisa Kit was purchased from Millipore (Madrid, Spain).

Preparation of Cells. Adrenal glands were obtained from the city slaughterhouse under the supervision of the local veterinary service. Bovine adrenal medullary chromaffin cells were isolated as described previously (Livett, 1984), with some modifications (Moro et al., 1990). We used the Percoll gradients for the cell isolation procedure; thus, we had in our cultures a mixture of adrenergic (60–70%) and noradrenergic cells (30–40%). Cells were suspended in DMEM supplemented with 5% fetal bovine serum, 50 IU/ml penicillin, and 50 μg/ml streptomycin. Cells were preplated for 30 min and proliferation inhibitors (10 μM cytosine arabinoside, 10 μM fluorodeoxyuridine, and 10 μM leucine methyl ester) were added to the medium to prevent excessive growth of fibroblasts that would mask the chromaffin cell death measurements. The total cell number was determined as described previously. For cell death studies, cells were plated at a density of 5×10^5 cells/well on 24-well dishes. Cultures were maintained in an incubator at 37°C in a water-saturated atmosphere with 5% CO_2. Cell treatments were performed in DMEM free of serum, because serum interferes with LDH measurements.

Measurement of Cell Death through LDH Activity. Samples of incubation media were collected at the end of the 24-h period of veratridine, CGP, and veratridine/CGP exposure to estimate extracellular LDH, an indication of cell death (Koh and Choi, 1987). LDH activity was measured in the cells (5×10^5 per well) after treatment with 10% Triton X-100 (intracellular LDH). LDH activity was measured spectrophotometrically at 490 to 620 nm with use of a microplate reader (iEMS reader MF; Thermo Fisher Scientific, Waltham, MA). Total LDH activity was defined as the sum of intracellular plus extracellular LDH activity. Released LDH was defined as the percentage of extracellular compared with total LDH activity (Cano-Abad et al., 1998).

Measurement of Cell Viability with MTT. To determine viable cells we used MTT formazan probe prepared at a concentration of 5 mg/ml. Cells were plated at a density of 5×10^5 in 24-well dishes for 48 h and maintained in the incubator at 37°C. After this time, cells were treated with CGP, CGP + veratridine, or veratridine alone for 24 h. Then, 50 μl of MTT formazan solution was added to each well and cells were incubated for 3 h. We removed with care all solution in each well and added 500 μl of DMSO and shook this solution for 1 h. Finally we took a 100-μl aliquot and measured absorbance at 540 by use of a microplate reader (iEMS reader MF; Thermo Fisher Scientific).

ROS Measurement. To measure cell production of reactive oxygen species (ROS), we used the fluorescence probe H_2DCFDA (Ha et al., 1997). Bovine chromaffin cells were plated at a density of 5×10^5 in 24-well dishes and maintained in the incubator at 37°C for 2 days. Then, they were treated for 3 h with CGP, CGP + veratridine, or veratridine alone, after the cells were loaded with 5 μM CM-H_2DCFDA for 30 min at 37°C. CM-H_2DCFDA crosses the cell membrane and is hydrolyzed by intracellular esterase to the nonfluorescent form, dichlorodihydrofluorescein; the latter reacts with intracellular H_2O_2 to form dichlorofluorescein, a green fluorescent dye. Fluorescence was measured in an inverted fluorescence microscope (Nikon eclipse TE300; Nikon Instruments Europe, Badhoevedorp, Netherlands).

Measurement of the Mitochondrial Membrane Potential. Chromaffin cells were plated at a density of 5×10^5 cells/well in 24-well dishes for 2 days. Cells were treated with CGP, veratridine, and CGP + veratridine for 3 h in an incubator at 37°C. Cells were then loaded with 5 μM JC-1 for 30 min at 37°C. Fluorescence was measured with an inverted fluorescence microscope (Nikon eclipse TE300; Nikon Instruments Europe, Badhoevedorp, Netherlands). JC-1 exhibits potential-dependent accumulation in mitochondria, indicated by a fluorescence emission shift from green (~529 nm) to red (~590 nm). Consequently, mitochondrial depolarization was indicated by an increase in the green/red fluorescence intensity ratio. The potential-sensitive color shift was due to concentration-dependent formation of red fluorescent J-aggregates (Smiley et al., 1991).

Measurement of Cytochrome *c* Levels in Bovine Chromaffin Cells. Cells were plated at 1×10^6 on six-well plaques and maintained in an incubator for 2 days at 37°C in a water-saturated atmosphere with 5% CO_2. After this time, cells were collected and treated with veratridine, CGP, and veratridine + CGP for 3 h. Cells were collected and suspended at the concentration of 5×10^6 cells/ml with cold buffer-1 (10 mM Tris-HCl, pH 7.5, 0.3 M sucrose, 10 μM aprotinin A, 10 μM pepstatin, 10 μM leupeptin, 1 mM phenylmethylsulfonyl fluoride). The cell suspension was homogenized 5 to 10 times in ice, and subsequently centrifuged at 10,000*g* for 1 h at 4°C. The supernatant was used as the cytosolic fraction. The pellet was resuspended with cold buffer-2 (10 mM Tris-HCl, pH 7.5, 1% Triton X-100, 150 mM NaCl, 10 μM aprotinin A, 10 μM pepstatin, 10 μM leupeptin, 1 mM phenylmethylsulfonyl fluoride); the suspension was sonicated three times on ice, centrifuged at 10,000*g* for 30 min at 4°C, and the supernatant was the mitochondrial fraction. Finally, we added 100 μl of sample to an anti-cytochrome *c* antibody-coated plate (Kluck et al., 1997).

An anti-cytochrome *c* monoclonal coating antibody was adsorbed onto a microtiter plate. Cytochrome *c* present in the sample or standard binds to the antibodies adsorbed on the plate; a biotin-conjugated monoclonal anti-cytochrome *c* antibody was added and binds to cytochrome *c* captured by the first antibody. After incubation, unbound biotin-conjugated anti-cytochrome *c* was removed during a wash step. Added streptavidin-horseradish peroxidase (HRP) binds to the biotin-conjugated anti-cytochrome *c*. Next, unbound streptavidin-HRP was removed during a wash step, and substrate

solution reactive with HRP was added to the wells (Narita et al., 1998). A colored product was formed in proportion to the amount of cytochrome *c* present in the sample. The reaction was terminated by addition of acid and absorbance was measured at 450 nm by use of a spectrophotometer microplate reader (iEMS reader MF; Thermo Fisher Scientific).

Current Recording, Data Acquisition, and Analysis. Sodium (Na^+) currents (I_{Na}) were recorded by use of the whole-cell configuration of the patch-clamp technique (Hamill et al., 1981). Whole-cell recordings were conducted with fire-polished electrodes (resistance, 2–5 MΩ) filled with an intracellular solution containing 160 mM Cs-methanesulfonate, 10 mM EGTA, 5 mM Mg-ATP, 0.3 mM Na-GTP, 10 mM HEPES/CsOH, pH 7.3.

Calcium (Ca^{2+}) currents (I_{Ca}) were recorded by use of the perforated patch configuration of the patch-clamp technique (Korn and Horn, 1989). To facilitate sealing, fire-polished patch pipettes of thin borosilicate glass (Kimax 51; Witz Scientific, Holland, OH) were dipped in a beaker with the intracellular solution that contained 9 mM NaCl, 145 mM Cs-glutamate, 1 mM $MgCl_2$, 10 mM HEPES, pH 7.3 with CsOH, and then back-filled with the same solution containing amphotericin B (50 μg/ml). Amphotericin B was dissolved in DMSO and stored at −20°C in stock aliquots of 50 mg/ml. Fresh pipette solution was prepared every 2 h. Recording started when the access resistance decreased below 25 MΩ, which usually happened within 10 min after sealing.

Electrodes were mounted on the headstage of an EPC-10 patch-clamp amplifier (HEKA Elektronik, Lambrecht, Germany), allowing cancellation of capacitative transients and compensation of series resistance. Data were acquired with sample frequency ranging between 5 and 20 kHz and filtered at 1 to 2 kHz. Recording traces with leak currents >25 pA or series resistance >25 MΩ were discarded. Bovine adrenal chromaffin cells were placed on an experimental chamber mounted on the stage of a Nikon eclipse T2000 or a Kikon diaphot inverted microscope. During the preparation of the seal with the patch pipette, the chamber was bathed with a control Tyrode's solution containing 137 mM NaCl, 5.3 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM HEPES, pH 7.4 with NaOH. Once the patch membrane was ruptured and the whole-cell configuration of the patch-clamp technique established, the cell being recorded was locally, rapidly, and continuously superfused with an extracellular solution of identical composition to the chamber solution (see *Results* for specific experimental protocols). External solutions (containing 1 μM TTX and no $CaCl_2$ when I_{Na} was recorded) were rapidly exchanged by use of electronically driven miniature solenoid valves coupled to a multibarrel concentration-clamp device. The common outlet of the perifusion system was placed within 100 μm of the cell to be patched, and the flow rate (1 ml/min) was regulated by gravity to achieve complete replacement of the cell surroundings within less than 200 ms. Data acquisition was performed with use of PULSE programs (HEKA Elektronik). The data analysis was performed with Igor Pro (Wavemetrics, Lake Oswego, OR) and PULSE programs (HEKA Elektronik). All experiments were performed at room temperature (22–24°C) on cells 2 to 4 days after culture.

Measurement of $[Ca^{2+}]_c$ Oscillations. Cells were plated on 25-mm-diameter poly(L-lysine)-coated glass coverslips at a density of 2×10^5 cells and maintained in an incubator for 3 days at 37°C in a water-saturated atmosphere with 5% CO_2. After this time, cells were loaded with 3 μM fluo-4AM and pluronic acid was dissolved in the standard recording Tyrode's solution containing 137 mM NaCl, 10 mM HEPES, 5.3 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, pH 7.4, for 45 min in an incubator. After this time, the coverslips were then washed twice and left for 15 min at room temperature to allow cytoplasmic esterase to cleave fluo-4 free of the AM group, thus rendering the molecule active for Ca^{2+} chelation and fluorescence. Finally, the coverslips containing the cells were placed on an experimental chamber mounted on the stage of a NIKON TMD inverted microscope with an oil immersion objective (Nikon 60xPlanApo; numerical aperture, 1.4) and a confocal laser scanning unit (MRC 1024; Bio-Rad Laboratories, Hemel Hempstead, UK), equipped with an Ar/Kr laser able to produce a 488-nm-wavelength beam. The chamber was continuously perfused with Tyrode's solution. The cell being recorded was locally, rapidly, and continuously superfused with an extracellular solution of composition identical to that of the chamber solution. External solutions were rapidly exchanged by use of electronically driven miniature solenoid valves coupled to a multibarrel concentration-clamp device. A region of interest bordering the whole cell and another outside the cell to record possible background changes were selected. The fluorescence recordings were started automatically by a trigger activated by the patch-clamp amplifier to better synchronize the recordings.

Data Analysis and Statistics. Data are given as means ± S.E. Differences between groups were determined by applying a one-way ANOVA followed by a Newman-Keuls test. When indicated, Student's *t* test was used to determine statistical significance between means of two homogeneous data sets. Differences were considered to be statistically significant when $p \leq 0.05$.

Results

Characteristics of the Cytotoxic Effects of Veratridine. In the experiments of Fig. 1, cells were exposed to increasing concentrations of veratridine in DMEM (serum-free) and maintained in the incubator at 37°C for 24 h. Figure 1A shows phase-contrast micrographs taken after the 24-h

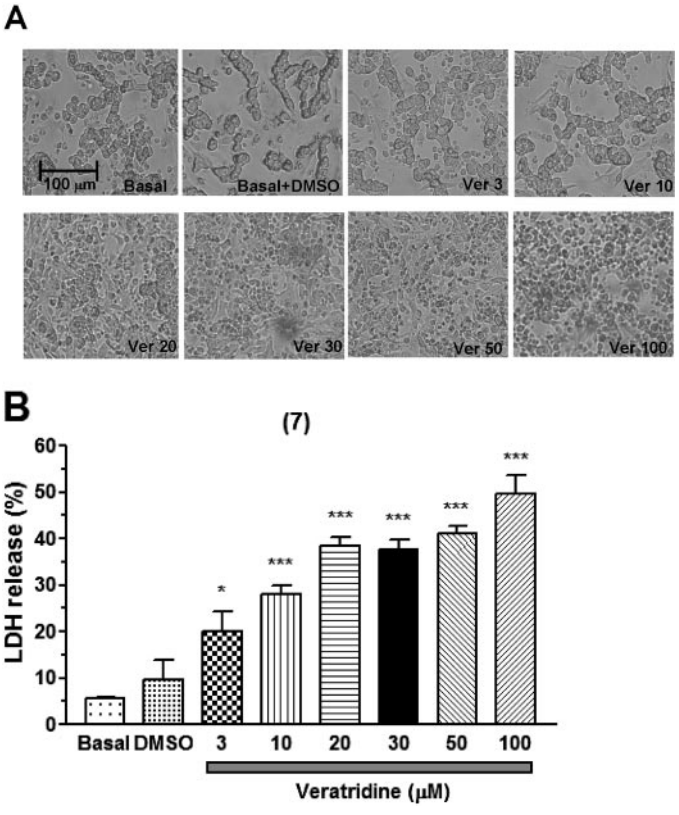

Fig. 1. Veratridine (Ver) caused chromaffin cell death in a concentration-dependent manner. A, phase-contrast micrographs of cells exposed to vehicle or to increasing concentrations of Ver for 24 h in DMEM (numbers at the bottom of each micrograph represent millimolar). B, Ver elicited release of LDH that was measured at the end of the 24-h incubation period. Cell death is expressed as the percentage of total LDH (LDH present in cells plus LDH present in the medium) (ordinate). DMSO, LDH release from cells incubated with the maximum DMSO concentration used as solvent for the 100 μM Ver concentration (0.1%). Data are means ± S.E. of seven experiments made in triplicate on cells from seven different cultures. *, $p < 0.05$; ***, $p < 0.001$, compared with basal.

incubation period; control cells adopted a typical disposition in clusters, although single round cells were also visible. Cells exhibited a sharp birefringent halo and well defined plasma membranes, with a homogeneous cytosol. Veratridine-treated cells showed a progressive deterioration as the drug concentration increased; thus, cells fused together, lost their birefringency, and presented irregular contours; the cytosol had a granular unstructured morphology.

Veratridine augmented LDH release in a concentration-dependent manner. At 3 μM, 20% of the total LDH was released into the medium, and at 20 to 30 μM, a maximum 40% LDH release was produced (Fig. 1B). An approximate EC_{50} of 10 μM for veratridine-elicited cell death was estimated graphically. Thus, we selected 30 μM as the concentration of veratridine that caused a reliable cell-damaging effect.

CGP Protected against the Cytotoxic Effects of Veratridine. We first tested whether CGP had any cell-damaging effect by itself. CGP concentrations in the range 0.3 to 30 μM did not augment LDH release above basal (approximately 5%) during a 24-h incubation period. However, at 100 μM, CGP itself exhibited a pronounced cytotoxic effect (approximately 75% LDH release) (Fig. 2A). Figure 2B shows the effects of increasing concentrations of CGP that protected the cells with an EC_{50} of 10 μM. With MTT as an indicator of mitochondrial function and cell viability, we found that CGP exerted a protection similar to that found with LDH (Fig. 2C).

Because the 30 μM concentration of CGP afforded a drastic cytoprotection with both LDH and MTT, we selected this concentration to perform the following experiments. The first protocol used consisted in preincubating the cells with 30 μM CGP during different time periods; it was then withdrawn and, thereafter, veratridine was immediately added and maintained for the remaining of the 24-h period. The results of this experiment are shown in Fig. 3A. With only 1 min of preincubation, CGP already afforded significant protection. This effect gradually augmented as the preincubation time increased, up to 45 min. An experiment similar to the previous one was performed with the difference that, after the CGP preincubation period, a 3-min wash period preceded the addition of veratridine; in this manner, it was assured that little CGP remained nearby the surrounding cells. Figure 3B shows that, under these conditions, CGP still afforded a significant protection that again depended on the length of the preincubation period. Control cells that were run in parallel were also preincubated with CGP during a similar time range (1–45 min), but CGP was also coincubated with veratridine during the remaining of the 24-h period. Obviously, under these conditions, CGP offered the maximal cytoprotection independently of the length of the preincubation period (Fig. 3C). In other experiments cells were incubated with veratridine for only 2 h. Zero to 30 min after veratridine washout, CGP (30 μM) was given and left for the remaining 22 h of the experiment. Under these conditions, CGP was still capable of affording significant protection (data not shown).

Effects of CGP on Cytotoxic Stimuli Other than Veratridine. As explained in the introduction, veratridine is a well established pharmacological tool to elicit cell death in neurons and chromaffin cells through a mechanism linked to Na^+ and Ca^{2+} overload. Thus, it was of interest to test whether CGP had cytoprotective effects on other cell toxicity

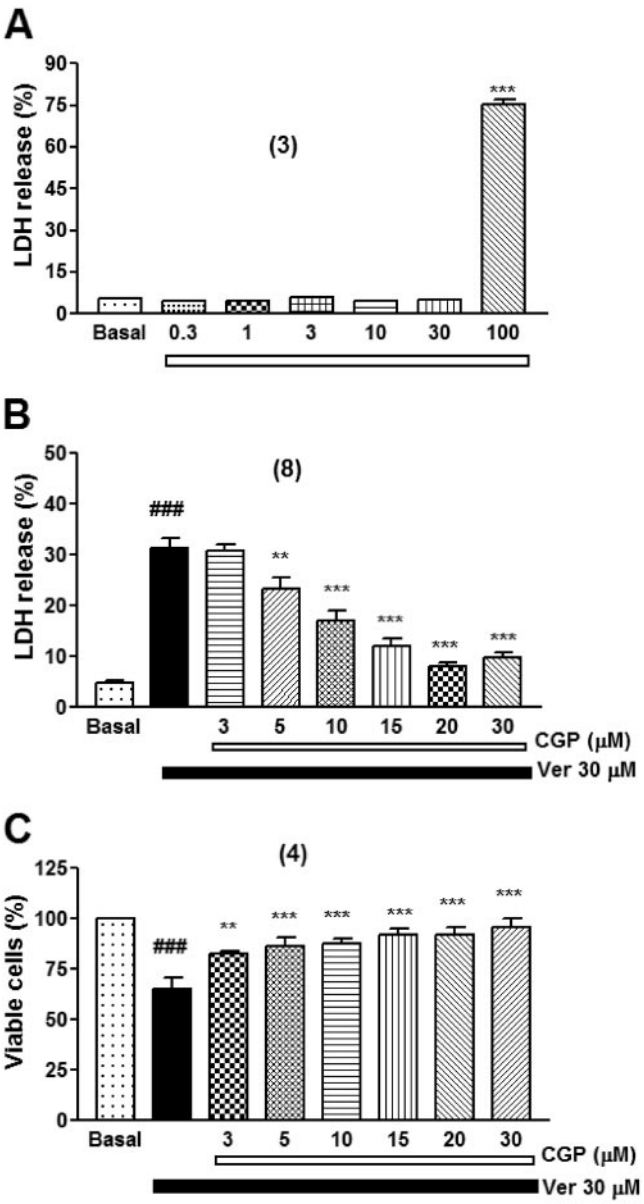

Fig. 2. Protection by CGP37157 (CGP) against the cytotoxic effects of veratridine (Ver). A, cells were incubated for 24 h with increasing concentrations of CGP. Basal, no drug added. B, cells were incubated first with CGP for 30 min followed by an incubation of 23.5 h with combined CGP (at the concentrations shown in the abscissa) and Ver (at 30 μM in all cases). C, cells were incubated first for 30 min (at the concentrations shown in the abscissa) followed by an incubation of 23.5 h with combined CGP and Ver (at 30 μM in all cases). In A and B, cell death was monitored by measuring in each individual plaque, the LDH present in the medium and in the cells at the end of the 24-h incubation period; these two parameters were added and named 100%, or total LDH. The released LDH (ordinates) elicited by each treatment was expressed as percentage of total LDH. In C, cell viability was measured with use of the mitochondrial probe MTT (see *Materials and Methods*). Data are means ± S.E. of the number of triplicate experiments shown in parentheses; each experiment was made in a different cell culture. ***, $p < 0.001$, with respect to basal (A); ###, $p < 0.001$, with respect to basal (B and C); **, $p < 0.01$, ***, $p < 0.001$, with respect to Ver alone (■) (B and C).

models. We had previously developed another model of chromaffin cell death through Ca^{2+} overloading by use of the L-type voltage-dependent Ca^{2+} channel activator FPL64176 (FPL), a mild K^+ depolarization (30 mM), and high extracellular [Ca^{2+}]. In this model, cell death was elicited by en-

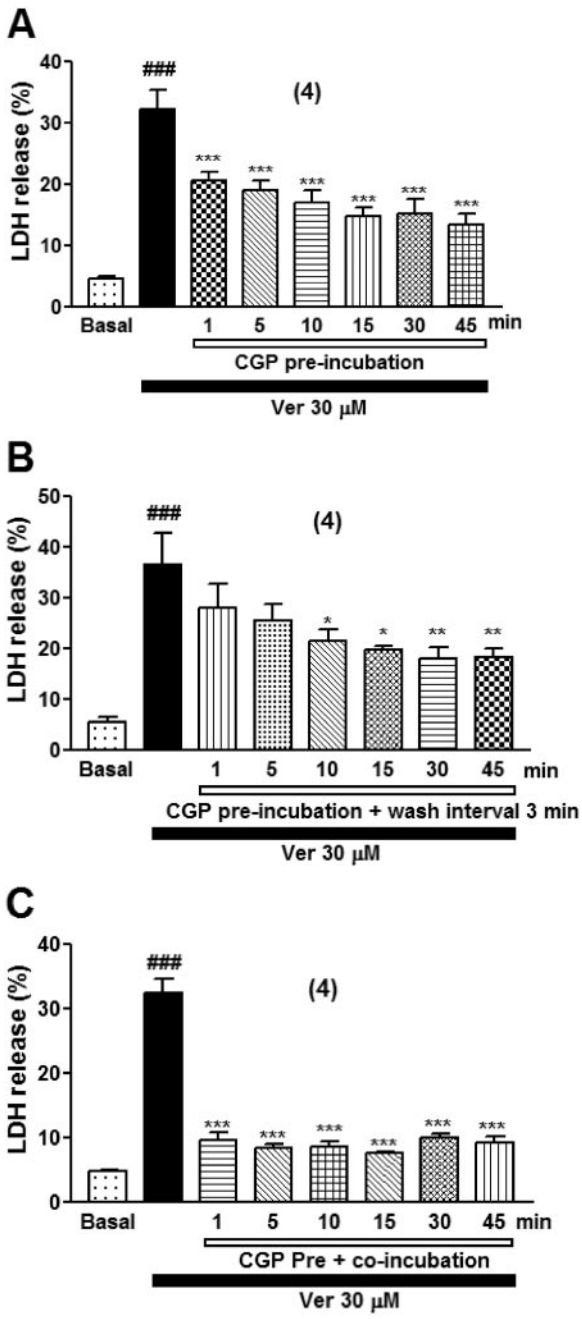

Fig. 3. The cytoprotection afforded by CGP37157 (CGP) has "memory." A, cells were preincubated for the indicated times (bottom horizontal bar) with 30 μM CGP; then, CGP was washed out and a new DMEM containing 30 μM veratridine (Ver) was added and left alone 24 h. B, cells were preincubated for the indicated times with 30 μM CGP; CGP was washed out and a 3-min interval (with fresh DMEM) was allowed before adding Ver. C, cells were preincubated for the indicated times with 30 μM CGP; then, this solution was replaced by a fresh DMEM containing CGP plus Ver. In all experiments, LDH release (ordinates) was measured after the 24-h incubation with Ver. Data are means ± S.E. of the number of triplicate experiments shown in parentheses; each experiment was performed in different cell cultures. ###, $p < 0.001$, with respect to basal; *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$, with respect to Ver alone (■).

hanced Ca^{2+} entry through L channels, because nimodipine afforded full protection (Cano-Abad et al., 2001). Hence, we tested whether, similar to nimodipine, CGP protected chromaffin cells against the cytotoxic effects of $30K^+$/FPL. These experiments were made in a Krebs-HEPES solution that allowed better ionic manipulations (Maroto et al., 1994; Cano-Abad et al., 2001). Greater basal LDH release was achieved (Fig. 4A) with respect to the veratridine experiments (Fig. 1B).

Figure 4A shows that cell incubation with $30K^+$/FPL solution for 24 h produced 30% LDH release, a figure similar to that obtained in our earlier experiments (Cano-Abad et al., 2001). When the cells were preincubated with increasing concentrations of CGP followed by incubation with CGP plus $30K^+$/FPL, CGP caused a clear-cut concentration-dependent cytoprotective effect. An estimated EC_{50} for this effect gave a value of 10 μM, similar to that obtained when using veratridine as a cytotoxic agent.

We resorted to a second cytotoxic stimulus unrelated to Ca^{2+}, i.e., blockade of the mitochondrial respiratory chain by combining 10 μM oligomycin that inhibits complex V, with 30 μM rotenone that blocks complex I (Olig/Rot). In a recent study we observed that incubation of bovine chromaffin cells with Olig/Rot for 24 h produced the release of 35 to 45% LDH (Egea et al., 2007), a cell death signal similar to that seen in our present experiments with veratridine (Fig. 1B). In the experiment of Fig. 4B, 24-h incubation with Olig/Rot caused

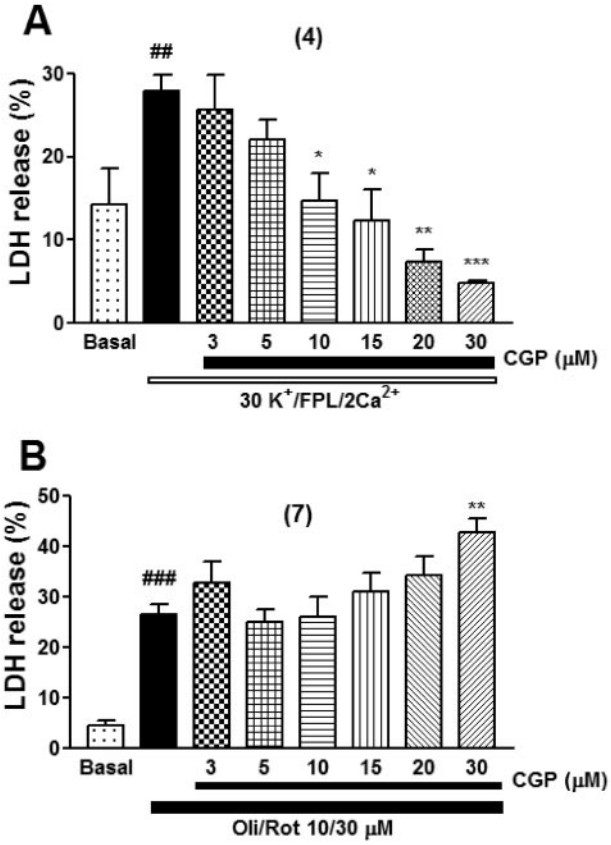

Fig. 4. Effects of CGP37157 (CGP) on the cytotoxic effects of FPL64176 (FPL), and combined oligomycin plus rotenone. A, cells were incubated 30 min with the CGP concentrations shown in the abscissa; then, the medium was changed by another containing the CGP concentrations plus 30 mM K^+, 0.3 μM FPL, and 2 mM Ca^{2+} ($30K^+$/FPL/$2Ca^{2+}$) and incubated during a 23.5-h period before LDH release was measured (ordinate). B, experimental protocol as in A but, here, 10 μM oligomycin and 30 μM rotenone (Olig/Rot) were used as cytotoxic agents. Data are means ± S.E. of the number of triplicate experiments shown in parentheses; each experiment was made with cells coming from different cell cultures. ##, $p < 0.01$, ###, $p < 0.001$, with respect to basal; *, $p < 0.05$, **, $p < 0.01$, with respect to the cytotoxic agent alone (■).

near 30% LDH release. In the presence of increasing concentrations of CGP (added 30 min before and present during cell incubation with Olig/Rot), LDH release did not change at 3 to 10 μM. At higher concentrations, CGP augmented the cell-damaging effects of Olig/Rot (near 50% LDH release at 30 μM).

Effects of Veratridine and CGP on Mitochondrial Function. Because a possible target of CGP is the mNCX, it seemed appropriate to explore whether veratridine affected the mitochondrial function and whether CGP was protecting against such damage. Three such functions were explored, i.e., production of ROS, the mitochondrial membrane potential, and the release of cytochrome *c*. Figure 5A shows the mean H_2DCFDA fluorescence (Ha et al., 1997) of 190 cells from three different cultures. Veratridine augmented 2.5-fold the ROS accumulation, an effect that was prevented by CGP.

We also determined the mitochondrial membrane potential variations ($\Delta\Psi_m$) with use of the fluorescence probe JC-1 (Smiley et al., 1991). Cells were incubated for 3 h with 30 μM veratridine, 30 μM CGP, or with a combination of both compounds. Then cells were loaded with JC-1 and their fluorescence was measured. By itself, CGP did not affect $\Delta\Psi_m$ but was capable of preventing the veratridine-elicited fluorescence loss. This is illustrated in a quantitative form in the bar diagram of Fig. 5B; veratridine caused a pronounced mitochondrial depolarization, and CGP prevented it.

A last parameter that we measured was the release into the cytosol of cytochrome *c*, an indication of mitochondrial disruption (Fig. 5C). Cells incubated with 30 μM veratridine for 3 h showed 6-fold increase of cytochrome *c* release with respect to basal; this increase was reduced by 70% when cells were coincubated with 30 μM CGP (added 30 min before veratridine) and 30 μM veratridine. By itself, 30 μM CGP had not effect on cytochrome *c* release (Fig. 5C).

Effects of CGP on Sodium Currents (I_{Na}) and Calcium Currents (I_{Ca}). We performed experiments in voltage-clamped cells to ascertain whether CGP was affecting I_{Na} and/or whether I_{ca} at 30 μM CGP caused a gradual inhibition of I_{Na}, which took approximately 2 to 3 min to block approximately 50% of the current (Fig. 6A). I_{Na} recovered promptly on CGP washout (80% recovery in approximately 20 s). The cell perifusion with 1 μM TTX produced full blockade of I_{Na}; the current gradually and fully recovered upon the toxin washout. Figure 6B shows original I_{Na} traces obtained during the depolarizing pulses indicated by small letters in Fig. 6A; I_{Na} exhibited a very fast activation and inactivation, reaching basal levels in approximately 2.5 ms. Figure 6C shows averaged current-voltage curves; the activation threshold was approximately −45 mV, I_{Na} peaked at −10 mV, and the apparent reversal potential was at +50 mV. At 30 μM, CGP reduced the peak I_{Na} by 60%; there was no shift in the *I/V* curve. Figure 6D shows a concentration-response curve in which a graph calculation gave an IC_{50} of 22 μM for I_{Na} inhibition by CGP.

The effects of CGP on I_{Ca} were tested in an additional series of experiments. Figure 7A shows the time course of I_{Ca} (measured as charge area, Q_{Ca}, once I_{Na} was suppressed) in a cell voltage-clamped at −80 mV and stimulated with test-depolarizing pulses given at 10-s intervals. In approximately a minute, 30 μM CGP reduced I_{Ca} by approximately 45%. Cadmium (Cd^{2+}, 100 μM) fully inhibited I_{Ca} in a reversible manner. Figure 7B shows original current traces from an example cell. Note the initial peak I_{Na} current with its typical fast inactivation, and the I_{Ca} currents that underwent a slow inactivation. The I_{Ca} trace-labeled with CGP was obtained 1 min after cell perifusion with 30 μM compound; CGP did not change the current inactivation. At 10 μM, CGP caused a gradual small decay of I_{Ca} (approximately 20%) that also recovered gradually and slowly on the compound washout (Fig. 7C). Example traces before and after 1 min of CGP

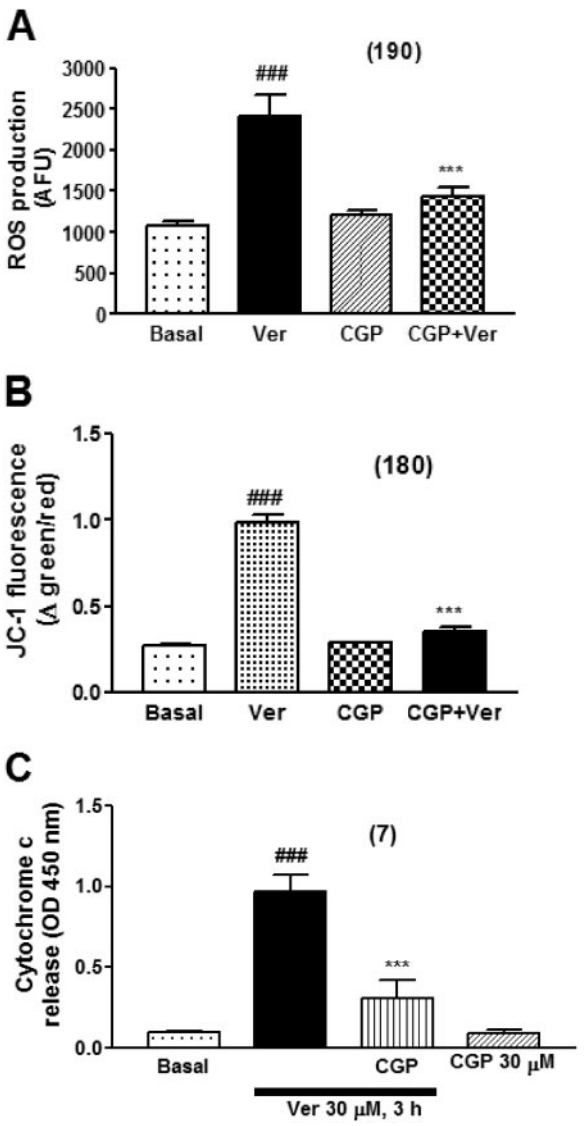

Fig. 5. Effects of veratridine (Ver) and CGP37157 (CGP) on mitochondrial function. A, blockade by CGP of the accumulation of ROS elicited by Ver; cells were exposed to vehicle (basal), 30 μM Ver, 30 μM CGP, or combined CGP + Ver for 3 h at 37°C and ROS accumulation was measured with the H_2DCFDA fluorescence probe. Data are expressed in arbitrary fluorescence units (AFU, ordinate) in 190 cells from three different cultures. B, blockade by CGP of mitochondrial depolarization elicited by Ver. Cells were incubated for 3 h with vehicle (basal), 30 μM Ver, 30 μM CGP, or combined CGP + Ver. The mitochondrial membrane ($\Delta\Psi_m$) was estimated with the fluorescence probe JC-1 (ordinate); data are means ± S.E. from 180 cells, from three different cultures. C, blockade by CGP of veratridine-elicited release of mitochondrial cytochrome *c* into the cytosol. Cells were incubated with vehicle (basal), Ver (30 μM), CGP (30 μM), or combined Ver + CGP. Data are means ± S.E of seven experiments from seven different cultures. ###, $p < 0.001$, with respect to basal; ***, $p < 0.001$, with respect to Ver alone.

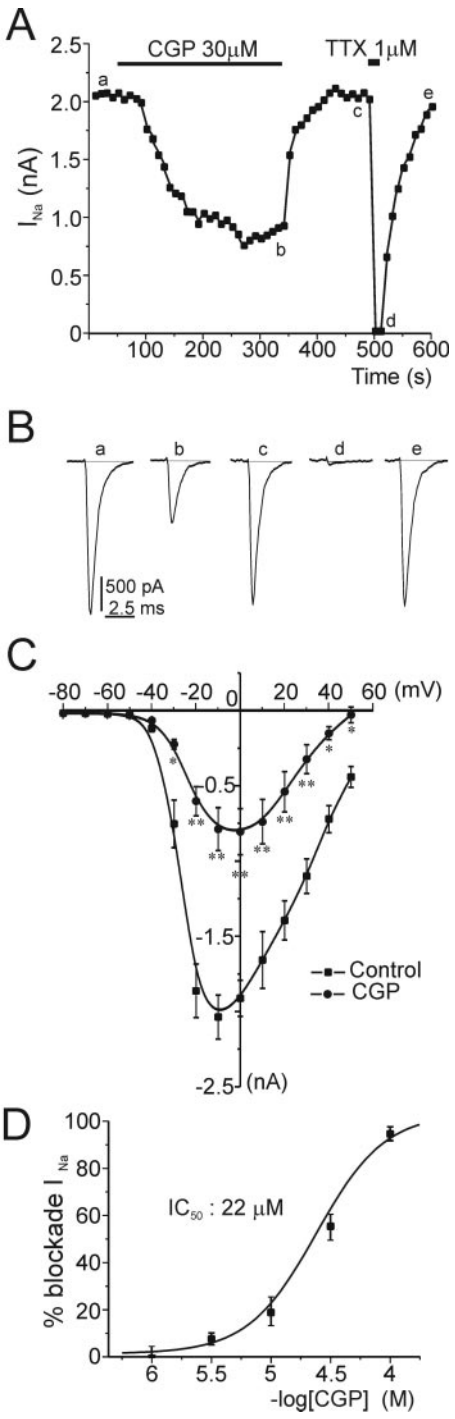

Fig. 6. Blockade by CGP37157 (CGP) of whole-cell sodium currents (I_{Na}). Cells were voltage-clamped at −80 mV. Upon breaking into the cell, 10-ms depolarizing pulses to various test potentials were regularly applied at 10-s intervals. I_{Na} stabilized after 5 to 10 pulses. A, time course of peak I_{Na} generated by the sequential application of test pulses to 0 mV. CGP and TTX were applied as indicated by the top horizontal bars. B, original I_{Na} traces taken at the points indicated by small letters in A. C, *I-V* curves constructed with the peak I_{Na} obtained on application of test pulses in 10-mV steps, given in the absence (control) and the presence of CGP. D, concentration-response curve for the inhibition by CGP of I_{Na}. Data in C and D are means ± S.E. The curve of D was constructed with data from 5 to 12 cells from different cultures. C, *, $p < 0.05$, **, $p < 0.01$ with respect to control.

perifusion are shown in Fig. 7D. Averaged results are shown in Fig. 7, E and F; 30 μM CGP blocked I_{Ca} by 60%, whereas 10 μM elicited approximately 18% blockade.

CGP Caused a Reversible Blockade of the $[Ca^{2+}]_c$ Oscillations Elicited by Veratridine. Fluo-4AM-loaded single cells were focally perifused with a Tyrode's solution. Under these conditions, spontaneous $[Ca^{2+}]_c$ oscillations were not seen during a 30-min recording period. To test the veratridine effects on $[Ca^{2+}]_c$, the cells shown in Fig. 8, A and B, were initially perifused with Tyrode's solution and, once a stable baseline was reached, veratridine (30 μM) was applied. $[Ca^{2+}]_c$ oscillations were elicited after a 1-min delay. The addition of CGP (5 μM) did not affect this $[Ca^{2+}]_c$ oscillatory pattern (Fig. 8B). Measurement of the number of oscillations during a 5-min period revealed the lack of effect of 5 μM CGP (Fig. 8D). In contrast, 30 μM CGP suppressed the $[Ca^{2+}]_c$ oscillations that quickly recovered on washout of the compound, as shown in the example cell of Fig. 8A. In 33 cells, 30 μM CGP inhibited the veratridine-elicited oscillations by 63% (Fig. 8C).

Discussion

In this study we have found that CGP affords cytoprotection against the cytotoxic effects of veratridine on chromaffin cells. We have also shown that CGP partially blocked I_{Na} and I_{Ca} in voltage-clamped cells, and the $[Ca^{2+}]_c$ oscillations elicited by veratridine. CGP has been demonstrated to inhibit the mNCX in various cell types since the discovery of its inhibitory properties on the cardiac mNCX (Vaghy et al., 1982). The question arises, therefore, as to whether CGP is protecting against veratridine-elicited cell death by acting on plasmalemmal Na^+ and Ca^{2+} channels and/or the mNCX (Fig. 9).

Inactivation delay of Na^+ channels elicited by veratridine (Ota et al., 1973) augments Na^+ entry (Kilpatrick et al., 1982) causing chromaffin cell depolarization (Lopez et al., 1995) opening of *L*, *N*, and *P*/*Q* voltage-dependent Ca^{2+} channels (Garcia et al., 2006), increased Ca^{2+} entry (Kilpatrick et al., 1982), and large $[Ca^{2+}]_c$ oscillations (Maroto et al., 1994, 1996; Lopez et al., 1995; Novalbos et al., 1999). This explains why Na^+ channel blockade by tetrodotoxin and blockade of *L*, *N*, and *P*/*Q* Ca^{2+} channels with flunarizine or a cocktail of ω-toxins and dihydropyridines, protect against Na^+ and Ca^{2+} overload and the ensuing cytotoxic effects of veratridine in neurons (Pauwels et al., 1989) and in bovine chromaffin cells (Maroto et al., 1994, 1996; Cano-Abad et al., 1998; Novalbos et al., 1999).

Although partial blockade of Na^+ and Ca^{2+} channels could explain the cytoprotective effects of CGP, some arguments suggest that an additional effect may also contribute: 1) The concentration that blocked by 50% I_{Na} (22 μM) caused more than 90% cytoprotection; 2) 10 μM CGP inhibited I_{Ca} by 20% but caused 50% cytoprotection; 3) full blockade of all Ca^{2+} channel subtypes with flunarizine or combined nimodipine and ω-conotoxin MVIIC are required to afford protection (Maroto et al., 1994, 1996; Cano-Abad et al., 1998; Novalbos et al., 1999); 4) 30 μM CGP afforded full protection despite the fact that the compound inhibited I_{Na} and I_{Ca} by only 50 to 60%; 5) blockade of I_{Na}, I_{Ca}, and $[Ca^{2+}]_c$ oscillations by CGP was reversed after 1-min washout, whereas its cytoprotective effects were present even on adding veratridine 3 min after CGP washout; and 6) when added 3 min after a veratridine cytotoxic pulse, conditions in which the Na^+ and Ca^{2+} chan-

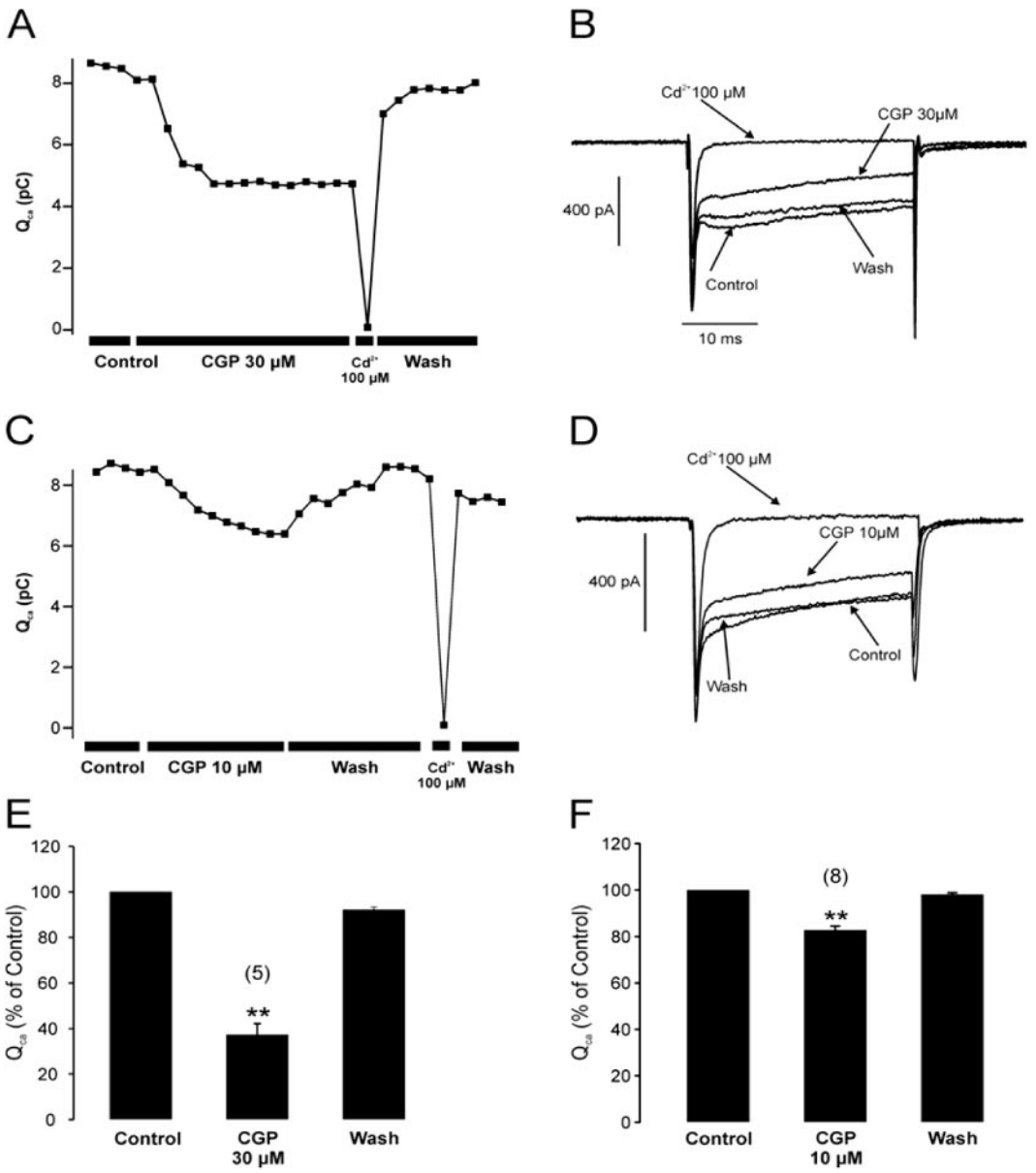

Fig. 7. Partial blockade by CGP37157 (CGP) of calcium currents (I_{Ca}). Whole-cell I_{Ca} was recorded under the perforated-patch configuration, using 2 mM Ca^{2+} as charge carrier. Cells were voltage-clamped at −80 mV; I_{Ca} was generated by 30-ms test pulses to 0 mV, given at 10-s intervals. A, time course of I_{Ca} measured as charge area (Q_{Ca}, ordinates), taking away the initial I_{Na} component, as shown in B. CGP and cadmium (Cd^{2+}) were perifused as indicated by the bottom horizontal bars. B, original I_{Na} and I_{Ca} traces taken from a sample cell, before (control) and 1 min after CGP perifusion. C, a representative experiment on the time course of Q_{Ca} before, during, and after 10 µM CGP. D, sample traces taken from C, after 1-min perifusion with 10 µM CGP. E and F, averaged results showing the blockade of Q_{Ca} by CGP in the number of cells shown in parentheses. **, $p < 0.01$ with respect to control.

nels are unlikely involved, CGP still afforded significant protection.

An additional previous finding from our laboratory involves the mNCX rather than Ca^{2+} channels, in the effects of CGP on Ca^{2+} handling by bovine chromaffin cells. In measurements of $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ (with mitochondria-targeted aequorin) we found a parallelism between the transients of $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ on challenging chromaffin cells with high K^+. At 20 µM, CGP slowed down (but did not stop) the mitochondrial Ca^{2+} efflux into the cytosol; however, the amplitude of the $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ transients elicited by K^+ were unchanged, indicating that 20 µM CGP did not block Ca^{2+} entry through Ca^{2+} channels. As commented above, this CGP concentration caused more than 90% cytoprotection.

In the light of the widely accepted hypothesis that mitochondrial Ca^{2+} overload leads to cell death (Duchen, 2000), it seems paradoxical that blockade of the mNCX by CGP produces cytoprotection. In fact, mutation of the mitochondrial protein PINK1, encoded by a gene believed to be involved in Parkinson's disease, caused a decrease of the mNCX activity; this results in mitochondrial Ca^{2+} overload that sensitizes the mitochondria to opening of the permeability transition pore, impairing respiration and rendering neurons vulnerable to cell death (Gandhi et al., 2009). However, the opposite view has also been suggested,

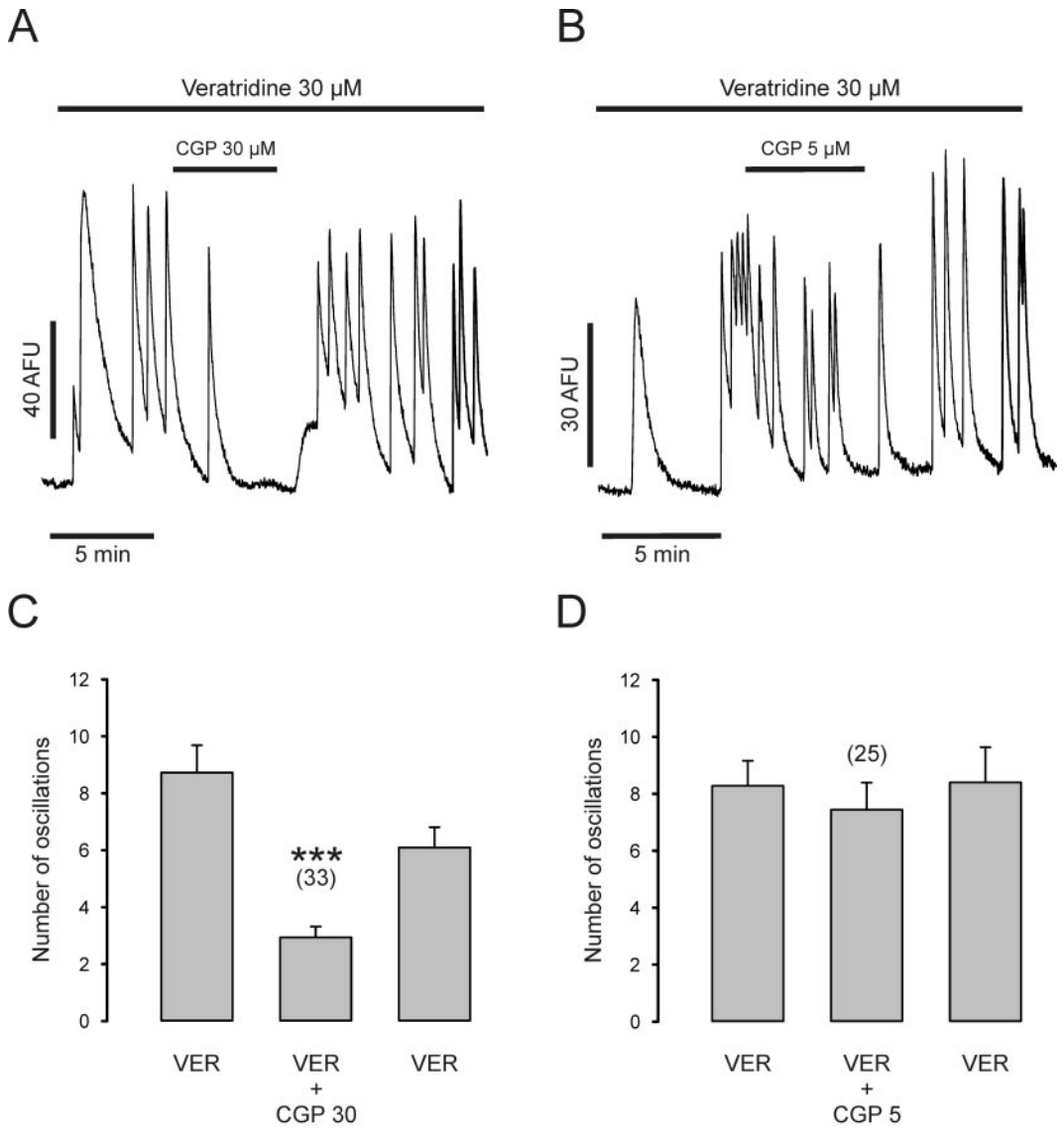

Fig. 8. CGP37157 (CGP) caused a reversible blockade of the veratridine (Ver)-evoked oscillations of the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$). A and C, single cells loaded with Fluo-4AM were initially perifused with Tyrode's solution. Once a stable baseline fluorescence was established, Ver was continuously and focally applied with a pipette onto the target cell, as indicated by the top long horizontal bars in A and C. CGP was perifused together with Ver during the period indicated by the top short horizontal bar. B and D, pooled results of the number of cells shown in parentheses. The number of oscillations induced by Ver was counted for 5 min before, in the presence of, and after application of CGP (ordinate). Data are means ± S.E. **, $p < 0.01$, ***, $p < 0.001$, compared with Ver. AFU, arbitrary fluorescence units.

i.e., by removing excess Ca^{2+} from the cytosol, mitochondria could display a neuroprotective activity (Nicholls, 1985; White and Reynolds, 1995). This fits well in the hypothesis that, beyond a critical physiological range, the $[Ca^{2+}]_c$ elevations become neurotoxic (Koike et al., 1989). In this context, a mild inhibition of the mNCX by CGP will slow down the delivery of Ca^{2+} into the cytosol to maintain the $[Ca^{2+}]_c$ within a physiological range.

This may be particularly relevant during veratridine treatment because enhanced $[Na^+]_c$ will further activate the mNCX (Duchen, 2000). Under these experimental conditions, CGP could contribute to mitigate large $[Ca^{2+}]_c$ transients, an effect that could be linked to its cytoprotective effects. Blockade of the mNCX by CGP could also explain the suppression of veratridine-elicited $[Ca^{2+}]_c$ oscillations by 30 μM compound; this concentration also blocks the spontaneous $[Ca^{2+}]_c$ oscillations of mouse fetal spinal cord ventral neurons (Fabbro et al., 2007). The high-capacity mitochondrial Ca^{2+} uptake pathway provides a mechanism that couples energy demand to increased ATP production through the Ca-dependent up-regulation of mitochondrial enzyme activity (Jouaville et al., 1999). Thus mitochondrial Ca^{2+} cycling may link the coupling between neuronal activity and energy production. In this manner, by slowing down Ca^{2+} efflux, the $[Ca^{2+}]_m$ will remain elevated longer to enhance dehydrogenases activity and ATP synthesis; this could account for the cardiac positive inotropic effects of CGP (Cox and Matlib, 1993), for its ability to enhance histamine-elicited ATP production in HeLa cells (Hernández-SanMiguel et al., 2006), and for the preservation of mitochondrial function and cell viability in the presence of veratridine (this work).

In conclusion, CGP is promoting chromaffin cell survival against the veratridine lethal effects by preventing 1) ROS overproduction, 2) mitochondrial depolarization, and 3) cyto-

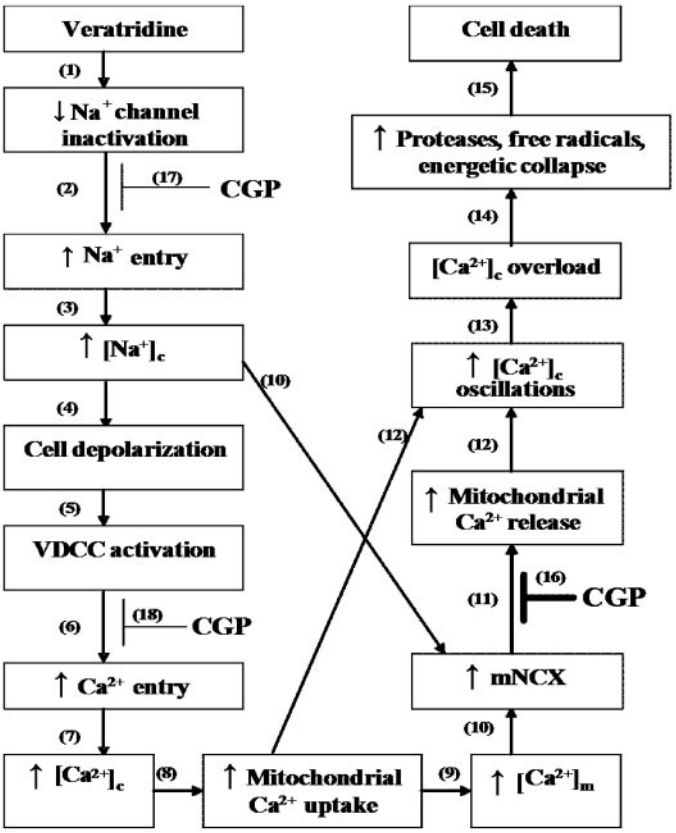

Fig. 9. A proposed sequence for the alteration of Na^+ and Ca^{2+} homeostatic mechanisms, leading to death of bovine chromaffin cells incubated with veratridine, and the mechanism of cytoprotection afforded by CGP37157 (CGP). (1) Veratridine delays the inactivation of Na^+ channels (Ota et al., 1973); (2) enhanced Na^+ entry through noninactivating Na^+ channels (Kilpatrick et al., 1982) augments $[Na^+]_c$ (3) and causes cell depolarization (4) (Lopez et al., 1995); this leads to cell opening of voltage-dependent Ca^{2+} channels (VDCCs) (5), enhanced Ca^{2+} entry (6), and $[Ca^{2+}]_c$ (7) (Maroto et al., 1994, 1996; Lopez et al., 1995); mitochondria see these enhanced $[Ca^{2+}]_c$ values and their uniporter augments mitochondrial Ca^{2+} uptake (8) (Montero et al., 2000) and the $[Ca^{2+}]_m$ (9) (Montero et al., 2000); augmented $[Na^+]_c$ and $[Ca^{2+}]_m$ increases the activity of the mNCX (10) (Uceda et al., 1995), leading to a more rapid Ca^{2+} release into the cytosol (11) that contributes to generate larger and longer $[Ca^{2+}]_c$ oscillations (Maroto et al., 1994, 1996; Novalbos et al., 1999) and Fig. 3 in this study. This will finally lead to cytosol Ca^{2+} overload (13), activation of proteases, protein misfolding, oxidative stress, energetic collapse (14), and cell death (15) (Sedlak and Snyder, 2006). Inhibition of the mNCX by compound CGP (16) will slow down the rate of mitochondrial Ca^{2+} release, thereby mitigating the brisk $[Ca^{2+}]_c$ changes and thus causing cytoprotection against veratridine toxicity. Partial blockade of I_{Na} (17) and I_{Ca} (18) could also contribute to the cytoprotective effects of CGP.

chrome *c* release from mitochondria. In this context, it will be interesting to design and synthesize novel benzothiazepine derivatives with more potency and selectivity to block the mNCX, without touching the Ca^{2+} channels or other structures contributing to cell Ca^{2+} homeostatic mechanisms. We are presently approaching this task, trying to synthesize a benzothiazepine compound that regulates Ca^{2+} fluxes through the mNCX; this effect may preserve the energetic capabilities of mitochondria, thus preventing cell death.

Acknowledgments

We thank Fundación Teófilo Hernando, Universidad Autónoma de Madrid, Spain, for continued support.

References

Cano-Abad MF, López MG, Hernández-Guijo JM, Zapater P, Gandía L, Sánchez-García P, and García AG (1998) Effects of the neuroprotectant lubeluzole on the cytotoxic actions of veratridine, barium, ouabain and 6-hydroxydopamine in chromaffin cells. *Br J Pharmacol* **124:**1187–1196.

Cano-Abad MF, Villarroya M, García AG, Gabilan NH, and López MG (2001) Calcium entry through L-type calcium channels causes mitochondrial disruption and chromaffin cell death. *J Biol Chem* **276:**39695–39704.

Cox DA and Matlib MA (1993) Modulation of intramitochondrial free Ca^{2+} concentration by antagonists of Na(+)-Ca^{2+} exchange. *Trends Pharmacol Sci* **14:**408–413.

Duchen MR (2000) Mitochondria and calcium: from cell signalling to cell death. *J Physiol* **529:**57–68.

Egea J, Rosa AO, Cuadrado A, García AG, and López MG (2007) Nicotinic receptor activation by epibatidine induces heme oxygenase-1 and protects chromaffin cells against oxidative stress. *J Neurochem* **102:**1842–1852.

Fabbro A, Pastore B, Nistri A, and Ballerini L (2007) Activity-independent intracellular Ca^{2+} oscillations are spontaneously generated by ventral spinal neurons during development in vitro. *Cell Calcium* **41:**317–329.

Gallo V, Kingsbury A, Balázs R, and Jørgensen OS (1987) The role of depolarization in the survival and differentiation of cerebellar granule cells in culture. *J Neurosci* **7:**2203–2213.

Gandhi S, Wood-Kaczmar A, Yao Z, Plun-Favreau H, Deas E, Klupsch K, Downward J, Latchman DS, Tabrizi SJ, Wood NW, et al. (2009) PINK1-associated Parkinson's disease is caused by neuronal vulnerability to calcium-induced cell death. *Mol Cell* **33:**627–638.

García AG, García-De-Diego AM, Gandía L, Borges R, and García-Sancho J (2006) Calcium signaling and exocytosis in adrenal chromaffin cells. *Physiol Rev* **86:**1093–1131.

Ha HC, Woster PM, Yager JD, and Casero RA Jr (1997) The role of polyamine catabolism in polyamine analogue-induced programmed cell death. *Proc Natl Acad Sci U S A* **94:**11557–11562.

Hamill OP, Marty A, Neher E, Sakmann B, and Sigworth FJ (1981) Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflugers Arch* **391:**85–100.

Hernández-SanMiguel E, Vay L, Santo-Domingo J, Lobatón CD, Moreno A, Montero M, and Alvarez J (2006) The mitochondrial Na+/Ca^{2+} exchanger plays a key role in the control of cytosolic Ca^{2+} oscillations. *Cell Calcium* **40:**53–61.

Jordán J, Galindo MF, Calvo S, González-García C, and Ceña V (2000) Veratridine induces apoptotic death in bovine chromaffin cells through superoxide production. *Br J Pharmacol* **130:**1496–1504.

Jouaville LS, Pinton P, Bastianutto C, Rutter GA, and Rizzuto R (1999) Regulation of mitochondrial ATP synthesis by calcium: evidence for a long-term metabolic priming. *Proc Natl Acad Sci U S A* **96:**13807–13812.

Kilpatrick DL, Slepetis RJ, Corcoran JJ, and Kirshner N (1982) Calcium uptake and catecholamine secretion by cultured bovine adrenal medulla cells. *J Neurochem* **38:**427–435.

Kluck RM, Bossy-Wetzel E, Green DR, and Newmeyer DD (1997) The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. *Science* **275:**1132–1136.

Koh JY and Cotman CW (1992) Programmed cell death: its possible contribution to neurotoxicity mediated by calcium channel antagonists. *Brain Res* **587:**233–240.

Koh JY and Choi DW (1987) Quantitative determination of glutamate mediated cortical neuronal injury in cell culture by lactate dehydrogenase efflux assay. *J Neurosci Methods* **20:**83–90.

Koike T, Martin DP, and Johnson EM Jr (1989) Role of Ca^{2+} channels in the ability of membrane depolarization to prevent neuronal death induced by trophic-factor deprivation: evidence that levels of internal Ca^{2+} determine nerve growth factor dependence of sympathetic ganglion cells. *Proc Natl Acad Sci U S A* **86:**6421–6425.

Korn SJ and Horn R (1989) Influence of sodium-calcium exchange on calcium current rundown and the duration of calcium-dependent chloride currents in pituitary cells, studied with whole cell and perforated patch recording. *J Gen Physiol* **94:**789–812.

Livett BG (1984) Adrenal medullary chromaffin cells in vitro. *Physiol Rev* **64:**1103–1161.

Lopez MG, Artalejo AR, Garcia AG, Neher E, and Garcia-Sancho J (1995) Veratridine-induced oscillations of cytosolic calcium and membrane potential in bovine chromaffin cells. *J Physiol* **482:**15–27.

Maroto R, De la Fuente MT, Artalejo AR, Abad F, López MG, García-Sancho J, and García AG (1994) Effects of Ca^{2+} channel antagonists on chromaffin cell death and cytosolic Ca^{2+} oscillations induced by veratridine. *Eur J Pharmacol* **270:**331–339.

Maroto R, de la Fuente MT, Zapater P, Abad F, Esquerro E, and García AG (1996) Effects of omega-conotoxin MVIIC on veratridine-induced cytotoxicity and cytosolic $Ca^{(2+)}$ oscillations. *Brain Res* **714:**209–214.

Martikainen P, Kyprianou N, Tucker RW, and Isaacs JT (1991) Programmed death of nonproliferating androgen-independent prostatic cancer cells. *Cancer Res* **51:**4693–4700.

Mattson MP (2007) Calcium and neurodegeneration. *Aging Cell* **6:**337–350.

Montero M, Alonso MT, Carnicero E, Cuchillo-Ibáñez I, Albillos A, García AG, García-Sancho J, and Alvarez J (2000) Chromaffin-cell stimulation triggers fast millimolar mitochondrial Ca^{2+} transients that modulate secretion. *Nat Cell Biol* **2:**57–61.

Moro MA, López MG, Gandía L, Michelena P, and García AG (1990) Separation and culture of living adrenaline- and noradrenaline-containing cells from bovine adrenal medullae. *Anal Biochem* **185:**243–248.

Narita M, Shimizu S, Ito T, Chittenden T, Lutz RJ, Matsuda H, and Tsujimoto Y (1998) Bax interacts with the permeability transition pore to induce permeability transition and cytochrome c release in isolated mitochondria. *Proc Natl Acad Sci U S A* **95:**14681–14686.

Nicholls DG (1985) A role for the mitochondrion in the protection of cells against calcium overload? *Prog Brain Res* **63:**97–106.
Novalbos J, Abad-Santos F, Zapater P, Cano-Abad MF, Moradiellos J, Sánchez-García P, and García AG (1999) Effects of dotarizine and flunarizine on chromaffin cell viability and cytosolic Ca^{2+}. *Eur J Pharmacol* **366:**309–317.
Orozco C, García-de-Diego AM, Arias E, Hernández-Guijo JM, García AG, Villarroya M, and López MG (2006) Depolarization preconditioning produces cytoprotection against veratridine-induced chromaffin cell death. *Eur J Pharmacol* **553:**28–38.
Ota M, Narahashi T, and Keeler RF (1973) Effects of veratrum alkaloids on membrane potential and conductance of squid and crayfish giant axons. *J Pharmacol Exp Ther* **184:**143–154.
Pauwels PJ, Van Assouw HP, Leysen JE, and Janssen PA (1989) Ca^{2+}-mediated neuronal death in rat brain neuronal cultures by veratridine: protection by flunarizine. *Mol Pharmacol* **36:**525–531.
Schanne FA, Kane AB, Young EE, and Farber JL (1979) Calcium dependence of toxic cell death: a final common pathway. *Science* **206:**700–702.
Sedlak TW and Snyder SH (2006) Messenger molecules and cell death: therapeutic implications. *JAMA* **295:**81–89.
Smiley ST, Reers M, Mottola-Hartshorn C, Lin M, Chen A, Smith TW, Steele GD Jr, and Chen LB (1991) Intracellular heterogeneity in mitochondrial membrane potentials revealed by a J-aggregate-forming lipophilic cation JC-1. *Proc Natl Acad Sci U S A* **88:**3671–3675.
Uceda G, García AG, Guantes JM, Michelena P, and Montiel C (1995) Effects of Ca^{2+} channel antagonist subtypes on mitochondrial Ca^{2+} transport. *Eur J Pharmacol* **289:**73–80.
Vághy PL, Johnson JD, Matlib MA, Wang T, and Schwartz A (1982) Selective inhibition of Na+-induced Ca^{2+} release from heart mitochondria by diltiazem and certain other Ca^{2+} antagonist drugs. *J Biol Chem* **257:**6000–6002.
White RJ and Reynolds IJ (1995) Mitochondria and Na+/Ca^{2+} exchange buffer glutamate-induced calcium loads in cultured cortical neurons. *J Neurosci* **15:** 1318–1328.

Address correspondence to: Dr. Antonio G. García, Instituto Teófilo Hernando, Facultad de Medicina, Universidad Autónoma de Madrid, C/ Arzobispo Morcillo, 4, 28029-Madrid, Spain. E-mail: agg@uam.es