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# Veratridine induces apoptotic death in bovine chromaffin cells through superoxide production

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**1** The molecular mechanisms involved in veratridine-induced chromaffin cell death have been explored.

**2** We have found that exposure to veratridine (30 $\mu$M, 1 h) produces a delayed cellular death that reaches 55% of the cells 24 h after veratridine exposure. This death has the features of apoptosis as DNA fragmentation can be observed.

**3** Calcium ions play an important role in veratridine-induced chromaffin cell death because the cell permeant $Ca^{2+}$ chelator BAPTA-AM and extracellular $Ca^{2+}$ removal completely prevented veratridine-induced toxicity.

**4** Following veratridine treatment, there is a decrease in mitochondrial function and an increase in superoxide anion production. Veratridine-induced increase in superoxide production was blocked by tetrodotoxin (TTX; 10 $\mu$M), extracellular $Ca^{2+}$ removal and the mitochondrial permeability transition pore blocker cyclosporine A (10 $\mu$M).

**5** Veratridine-induced death was prevented by different antioxidant treatments including catalase (100 IU $ml^{-1}$), N-acetyl cysteine (100 $\mu$M), allopurinol (100 $\mu$M) or vitamin E (50 $\mu$M).

**6** Veratridine-induced DNA fragmentation was prevented by TTX (10 $\mu$M).

**7** Veratridine produced a time-dependent increase in caspase activity that was prevented by $Ca^{2+}$ removal and TTX (10 $\mu$M). In addition, calpain and caspases inhibitors partially prevented veratridine-induced death.

**8** These results indicate that chromaffin cells share with neurons the molecular machinery involved in apoptotic death and might be considered a good model to study neuronal death during neurodegeneration.

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**Keywords:** Chromaffin cells; apoptosis; veratridine; free radicals; mitochondria; caspases

**Abbreviations:** $\Delta\Psi_m$, mitochondrial potential; AC-YVAD-CMK, Acetyl-tyr-val-ala-asp-chloromethylketone; AC-ZVAD-FMK, benzyloxycarbonyl-val-ala-asp-(O-methyl)-fluormethylketone; D-AP5, D(−)-2-amino-5-phosphono pentanoic acid; BAPTA-AM, 1,2-bis(2-aminophenoxy)-ethane-N,N,N′,N′-tetra-acetic acid tetrakis (acetoximethyl ester); CA, catecholamines; $[Ca^{2+}]_i$, intracellular free calcium concentration; CsA, cyclosporin A; DMSO, dimethyl sulphoxide; Het, hydroethidine; LDH, lactate dehydrogenase; MnTABP, Mn(III)tetrakis (4-benzoic acid) porphyrin chloride; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide); PBS, phosphate buffered saline; PT, permeability transition; ROS, reactive oxygen species; TTX, tetrodotoxin

## Introduction

Bovine chromaffin cells share many features in common with neurons. Some of these similarities include embryological origin (Le Dourain *et al*., 1981; Le Dourain, 1986) and the components of secretory machinery that allow them to secrete catecholamines (CA) (Roth & Burgoyne, 1994; Burgoyne & Williams, 1997). These common characteristics, together with the fact that chromaffin cells are easier to isolate and culture than neurons, have established chromaffin cells as a good model to study different aspects of cellular neurobiology including neurotransmitter secretion (Chow *et al*., 1994). However, one interesting aspect of chromaffin cell biology that has not been explored yet is the study of the mechanisms activated in these cells during death process induced by different noxious stimuli. Activation of common mechanisms in neurons and chromaffin cells during the death process might allow the use of chromaffin cells as a plausible model to study the mechanisms activated during neuronal death.

Chromaffin cells secrete CA in response to different stimuli including exposure to nicotinic agonists (Holz & Senter, 1981; Schneider *et al*., 1981) and passive membrane depolarization by increasing concentrations of extracellular $K^+$ (Ishikawa & Kanno, 1978; Baker & Rink, 1975). Following membrane depolarization, voltage-dependent $Ca^{2+}$ channels located in the plasma membrane open (Fenwick *et al*., 1982; Ceña *et al*., 1989) producing an increase in intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) (Kao & Schneider, 1986; Calvo *et al*., 1995). This increase in $[Ca^{2+}]_i$ is responsible for depolarization-induced CA secretion (Cheek *et al*., 1993; Finnegan & Wightman, 1995).

Veratridine is known to depolarize excitable cells by preventing inactivation of voltage-dependent $Na^+$ channels (Catterall & Coppersmith, 1981) keeping them open for long periods of time (Bönisch & Keller, 1983). This depolarizing effect causes an increase in both $Na^+$ and $Ca^{2+}$ influx (Amy & Kirshner, 1982) which induces CA release from chromaffin

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(Ceña *et al*., 1983; Sihra *et al*., 1984; Yokoo *et al*., 1998) and other secretory cells (Schoffelmeer *et al*., 1985).

Veratridine-treated neurons show increases in intracellular $Na^+$ concentration (Takei *et al*., 1989) and $[Ca^{2+}]_i$ (Ashley, 1986) resembling those changes observed during the early stages of ischaemia-induced neuronal death. In addition, veratridine has been shown to produce $Ca^{2+}$-dependent death in rat hipocampal neurons and rat cerebellar granular cells, suggesting that neuronal damage could be prevented by using $Ca^{2+}$ channel blockers (Pauwels *et al*., 1989). Recently, these findings on veratridine-induced toxicity were extended to chromaffin cells (Maroto *et al*., 1994).

To validate chromaffin cells as a model to study neuronal death, it would be necessary to identify the molecular mechanisms activated during chromaffin cell death and compare them with those activated during neuronal death. In this paper, we have explored such mechanisms, using veratridine-induced toxicity as a model of cellular death, and found that this alkaloid produces a decrease in mitochondrial function and a $Ca^{2+}$-dependent increase in superoxide ($O_2^-$) production. Further mechanisms involved in apoptopsis such as protease activation and DNA fragmentation also take place during veratridine-induced neuronal death, supporting the use of chromaffin cells as a good model to study the intracellular machinery activated during neuronal death.

## Methods

### *Chromaffin cell culture*

Bovine chromaffin cells were isolated as previously described (Greenberg & Zinder, 1982). Briefly, glands were washed with a $Ca^{2+}$-free Locke's solution (Locke medium) containing (in mM): NaCl 154, KCl 5.6, $MgCl_2$ 1, HEPES 10, glucose 10, pH 7 to remove remaining erythrocytes. Glands were then incubated with $Ca^{2+}$-free Locke's medium containing 0.2% collagenase (Boehringer-Mannheim, Indianapolis, IN, U.S.A.) and 0.5% bovine serum albumin (Calbiochem, La Jolla, CA, U.S.A.) for 45 ($3 \times 15$) min. Glands were then opened, the medulla separated from the cortex and incubated with collagenase for an additional 30 min. After filtering through a nylon mesh, chromaffin cells were separated from erythrocytes in a Percoll gradient. Cells were plated, at different densities ($0.75 \times 10^6$ cells well $^{-1}$ in 24-multiwell plates, $2 \times 10^6$ cells ml$^{-1}$ in culture flasks or at $2 \times 10^5$ on poly-L-lysine covered coverslips), in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal calf serum, penicillin (100 IU ml$^{-1}$) and streptomycin (50 $\mu$g ml$^{-1}$). Cells were incubated at 37°C under an atmosphere of 5% $CO_2$.

### *Cell viability experiments*

Coverslips containing chromaffin cells were rinsed twice with Krebs HEPES buffer (K-H) with the following ionic composition (in mM): NaCl 140, KCl 5.9, $MgCl_2$ 1.2, HEPES 15, glucose 10, $CaCl_2$ 2.5, pH 7.4, incubated for different times either in K-H or in K-H containing the indicated drugs, and exposed for 1 h to veratridine (30 $\mu$M) at room temperature. Exposure was terminated by washing the cells three times with K-H solution. Cellular death was determined using fluorescein diacetate/propidium iodide double staining procedure (Jordan *et al*., 1997). Twenty-four hours after exposure to veratridine, chromaffin cells were incubated for 45 s at 22–25°C with 15 $\mu$g ml$^{-1}$ fluorescein diacetate (Sigma) and (4.6 $\mu$g ml$^{-1}$) propidium iodide (Molecular Probes, Inc. Eugene, OR, U.S.A.) in PBS (ionic composition in mM: $Na_2HPO_4$ 100, $NaH_2PO_4$ 100, NaCl 140, pH 7.4). The stained cells were examined with a standard epi-illumination fluorescence microscope (Axiophot, Zeiss, Germany). Cells stained with propidium iodide represented dead cells, while cells stained with fluorescein diacetate represented live cells. A blinded observer counted the number of dead and alive cells in five microscopic fields (under 40× magnification) reaching approximately 300–450 cells for each coverslip and the mean was regarded as the representative value for the coverslip. The percentage of surviving cells was determined in three or four coverslips for each condition and normalized to controls examined in parallel. The average per cent survival, relative to control, from at least three separate experiments for each condition is expressed in the text and figures as the mean ± s.e.mean. Statistical significance was determined by Student's *t*-test.

Chromatin state was analysed by staining chromaffin cells with the dye Hoechst 33342 (Molecular Probes, Inc., Eugene, OR, U.S.A.). Cultures were rinsed three times with PBS and then incubated with 1 ng ml$^{-1}$ Hoechst 33342 for 20 min at room temperature. After two rinses with PBS, cell staining was analysed using a fluorescent microscope.

### *Fluorimetric determinations*

All experiments were performed at room temperature on the stage of a Nikon Diaphot inverted microscope equipped with a 75 W Xenon lamp and a Nikon 40×, 1.3 numerical aperture, epifluorescence oil immersion objective. Images were acquired with a CCD camera and analysed using commercial software (Life Sciences Ltd., U.K.).

Superoxide production was monitored with hydroethidine (HEt, Molecular Probes) as previously described (Bindokas *et al*., 1996). Background was subtracted, and fluorescence recorded using an excitation filter of 535 nm and an emission filter of 635 nm (Omega Optical Inc., Brattleboro, VT, U.S.A.). Fluorescent images were recorded every 10 s over a 7 min period. Linear regression of fluorescence data was obtained for each condition and the slope of the best fitting line was taken as an index of $O_2^-$ production.

### *Measurement of mitochondrial function*

Mitochondrial function in chromaffin cell cultures was studied using a modification of the method described by Liu *et al*. (1997). Briefly, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added to the cells at a final concentration of 2.5 mg ml$^{-1}$ and incubated for 1 h to allow the conversion of MTT into purple formazan crystal. Cells were then washed with PBS, and lysed with 0.04 M HCl in isopropanol. Absorbance (595 nm) was quantified using a microplate reader (BioRad, Hercules, CA, U.S.A.). Results were expressed as percentages of the absorbance measured in vehicle treated cells.

### *DNA isolation and analysis*

For detection of DNA fragmentation, $20 \times 10^6$ untreated control cells or cells treated with veratridine (30 $\mu$M, 1 h), paraquat (methyl viologen, 1 mM, 24 h) or $H_2O_2$ (100 $\mu$M, 24 h) were collected, 24 h later. DNA was extracted by a modification of a previously described method (Rokhlin *et al*., 1997). Cells were rinsed twice in PBS and, after centrifugation, the pellet lysed in 200 $\mu$g ml$^{-1}$ proteinase K (Sigma, St. Louis, MO, U.S.A.), 5 mM Tris (pH 7.4) 5 mM EDTA and 0.5%

sodium dodecyl sulphate for 2 h at 50°C. DNA was then extracted with phenol-chloroform-isoamyl alcohol (25:24:1) and precipitated overnight at −80°C with 3 M sodium acetate and 2.5 volumes of ethanol. Finally samples were treated with 3 mg $ml^{-1}$ boiled pancreatic Ribonuclease A (Boehringer Mannheim) for 1 h at 50°C and the DNA loaded onto a 1.5% agarose gel with 0.3 $\mu$g $ml^{-1}$ ethidium bromide and run in TAE buffer (Tris-Acetate 40 mM; EDTA 5 mM; pH 8.0).

### *Assay of caspase enzymatic activity*

Four, 8 and 20 h after treatment with 30 $\mu$M veratridine, bovine chromaffin cells were collected in a buffer with the following composition (in mM): HEPES 25, EDTA 5, EGTA 1, $MgCl_2$ 5, DTT 5, PMSF 1 and 10 $\mu$g $ml^{-1}$ each of pepstatin and leupeptin, pH 7.5. The cellular material was left for 20 min on ice and then was sonicated in ice. The lysate was centrifuged for 20 min at $10{,}000 \times g$ and the supernatant was quickly frozen in a methanol dry ice bath and stored at −80°C. Protein concentration of the lysates was quantified using the BCA Protein Assay (Pierce, IL, U.S.A.). Lysates (30 $\mu$g protein) were incubated at 37°C in a buffer containing 25 mM HEPES (pH 7.5), 10% sucrose, 0.1 CHAPS and 10 mM DTT with the fluorogenic substrate DEVD-AFC (15 $\mu$M in DMSO, Calbiochem System Products) (Stefanis *et al*., 1996). Substrate cleavage emitted a fluorescent signal that was quantified in a fluorometer Perkin-Elmer (luminiscence-spectrophotometer LS50B) (excitation 400 nm, emission 505 nm). Enzymatic activity is expressed as arbitrary fluorescent units (A.F.U.). TTX (10 $\mu$M) was added or $Ca^{2+}$ was removed 30 min before and maintained during veratridine (30 $\mu$M; 1 h) treatment.

### *Reagents*

Acetyl-tyr-val-ala-asp-chloromethylketone (Ac-YVAD-CMK, Bachem, Torrance, CA, U.S.A.), and benzyloxycarbonyl-val-ala-asp-(O-methyl)-fluormethylketone (Z-VAD-FMK, Enzyme System Products, Dublin, CA, U.S.A.) were dissolved in dimethylsulphoxide (DMSO, Sigma). MDL 28,170 (Mation Merrell Dow, Cincinnati, OH, U.S.A.) in 95% ethanol; 1,2-bis(2-aminophenoxy)-ethane-N,N,N′,N′-tetra-acetic acid tetrakis (acetoximethyl ester) (BAPTA-AM) was obtained from Molecular Probes. Catalase, tetrodotoxin (TTX), paraquat and vitamin E were obtained from Sigma. All the other reagents were obtained from commercial sources and were of the maximal available purity.

## Results

### *Veratridine-induced neuronal death requires an increase in intracellular calcium*

Exposure of bovine chromaffin cells to veratridine (30 $\mu$M) for different times increased cellular death, measured 24 h later, that reached about 30% after 30 min exposure and about 55% following 1 h exposure to veratridine (Figure 1). When the alkaloid was present for 24 h, about 90% of the cells were dead.

In chromaffin cells, veratridine (1 h) causes $[Ca^{2+}]_i$ oscillations and certain $Ca^{2+}$ channel blockers can prevent veratridine-induced toxicity (Maroto *et al*., 1994; Lopez *et al*., 1995). In agreement with these reports, either $Na^+$ or $Ca^{2+}$ removal from the extracellular medium completely prevented veratridine-induced cellular death (Figure 2). As expected, the known blockers of voltage dependent $Na^+$ channels TTX (10 $\mu$M) (Hagiwara & Nakajima, 1966; Hille, 1966) or riluzole (Yokoo *et al*., 1998) almost completely prevented veratridine-induced cell death (Figure 2). The next set of experiments was addressed to study whether this increase in $[Ca^{2+}]_i$ played a role in veratridine-induced chromaffin cell death. To overcome the problem of the different voltage-activated $Ca^{2+}$ channels as possible pathways for $Ca^{2+}$ entry into chromaffin cells, we used an intracellular $Ca^{2+}$ chelator to prevent the actions triggered by an increase in $[Ca^{2+}]_i$ levels. BAPTA-acetoxymethyl ester (BAPTA-AM) is a cell membrane permeable chelator that is metabolized to a non-permeable free form by cellular esterases (Adler *et al*., 1991; Tymianski *et al*., 1993). Pre-treatment of chromaffin cells with BAPTA-AM (10 $\mu$M; 1 h) prior to veratridine exposure reduced veratridine-induced neuronal death by more than 80% (Figure 2).

### *Veratridine induces a $Ca^{2+}$-dependent superoxide production*

Increases in $[Ca^{2+}]_i$ might lead to a collapse in mitochondrial potential ($\Delta\Psi_m$) (Ankarcrona *et al*., 1996; Kruman *et al*., 1998). This collapse in $\Delta\Psi_m$ precedes apoptosis and might represent an early signal leading to cellular death (Ellerby *et al*., 1997; Wadja *et al*., 1998). We determined the functional status of chromaffin cell mitochondria by monitoring the ability to reduce MTT and found that 1 h after exposure to veratridine (30 $\mu$M, 1 h) the ability of chromaffin cells to reduce MTT was decreased by about 20% without apparent morphological changes (data not shown).

Mitochondrial potential collapse is considered to be the main source of free radicals such as $O_2^-$ anion in neurons (Keller *et al*., 1998) and reactive oxygen species (ROS) production has been associated with cellular death in several models of neuronal toxicity (Mattson *et al*., 1995a; Seaton *et al*., 1997). We have explored whether ROS were involved in veratridine-induced chromaffin cell death. To address this issue, we monitored $O_2^-$ production by a microfluorimetric assay as stated in Methods. Bovine chromaffin cells in culture had a basal $O_2^-$ production rate of $3.94 \pm 0.71$ A.F.U. $min^{-1}$ ($n = 76$; Figure 3). Veratridine (30 $\mu$M) caused an increase in the rate of $O_2^-$ production (Figure 3) reaching a value of $15.37 \pm 1.62$ A.F.U. $min^{-1}$ ($n = 51$). TTX (10 $\mu$M) completely

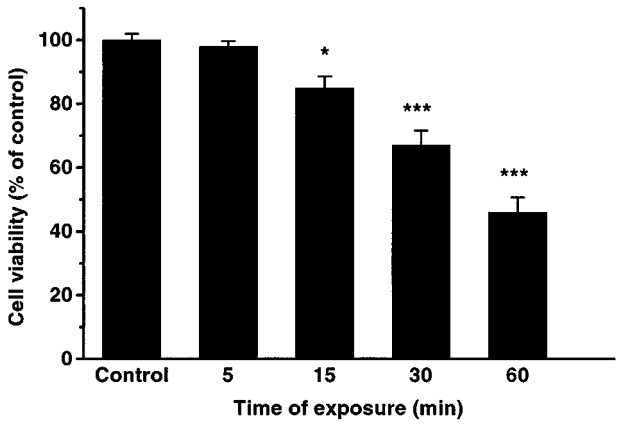


**Figure 1** Delayed death induced by veratridine in bovine chromaffin cells. Bovine chromaffin cells were exposed to veratridine (30 $\mu$M) for the indicated times and cellular viability measured 24 h later. The percentage of dead cells was determined by double staining with fluorescein diacetate and propidium iodide as stated in Methods. Data represent mean ± s.e.mean from at least nine different coverslips obtained from three different cultures. $*P < 0.05$; $***P < 0.001$ as compared to control cells.

blocked the observed increase in $O_2^-$ production ($5.16 \pm 0.64$ A.F.U. $min^{-1}$; $n=12$). Veratridine-induced increase in $O_2^-$ production rate was markedly dependent on $Ca^{2+}$ influx into chromaffin cells as extracellular $Ca^{2+}$ removal completely prevented veratridine-induced increase in $O_2^-$ anion production ($4.14 \pm 0.93$ A.F.U. $min^{-1}$; $n=32$; Figure 3). Supraphysiological increases in $[Ca^{2+}]_i$ levels cause permeability transition (PT) pore opening (Schinder *et al*., 1996; White & Reynolds, 1996) which has been proposed as a non-return point in the execution phase of cell death cascade (Ankarcrona *et al*., 1996). Further experiments were designed to evaluate the role of PT pore in veratridine-induced death. To address this question we treated chromaffin cells for 15 min before and during veratridine exposures with cyclosporin A (10 $\mu$M), a well known PT pore blocker (Schinder *et al*., 1996). As shown in Figure 3, cyclosporine A blocked veratridine-induced rise in $O_2^-$ production rate ($4.63 \pm 0.53$ A.F.U. $min^{-1}$; $n=26$) pointing to mitochondria as the main source of $O_2^-$.

To further explore the role that increased $O_2^-$ production plays in veratridine-induced death we attempted to prevent death by using different drugs that interfere with $O_2^-$ deleterious activity. MnTABP is a cell-permeant manganese porphyrin with superoxide dismutase (SOD) mimetic activity. As shown in Figure 4, pre-treatment for 12 h, to allow access to cell interior, with MnTABP (10 nM) produced a significant protective effect against veratridine (30 $\mu$M)-induced death.

Superoxide anions might induce chromaffin cell death through their metabolite $H_2O_2$ as catalase (100 IU $ml^{-1}$) addition at the same time as veratridine significantly decreased cell death (Figure 4). In addition, treatment of chromaffin cells with non-specific antioxidants like vitamin E (50 $\mu$M) or N-acetyl cysteine (NAC; 100 $\mu$M) also prevented veratridine-induced death (Figure 4). It is also possible that following veratridine, $O_2^-$ might be generated by xanthine oxidase activity, which is induced by increases in $[Ca^{2+}]_i$. In our experiments, allopurinol showed some protective action, suggesting a role for xanthine oxidase in $O_2^-$ generation during veratridine-induced death. However, it could not be excluded that allopurinol might act as a non-specific scavenging agent as it has been proposed (Moorhouse *et al*., 1987). This set of data points to a role for ROS, in general, and, more specifically, for $O_2^-$ as mediators of veratridine-induced death in bovine chromaffin cells.

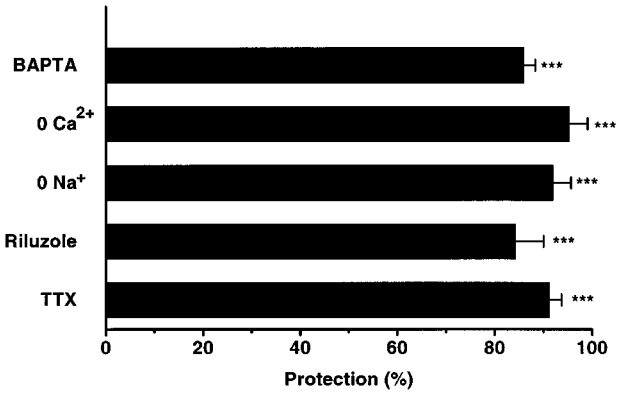


**Figure 2** Effect of different agents on chromaffin cell survival 24 h after exposure to veratridine. Chromaffin cells were treated with veratridine (30 $\mu$M; 1 h) and cell viability determined 24 h later. The experimental protocol was the same as in Figure 1. TTX (10 $\mu$M) and riluzole (10 $\mu$M) were added 5 min before veratridine and maintained during the experiment. BAPTA-AM (10 $\mu$M) was added to the cells 1 h prior to veratridine. Cells were bathed in a $Na^+$-free or $Ca^{2+}$-free medium for 10 min prior to veratridine and kept in that medium for an additional 1 h. Data represent mean $\pm$ s.e.mean of the percentage of living cells after each treatment related to control untreated cells. At least nine coverslips obtained from three different cultures were used for each condition. $^{***}P<0.001$ as compared with control cells.

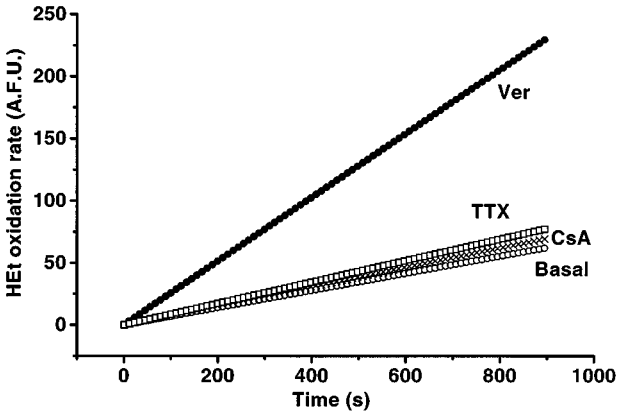


**Figure 3** Rate of veratridine-induced superoxide anion production. Ethidium fluorescence production following veratridine (30 $\mu$M) was determined as indicated in Methods. The slopes of the lines fitting the fluorescence intensity changes (an index of the rate of superoxide production) were individually calculated and averaged. The mean slope values were used to generate a theoretical line using the following equation: $y=ax$. The figure represents the generated lines for superoxide production in untreated cells and in the presence of veratridine alone or with TTX (10 $\mu$M) or cyclosporin A (10 $\mu$M).

## *Veratridine activates caspases in bovine chromaffin cells*

One common downstream mechanism in apoptotic death is caspase activation (Posmantur *et al*., 1997). Exposure of chromaffin cells to veratridine (30 $\mu$M) for 1 h, induced a time-dependent increase in caspase activity that was evident 4 h after the beginning of the treatment and reached a plateau at 8 h (Figure 5A). This veratridine-induced caspase activation was blocked by $Ca^{2+}$ removal or the presence of TTX (10 $\mu$M) (Figure 5B). In addition, we have explored protease activation following veratridine treatment in chromaffin cells by studying

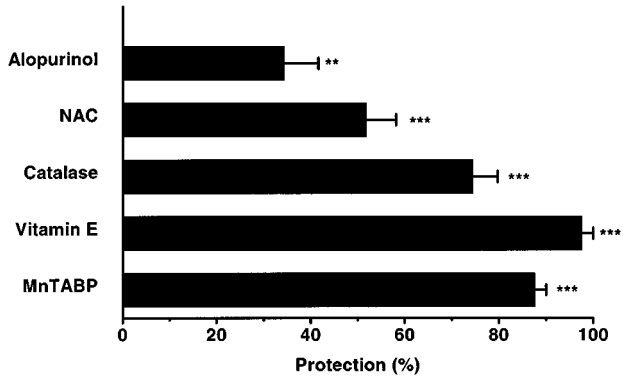


**Figure 4** Effect of different antioxidant drugs on chromaffin cell survival after veratridine treatment. The protocol was the same as in Figure 1. MnTABP (10 nM) and N-acetyl cysteine (NAC; 100 $\mu$M) were present 12 h before veratridine and maintained during the extent of the experiment; vitamin E (50 $\mu$M) and allopurinol (100 $\mu$M) were present 1 h before veratridine and maintained during the experiment. Catalase (100 IU $ml^{-1}$) was present only during veratridine treatment. Data represent mean $\pm$ s.e.mean of the percentage of living cells after each treatment related to control untreated cells. At least nine different dishes obtained from three different cultures were used for each condition. $^{**}P<0.01$; $^{***}P<0.001$ as compared with control cells.

the protective effect of specific inhibitors of calpain (MDL-28170) (Wang, 1990) and caspases (Ac-YVAD-CMK and Ac-ZVAD-FMK) (Nicholson *et al*., 1995; Nath *et al*., 1998) on veratridine-induced death. As can be seen in Figure 6, both MDL-28170 (50 $\mu$M) and Z-VAD-FMK (100 $\mu$M) partially protected against veratridine-induced neuronal death, being Ac-YVAD-CMK (100 $\mu$M) less efficient.

### *Veratridine causes DNA breakdown in chromaffin cells*

One of the hallmarks of apoptotic death is DNA fragmentation (Tobita *et al*., 1995; Chen *et al*., 1997). As can be seen in Figure 7A, untreated chromaffin cells stained with Hoechst 33342 showed no signs of DNA fragmentation. However, 24 h after exposure to veratridine (30 $\mu$M; 1 h) signs of DNA fragmentation were evident in some cells (Figure 7B). In addition, veratridine also induced the appearance of a typical ladder pattern in chromaffin cell DNA suggesting the presence of apoptotic death (Figure 7C). In good agreement with the pharmacology of the observed cellular death, veratridine-induced DNA fragmentation was prevented by TTX (10 $\mu$M). On the other hand, paraquat (1 mM, 24 h) a compound that generates superoxide was also able to induce, by itself, DNA fragmentation (Figure 7C). Similarly, $H_2O_2$ (100 $\mu$M, 24 h) also caused DNA fragmentation (data not shown).

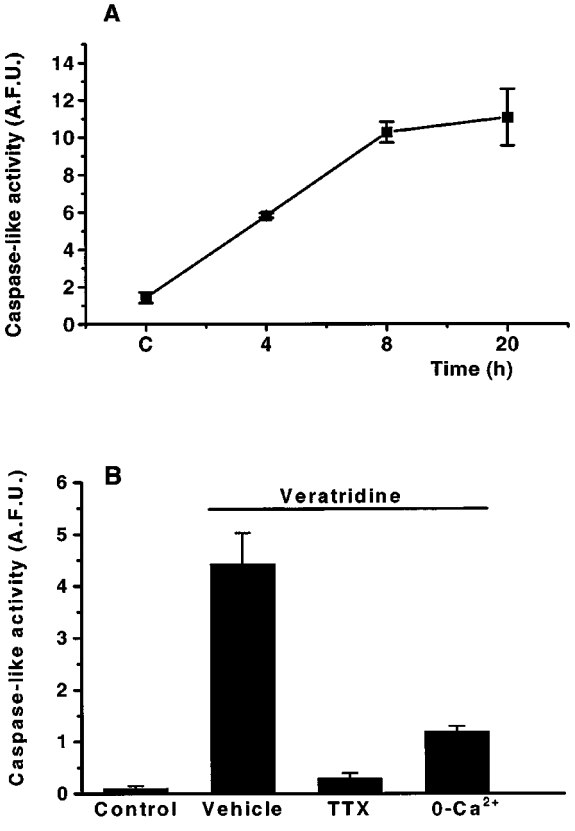


**Figure 5** Veratridine-induced caspase-like activity. (A) Veratridine (30 $\mu$M, 1 h) induced a time-dependent increase in caspase-like activity measured as arbitrary fluorescent units (A.F.U.). The effect was evident at 4 h and reached a plateau at 8 h. Data represent mean$\pm$s.e.mean of three experiments carried out in duplicate. (B) Effect of TTX (10 $\mu$M) addition or extracellular $Ca^{2+}$ removal on veratridine (30 $\mu$M, 1 h)-induced caspase-like activity measured 8 h after veratridine addition. TTX was added or $Ca^{2+}$ was removed 30 min before and maintained during veratridine exposure. Data represent mean$\pm$s.e.mean of three experiments done in duplicate.

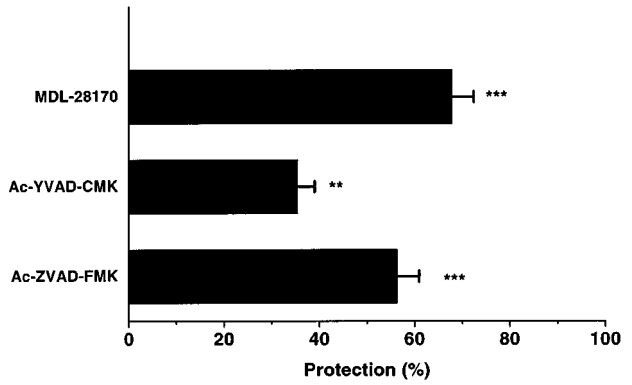


**Figure 6** Effect of protease inhibitors on chromaffin cell death after veratridine treatment. Cultures were incubated with a calpain inhibitor (MDL-28170; 50 $\mu$M) or two caspase inhibitors, (Ac-VAD-CMK; 100 $\mu$M or Z-VAD-FMK; 100 $\mu$M), as indicated in Methods. Twenty-four hours after veratridine (30 $\mu$M; 1 h), cell viability was determined as indicated in the legend to Figure 1. Data represent mean$\pm$s.e.mean of the percentage of living cells after each treatment related to control untreated cells. At least nine different dishes obtained from three different cultures were used for each condition. $^{**}P<0.01$; $^{***}P<0.001$ as compared with control cells.

## Discussion

In this study, we have examined the mechanisms activated by veratridine to induce cell death in bovine chromaffin cells. We have confirmed the described neurotoxic actions of continuous exposure to veratridine for 24 h (Maroto *et al*., 1994; 1996) and found that short (1 h) exposures to veratridine induced a significant degree of delayed chromaffin cell death. In addition, we have gained some insight on the mechanisms activated by veratridine in chromaffin cells. Our data indicate that, following $[Ca^{2+}]_i$ increase, there is a mitochondrial disfunction and a corresponding increase in the rate of $O_2^-$ production. This would lead to caspase activation and DNA fragmentation suggesting that veratridine-induced death in chromaffin cells requires activation of a death program and, likely, apoptotic death.

An increase in $[Ca^{2+}]_i$ has been proposed as a common mechanism mediating neurotoxicity induced by different insults like cerebral hypoxia, epilepsy and neurodegenerative diseases (Choi, 1988). Veratridine depolarizes chromaffin cells (Kilpatrick, 1983; López *et al*., 1995), opens voltage-dependent $Ca^{2+}$ channels and elevates $[Ca^{2+}]_i$ (Maroto *et al*., 1994) suggesting that this increase is a key factor in veratridine-induced toxicity. In good agreement with this view, either $Ca^{2+}$ removal from extracellular medium or pretreatment with the cell-permeant fast $Ca^{2+}$ chelator BAPTA-AM rendered cultured cells resistant to veratridine. Protective actions of $Ca^{2+}$chelators on veratridine-induced chromaffin cell death are similar to those described for other models of neurodegeneration (Tymianski *et al*., 1993). Moreover, the known $Na^+$ channel blockers TTX (Hille, 1966; Almers & Levinson, 1975) and riluzole (Yokoo *et al*., 1998) exert their protective actions against veratridine-induced toxicity acting upstream of $Ca^{2+}$ entry.

Mitochondrion is the most important intracellular organella removing $Ca^{2+}$ from the cytosol. However, supraphysiological increases in $[Ca^{2+}]_i$ levels cause PT pore opening (Schinder *et al*., 1996; White & Reynolds, 1996). Our data show that veratridine impairs mitochondrial function in chromaffin cells as demonstrated by its diminished ability to reduce MTT. It

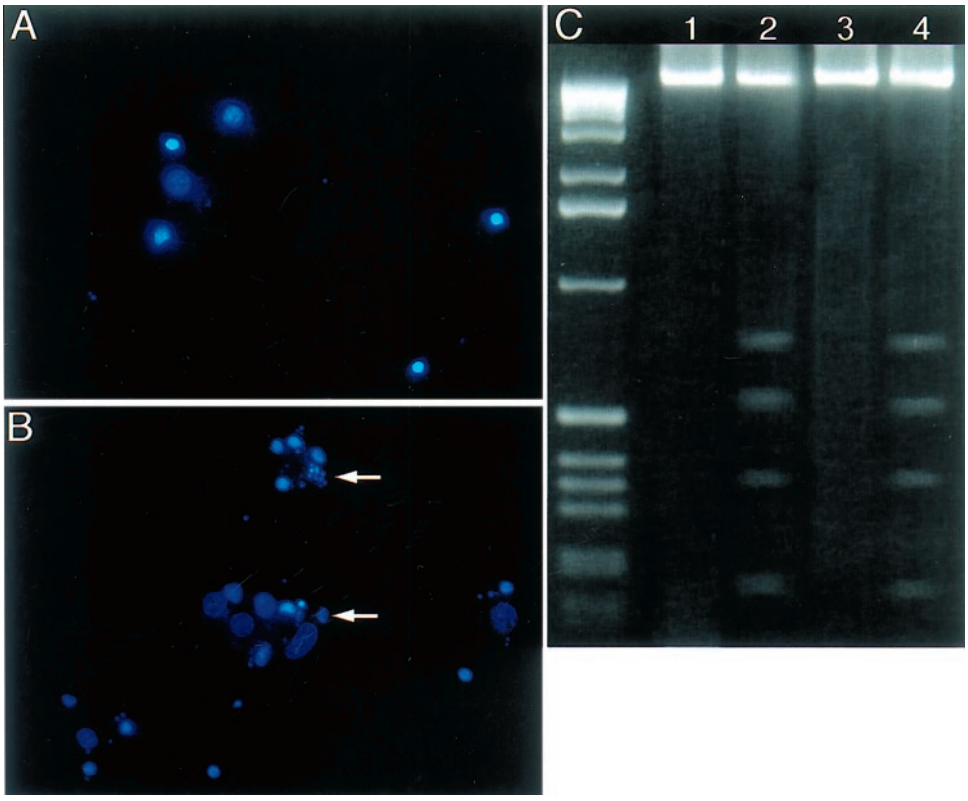


**Figure 7** Veratridine induces DNA fragmentation in chromaffin cells. (A) Untreated chromaffin cells stained with the dye Hoechst 33342 as indicated in Methods. Normal nuclei without signs of chromatin fragmentation can be observed. (B) Cells of the same culture exposed to veratridine (30 $\mu$M; 1 h) and stained with Hoechst 24 h later. Chromatin fragmentation is evident in some cells (arrows). (C) Chromaffin cell DNA was isolated and loaded on an ethidium bromide agarose gel as indicated in Methods. Untreated cells (lane 1) did not show DNA fragmentation. In contrast, chromaffin cells exposed to veratridine (30 $\mu$M; 30 min) showed the typical ladder pattern indicating DNA internucleosomal breakdown (lane 2). This DNA degradation was prevented by TTX (10 $\mu$M) addition (lane 3). In addition, paraquat (1 mM, 24 h) also caused DNA fragmentation (lane 4).

has been proposed that PT pore opening and $\Delta\Psi_m$ collapse might be a non-return point in the execution phase of cell death cascade (Ankarcrona *et al*., 1996) due to the release of some pro-apoptotic factors that, besides other effects, might contribute to caspase activation (Nagata, 1997). This $\Delta\Psi_m$ collapse would also lead to ROS production (Reynolds & Hastings, 1995) that might activate different signalling pathways leading to neuronal death (Greenlund *et al*., 1995; Mattson *et al*., 1995b; Prehn *et al*., 1997). The complete inhibition of veratridine-induced increase in $O_2^-$ production with the known blocker of PT pore, cyclosporin A, suggested a key role for PT pore opening in veratridine-induced death process. The ability of cyclosporin A to block $O_2^-$ production suggests that veratridine induces a sequential cascade including perturbed $[Ca^{2+}]_i$ homeostasis, $\Delta\Psi_m$ collapse, PT pore opening and $O_2^-$ production.

A dynamic equilibrium exists *in vivo* between the oxidative damage potential and the antioxidant defensive capacity at all times (Floyd & Carney, 1992). Our findings suggest that, following veratridine treatment, this equilibrium is displaced toward the oxidative state in bovine chromaffin cells. In neurons, it has been described that following $\Delta\Psi_m$ collapse there is an increase in ROS due to incomplete electron transfer (Prehn *et al*., 1997). Veratridine produced an increase in the rate of $O_2^-$ production in chromaffin cell cultures. In good agreement with the hypothesis of a key role for an increase in $[Ca^{2+}]_i$ during veratridine-induced cellular death, $O_2^-$ production was blocked by $Ca^{2+}$ removal from the extracellular medium or by the addition of the specific $Na^+$ channel blocker TTX. The role of $O_2^-$ in veratridine-induced chromaffin cell death is similar to that observed during excitotoxic death in neurons where glutamate receptor activation has been involved in $O_2^-$ generation (Patel, 1996; Bindokas *et al*., 1996) and also agrees with a report indicating that there is a significant decrease in ROS generation after NMDA receptor blockade with D(−)2-amino-5-phosphonopentanoic acid (d-AP-5) following ischaemia (Pérez *et al*., 1997).

Once produced, $O_2^-$ radical is processed through a pathway that includes $H_2O_2$ generation and hydroxyl radical production, being this last one a highly reactive and toxic free radical (Ayata *et al*., 1997). Evidence favouring activation of this pathway during veratridine-induced toxicity is supported by experiments using the SOD mimetic Mn-TABP that catallytically degrades $O_2^-$. Mn-TABP has been shown to have neuroprotective action in motor neuronal cultures following growth factor withdrawal (Estevez *et al*., 1998) and in cortical neurons after NMDA treatment (Patel *et al*., 1996). Similarly, MnTABP showed a protective action against veratridine-induced toxicity in chromaffin cells. Although our data point to mitochondria as the main source of $O_2^-$ radical, it is also possible that, following veratridine, xanthine oxidase activity, which is activated by increases in $[Ca^{2+}]_i$, and has been related to excitotoxicity (Facchinetti *et al*., 1992), might contribute to $O_2^-$ generation because, under our experimental conditions, allopurinol showed a protective action against veratridine-induced chromaffin cell death, although a non-specific scavenger action of allopurinol can not be excluded (Moorhouse *et al*., 1987). Nevertheless, complete rescue of

veratridine-induced death could not be achieved by either catalase or NAC. The use of suboptimal concentrations of the different drugs is unlikely because higher concentrations rescued the same percentage of cells from veratridine-induced death indicating scavenger saturation. It is more likely that multiple free radical-related pathophysiological pathways are involved in veratridine-induced death.

A final effector in the programmed death pathway is protease activation (Chen *et al*., 1998; Pettmann & Henderson, 1998). This mechanism seems also to be activated in chromaffin cells because: (a) there is a time-dependent increase in caspase activation following veratridine; (b) veratridine-induced caspase activation is prevented by either TTX addition or extracellular $Ca^{2+}$ removal; and (c) inhibitors of both calpain and caspases might partially prevent veratridine toxicity. Caspase activation suggests the presence of a cell death programme and an apoptotic death. This view was further supported by the fact that DNA fragmentation which is related to apoptosis in neural cells (Itano & Nomura, 1995; Jänicke *et al*., 1998) was evident following chromaffin cell exposure to veratridine. Both veratridine-induced caspase activation and DNA fragmentation can be blocked by extracellular $Ca^{2+}$ removal and TTX suggesting that an increase in $[Ca^{2+}]_i$ is one of the early events leading to activation of a programmed death in chromaffin cells. Furthermore, free radicals seem to play an important role in the signalling cascade leading to chromaffin cell death following veratridine because paraquat (an $O_2^-$ generator) and $H_2O_2$, that is produced during $O_2^-$ processing, are able to induce DNA fragmentation in chromaffin cells.

The results presented here indicate that veratridine activates a cell death programme in chromaffin cells by sequential mechanisms that include increase in $[Ca^{2+}]_i$, mitochondrial dysfunction, PT pore opening and $O_2^-$ production leading to caspase activation and DNA degradation. The similarity between these mechanisms activated during chromaffin cell death and those responsible for excitotoxic neuronal death (Pettmann & Henderson, 1998) suggests that chromaffin cells might be a useful model to study the molecular mechanisms activated during neuronal death.

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