Mitochondrial Na+/Ca2+-Exchanger Blocker CGP37157 Protects against Chromaffin Cell Death Elicited by Veratridine

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Received April 6, 2009; accepted June 8, 2009

ABSTRACT

Mitochondrial calcium (Ca2+) dyshomeostasis constitutes a critical step in the metabolic crossroads leading to cell death. Therefore, we have studied here whether 7-chloro-5-(2-chloro-phenyl)-1,5-dihydro-4,1-benzothiazepin-2(3H)-one (CGP37157; CGP), a blocker of the mitochondrial Na+/Ca2+-exchanger (mNCX), protects against veratridine-elicited chromaffin cell death, a model suitable to study cell death associated with Ca2+ 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 EC50 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 Ca2+ currents by 50 to 60% and the veratridine-elicited oscillations of cytosolic Ca2+. 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 Ca2+ concentrations ([Ca2+]c), leads to excitotoxic neuronal death (Schanne et al., 1979) and to neurodegeneration (Mattson, 2007). However, Ca2+ may behave as both a cell survival supporter and a cell death inducer. For instance, cell depolarization and subsequent Ca2+ 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 [Ca2+]c by ionophores induces apoptosis (Martikainen et al., 1991). The opposite is also true, i.e., Ca2+ antagonists that reduce [Ca2+]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 [Ca2+]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 Ca2+ accumulation into mitochondria could play a neuropro-...
ective 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 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-diphenylylformazan, 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 Cientifica, Spain). Dulbecco’s modified Eagle’s medium (DMEM), fetal calf serum, and penicillin/streptomycin were purchased from Invitrogen (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 \(\mu\)g/ml streptomycin. Cells were preplated for 30 min and proliferation inhibitors (10 \(\mu\)M cytosine arabinoside, 10 \(\mu\)M fluoride-oxycyridine, and 10 \(\mu\)M 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 \(5 \times 10^5\) cells/well on 24-well dishes. Cultures were maintained in an incubator at 37°C in a water-saturated atmosphere with 5% CO\(_2\). 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 \(5 \times 10^6\) cells/ml with cold buffer-1 (30 mM Tris-HCl, pH 7.5, 0.3 M sucrose, 10 \(\mu\)M aprotinin A, 10 \(\mu\)M pepstatin, 10 \(\mu\)M leupeptin, 1 mM phenylmethylsulfonyl fluoride). The cell suspension was homogenized 5 to 10 times in ice, and subsequently centrifuged at 10,000g 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 \(\mu\)M aprotinin A, 10 \(\mu\)M pepstatin, 10 \(\mu\)M leupeptin, 1 mM phenylmethylsulfonyl fluoride); the suspension was sonicated three times on ice, centrifuged at 10,000g for 30 min at 4°C, and the supernatant was the mitochondrial fraction. Finally, we added 100 \(\mu\)l 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. Avidin-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 (\(I_{\text{Na}}\)) 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 M\(\Omega\)) 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 (\(I_{\text{Ca}}\)) 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 \(\mu\)g/ml). Amphotericin B was dissolved in DMSO and stored at \(-20^\circ\text{C}\) in stock aliquots of 50 mg/ml. Fresh pipette solution was prepared every 2 h. Recording started when the access resistance decreased below 25 M\(\Omega\), 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 M\(\Omega\) were discarded.

Bovine adrenal chromaffin cells were placed on an experimental chamber mounted on the stage of a Nikon eclipse T2000 or a Nikon 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 \(\mu\)M TTX and no CaCl\(_2\) when \(I_{\text{Na}}\) 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 perfusion system was placed within 100 \(\mu\)m 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\(^{2+}\)] Oscillations.** Cells were plated on 25-mm-diameter poly(t-lysine)-coated glass coverslips at a density of \(2 \times 10^5\) cells and maintained in an incubator for 3 days at 37°C in a water-saturated atmosphere with 5% CO\(_2\). After this, the cells were loaded with 3 \(\mu\)M 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 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 \(p < 0.05\).

**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
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 EC₅₀ 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 EC₅₀ 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 Ca²⁺ overload. Thus, it was of interest to test whether CGP had cytoprotective effects on other cell toxicity models. We had previously developed another model of chromaffin cell death through Ca²⁺ overloading by use of the L-type voltage-dependent Ca²⁺ channel activator FPL64176 (FPL), a mild K⁺ depolarization (30 mM), and high extracellular [Ca²⁺]. In this model, cell death was elicited by en-
enhanced 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 \(\mu\)M, 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 \(\mu\)M oligomycin that inhibits complex V, with 30 \(\mu\)M 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
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$_2$DCFDA 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 (ΔΨ$_m$) 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 ΔΨ$_m$ 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 no 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 perfusion 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-depoloarizing 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 perfusion 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...
CGP Caused a Reversible Blockade of the [Ca^{2+}]_c Oscillations Elicited by Veratridine. Fluo-4AM-loaded single cells were focally perfused 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 perfused 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. 8D). 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 Ca2+ 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 Ca2+ channels (Garcia et al., 2006), increased Ca2+ 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 \( P/Q \) Ca2+ channels with flunarizine or a cocktail of \( \omega \)-toxins and dihydropyridines, protect against Na+ and Ca2+ 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 Ca2+ 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 Ca2+ channel subtypes with flunarizine or combined nimodipine and \( \omega \)-conotoxin MVIIIC 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 Ca2+ chan-
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,
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 \(\mu\)M 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-

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**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 perfused 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 perfused 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.
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 CGP7157 (CGP). (1) Veratridine delays the inactivation of Na\(^+\) channels (Ota et al., 1973); (2) enhanced Na\(^+\) entry through nonactivating Na\(^+\) channels (Kilpatrick et al., 1982) augments [Na\(^+\)], (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\(^{2+}\)]\(_{c}\) values and their uniporter augments mitochondrial Ca\(^{2+}\) uptake (8) (Montero et al., 2000) and the [Ca\(^{2+}\)]\(_{i}\) (9) (Montero et al., 2000); augmented [Na\(^+\)] and [Ca\(^{2+}\)]\(_{i}\) increases the activity of the mNCX (10) (Ueda et al., 1995), leading to a more rapid Ca\(^{2+}\) release into the cytoplasm (11) that contributes to generate larger and longer [Ca\(^{2+}\)]\(_{c}\) oscillations (Maroto et al., 1994, 1996; Lopez et al., 1995); mitochondria see these enhanced [Ca\(^{2+}\)]\(_{i}\), values and their uniorbiter augment mitochondrial Ca\(^{2+}\) uptake (8) (Montero et al., 2000) and the [Ca\(^{2+}\)]\(_{i}\) (9) (Montero et al., 2000); augmented [Na\(^+\)] and [Ca\(^{2+}\)]\(_{i}\) increases the activity of the mNCX (10) (Ueda et al., 1995), leading to a more rapid Ca\(^{2+}\) release into the cytoplasm (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, energy 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\(^{2+}\)]\(_{i}\) changes and thus causing cytoprotection against veratridine toxicity. Partial blockade of I\(_{\text{Na}^{+}}\) (17) and I\(_{\text{Ca}^{2+}}\) (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


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