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## INTRODUCTION

This study was based on reports that pyridostigmine bromide (PB) either alone or in combination with other compounds can produce nerve damage and neuropsychological impairment (Loewenstein-Lichtenstein *et al.*, 1995; Abou-Donia *et al.*, 1996a,b). These results were surprising in view of the fact that PB has a quaternary chemical structure and would not be expected to penetrate into the brain. PB given twice daily to rats for 4 days caused brain apoptosis, especially in cerebral cortex. These data were in agreement with a report that PB can indeed enter the brain under stressful conditions and inhibit brain cholinesterase (Friedman *et al.*, 1996). Further experiments revealed that PB could also cause apoptosis in primary cultures from rat brain. Studies were then conducted to identify the molecular mechanisms involved in the apoptotic destruction of brain cells by PB. The results identify surface receptors on brain cells which mediate the neurotoxic action of PB. Stimulation of these receptors is followed by activation of enzymes which carry out the programmed cell death.

## BODY

### Characterization of PB-induced Neuronal Apoptosis in Rats:

PB given ip twice daily for 4 days caused a dose-related apoptosis in rat brain (Li *et al.*, 2000). With 1.85 mg/kg, 3 of 4 rats exhibited extensive apoptosis in cortex, striatum and hippocampus. At a dose of 1.5 mg/kg 3 of 4 rats showed apoptotic cell death only in cortex and striatum. At 1 or 0.5 mg/kg apoptosis was seen in 1 of 4 animals and then only in the cortex. These data demonstrate that PB can cause brain damage when given systemically but the effect is not seen in every animal. Also the cerebral cortex is the most susceptible brain area to the neurotoxic action of PB.

### **PB Dosage Indexing by Measurement of Cholinesterase:**

PB is used clinically as a cholinesterase inhibitor in the treatment of myasthenia gravis and as prophylaxis against nerve gas poisoning. Since a major pharmacological effect of PB is cholinesterase inhibition, cholinesterase levels were measured in rats treated with doses employed in the following neurotoxicity studies to ensure that effective concentrations were delivered. Plasma cholinesterase activity was significantly decreased after all doses of PB employed. Despite the quaternary nature of PB, estimates of brain cholinesterase were obtained after single (Fig. 1) or repeated (4 days) dosing with PB, 1.85 mg/kg (Fig. 2). Although no significant effect was seen, the mean was consistently less in treated animals (Fig. 1 & 2) and some treated individuals had low brain levels. Thus it appears that PB enters the brains of some animals and that brain cholinesterase inhibition may reflect the CNS activity of PB in some individuals.

That cholinesterase inhibition is part of the toxic action of PB is suggested by the fact that atropine pretreatment will diminish brain cell apoptosis caused by PB (Li *et al.*, 2000). Thus it appears that acetylcholine accumulation and excessive activation of muscarinic receptors is involved in PB-induced apoptosis of brain cells

### **Characterization of a Delayed Progressive Effect of PB:**

Apoptosis revealed by TUNEL staining occurs at low levels in normal brain and apoptotic cells are removed in a few hours by macrophages and neighboring phagocytic cells (Bursch *et al.*, 1990). To assess the duration of the apoptotic response to PB, rats were sacrificed 5, 10, 20 and 30 days after a 4 day treatment with PB, 1.85 mg/kg. TUNEL staining revealed enhanced apoptosis in the cortex of these animals even after an interval of 30 days following the

last dose of PB (Li *et al.*, 2000). It appears that PB initiates a cell death process that continues for an extended period of time after termination of dosing.

#### **PB-induced Apoptosis in Cultured Cells:**

Apoptotic cell death was observed in cultured rat brain cortical cells after PB exposure confirming that PB can initiate brain cell apoptosis independent of contributing peripheral effects in the animal (Li *et al.*, 2000).

After establishing brain cell destruction by PB *in vivo*, mechanisms of this effect were studied *in vitro*. A concentration-response to PB in cultured brain cells showed that necrotic cell death ((LDH release) occurs when amounts in excess of 250  $\mu\text{M}$  are used (Fig. 4). Below 250  $\mu\text{M}$ , PB caused a concentration-related increase in apoptosis in granule cells (Fig. 5). Thus concentrations of PB from 10 to 250  $\mu\text{M}$  all cause the same type of cell death and presumably initiate the same toxic mechanism in cultured brain cells. Therefore a concentration of 250  $\mu\text{M}$  PB was chosen to ensure a strong and consistent apoptosis in primary cultures of brain cells. Programmed cell death by TUNEL staining was confirmed in cerebellar granule cells using electron microscopy and DNA laddering (Li *et al.*, 2001).

#### **PB-induced Generation of Intracellular Reactive Oxygen Species:**

Although many factors can induced apoptosis, reactive oxygen species (ROS) are common mediators of this process (Jabs, 1999) especially in brain (Sato *et al.*, 1998). The brain has a high oxidative metabolism rate and a low antioxidant capacity (Facchinetti *et al.*, 1998) which predisposes this organ to oxidative damage. It was likely that PB-induced apoptosis of brain cells was mediated by ROS.

To measure ROS generation, cultured cells were loaded with oxidant-sensitive fluorescent dye (2,7-dichlorofluorescein diacetate) (Gunasekar *et al.*, 1995). After washing away

extracellular dye, PB was added and ROS generation determined by measuring increased fluorescence. Figure 6 shows PB induces a rapid, dose-dependent increase in intracellular ROS. Even a low PB concentration (10  $\mu$ M) caused a significant increase in ROS in cerebellar granule cells (Li *et al.*, 2001).

To verify that our method actually reflected oxidative species generation, antioxidants were employed. PBN (phenyl-N-tert-butyl nitron) is a cell permeable antioxidant and catalase (CAT) and superoxide dismutase (SOD) are enzymes which catalyse breakdown of different ROS. In the presence of these antioxidants, PB-induced ROS generation was attenuated (Fig. 7). Clearly, a variety of oxidative species are rapidly generated in cultured brain cells after PB treatment.

To determine which cell surface receptors are activated by PB to initiate ROS generation, specific blockers were used. Atropine, which was effective *in vivo* was also effective *in vitro* (Fig. 8). Furthermore MK-801, a blocker of the glutamatergic NMDA receptor, strongly inhibited PB-induced ROS generation (Fig. 8). It appears that both muscarinic and NMDA surface receptors are involved in PB-induced generation of ROS in brain cells.

So muscarinic and NMDA receptors mediate ROS generation by PB, do they also mediate apoptosis? Figure 9 shows that atropine or MK-801 decreases the apoptotic response to PB in cerebellar granule cells. Thus activation of these two receptors by PB then generates ROS and also mediates programmed cell death. It appears that receptor activation by PB is followed by increased ROS production and subsequently the ROS initiate the apoptotic process. Blockade of either surface receptor or use of antioxidants is able to inhibit PB-induced apoptosis (Fig. 9).

When both MK-801 and atropine were added to granule cell cultures prior to PB, no further protection against enhanced ROS generation (Fig. 8) or apoptosis was seen (data not

shown). This result suggests that these two antagonists affect the same process. It is possible that muscarinic receptor activation leads to glutamate release which then activates NMDA receptors. Thus both receptors may be involved in the response and may operate sequentially to cause the oxidative stress and the apoptosis.

If PB enters the brain and inhibits brain cholinesterase in some individuals, then acetylcholine may accumulate and activate not only muscarinic but nicotinic receptors as well. To determine whether nicotinic receptors can mediate the toxic effects of PB in cerebellar granule cells, hexamethonium was employed to block these receptors. In a concentration of 300  $\mu\text{M}$ , hexamethonium had no significant effect on PB-induced TUNEL staining (N=6), DNA laddering or DCF-fluorescence (N=3). Also the nicotinic agonist tetramethylammonium at 200 or 400  $\mu\text{M}$  did not significantly increase PB-induced TUNEL staining (N=6), DNA laddering or DCF-fluorescence (N=3) in cerebellar granule cells. Any PB-induced acetylcholine accumulation in brains of certain individuals with subsequent activation of nicotinic receptors appears to be harmless to neurons and is not part of the toxic action of PB.

#### **Mechanisms of PB-induced Apoptosis:**

The evidence suggests that two surface receptors are involved in PB-induced apoptosis, the muscarinic and the NMDA glutamate receptor. Mechanisms linking the cell surface effect of PB to its apoptotic action are critical for understanding the mechanism underlying the cytotoxic response.

Some surface receptors mediate apoptosis by pathways independent of mitochondria. The granzyme system mediates apoptosis when cytotoxic lymphocytes (CtL) bind to CtL receptors and introduce the protease granzyme B into the target cell (Israels & Israels, 1999). Granzyme B then triggers procaspase activation and caspase initiates a restricted peptide

cleavage involving cytoskeletal proteins, nuclear membrane, integrins, and cleavage of DNA nuclease from its associated protein inhibitor. Another mitochondrial-independent apoptotic mechanism involves Fas (CD95) receptor and can be activated by tumor necrosis factor (Israels & Israels, 1999). The Fas receptor then activates intracellular proteins which turn on procaspase-8 to begin the limited proteolysis of programmed cell death. Other apoptotic processes do not involve surface receptors but cause cell death by interfering with mitochondrial function, e.g. after ischemia (Fujimura *et al.*, 1999, 2000; Ciolo *et al.*, 2000), flavonoids (Wang *et al.*, 1999) and other cancer preventatives (Suzuki *et al.*, 1999), colchicine (Gorman *et al.*, 1999) and other toxic agents (Zhuang & Cohen, 1998). It remained to be determined whether mitochondria were involved in PB-induced apoptosis.

#### **PB-induced Mitochondrial Dysfunction:**

Release of cytochrome c after loss of mitochondrial membrane potential is critical in some types of programmed cell death. Changes in the outer mitochondrial membrane cause release of intermembrane contents including cytochrome c. In turn cytochrome c binds to Apaf-1 which is followed by activation of procaspase-9 (Israels & Israels, 1999). Caspase-9 activates other caspases including caspase-3 which is a selective protease and causes characteristic apoptotic changes.

Thus to examine the process of PB-induced apoptosis, mitochondrial membrane potential was evaluated after PB addition to cerebellar granule cells. The fluorescent dye rhodamine 123 was used to measure changes in mitochondrial membrane potential. Rhodamine 123 is a lipophilic cation which accumulates in mitochondria in response to the negative membrane potential of these organelles (Johnson *et al.*, 1980, Chen, 1989). The dye binds to mitochondrial

matrix and thus decreases in fluorescence. Mitochondrial depolarization releases dye into the cytoplasm and an increase in rhodamine 123 fluorescence occurs.

Cultured 7-8 day granule cells were loaded with rhodamine 123 (10  $\mu\text{M}$ ) in KR buffer at 37°C for 20 min. Coverslips with granule cells loaded with rhodamine 123 were then moved to a cell chamber (Medical System, Inc., Greenvale, NY) mounted on a heated (37°C) microscope stage after double washing with KR buffer. Changes in fluorescence were monitored using a SLM-8000-TMC spectrofluorometer attached to a Nikon diaphot TMD microscope over a 10 min period after different treatments at wavelengths of 475 nm (excitation) and 525 nm (emission).

Figure 10 shows that 50, 100 and 250  $\mu\text{M}$  PB increase rhodamine 123 fluorescence in cerebellar granule cells indicating that a substantial loss of mitochondrial membrane potential occurs quickly after cells are treated with PB. A high dose of PB which leads to necrosis shows no change in mitochondrial membrane potential (Fig. 10). These results indicate that PB decreases mitochondrial membrane potential within a few minutes in concentrations which cause apoptosis and suggest that mitochondria are indeed involved in the apoptotic action of PB on brain cells.

Further studies show that the PB-induced change in mitochondrial membrane potential can be inhibited using atropine or MK-801 (Fig. 11). Thus blockers which diminish the apoptotic effect of PB also block PB's action on mitochondria suggesting that the two actions are related.

Noted changes in potential suggested that mitochondrial permeability is increased by PB and that cytochrome c levels should be increased in granule cell cytoplasm by this treatment. Accordingly mitochondria-free samples of PB treated cells were assayed for cytochrome c.

Cytochrome c release was measured using western blots. Granule cells were washed with ice-cold PBS and harvested by centrifugation at 1000 rpm for 5 min. Mitochondria-free cytosolic extracts were prepared by suspending the cell pellets in a buffer containing 220 mM mannitol, 68 mM sucrose, 20 mM HEPES, pH 7.4, 50 mM KCl, 5 mM EGTA, 1 mM EDTA, 2 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, and protease inhibitors on ice and homogenized. The homogenates were centrifuged at 15,000 g for 40 min, and the mitochondria-free cytosolic supernatants were frozen at -70°C until further analysis.

The protein content of the mitochondria-free cytosolic supernatant was determined by the Bradford assay (Bio-Rad). The samples containing 25 µg protein were boiled in Laemmli buffer for 5 min and subjected to electrophoresis in 12% SDS-polyacrylamide gel, followed by transfer to a nitrocellulose membrane. After blocking with phosphate-buffered saline containing 5% nonfat dry milk and 0.1% Tween 20, the membrane was exposed to the primary antibody for 3 hr at room temperature on a rotator. Reactions were detected with the fluorescein-linked anti-mouse Ig (second antibody) conjugated to horseradish peroxidase using enhanced chemiluminescence.

Figure 12 shows that increased levels of cytochrome c appear in granule cell cytoplasm within 2 hr after PB treatment. Thus a rapid release of cytochrome c from mitochondria occurs after PB exposure. Furthermore, quite low concentrations of PB down to 10 µM are highly effective in increasing PB-induced cytochrome c release from mitochondria (Fig. 13). Finally, release of cytochrome c from mitochondria by PB is blocked by atropine or MK-801 (Fig. 14) which also block the apoptotic response to PB (Fig. 9). These data show an important toxic action of PB on cultured brain cells and it is likely that release of cytochrome c is a key event in the cell damage caused by PB.

When cytochrome c is released into the cytoplasm, it activates cytoplasmic protein, Apaf-1 (apoptotic protease activating factor-1) (Israels & Israels, 1999). The cytochrome c/Apaf-1 complex then activates caspase-9 which in turn activates other caspases including caspase-3 which is the enzyme which causes the damage to cell structures seen in apoptosis. Microinjection of cytochrome c can induce caspase-dependent cell death in some lines (Li *et al.*, 1997). Thus it was important to determine whether or not PB-induced apoptosis involves caspase-3.

Caspase-3 like protease activity was measured by spectrophotometric assay. After different treatments, granule cells were washed with PBS and suspended in buffer containing 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 10 mM EGTA, and then incubated with 10  $\mu$ M digitonin at 37°C for 10 min. Lysates were centrifuged at 15,000 rpm for 3 min and the supernatants containing 30  $\mu$ g of protein were incubated with 50  $\mu$ M caspase-3 substrate Ac-DEVD-MCA at 37°C for 1 hr. Levels of 7-amino-4-methylcoumarin, which is released by active caspase-3 from Ac-DEVD-MCA was measured by Hitachi F-2000 spectrofluorometer with excitation and emission wavelength of 380 and 460 nm, respectively. Caspase-3 activity is expressed as fluorescent units/min/mg protein.

Figure 15 shows a steady increase in caspase activity over 24 hrs after exposure of granule cells to PB. Both 100 and 250  $\mu$ M caused a substantial increase in caspase-3 activity (Fig. 16). Furthermore treatments which prevented PB-induced apoptosis (PBN, atropine, MK-801) also inhibited the increase in caspase-3 activity caused by PB (Fig. 9 & 17). Finally, an inhibitor of caspase-3 (zVAD) decreased the incidence of apoptotic cell death after PB treatment (Fig. 18) showing a direct relationship between PB-induced cell death and caspase-3 activity. Taken together these data indicate that PB does activate a cysteine protease known to effect

programmed cell death and when this enzyme is blocked, apoptosis induced by PB is much diminished.

#### **PB-induced Activation of Redox-Sensitive Transcription Factors:**

The final series of experiments involved the protein NF $\kappa$ B (nuclear factor-kappa B). This protein is ubiquitous in cell cytoplasm and is activated by inflammation or oxidative stress (Siebenlist *et al.*, 1994; Baldwin, 1996). Upon activation NF $\kappa$ B is separated from its protein inhibitor, I $\kappa$ B, and migrates to the nucleus where it can activate transcription of proteins which protect cells from death or which promote apoptosis, depending on cell type and the stimulus involved.

To determine whether PB can activate NF $\kappa$ B, granule cells were exposed to PB and then NF $\kappa$ B was measured in cell lysates using the electrophoretic mobility shift assay (EMSA). Granule cells were harvested and suspended in 800  $\mu$ l ice-cold hypotonic buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 2 mM MgCl<sub>2</sub>, 0.1 mM EDTA) with protein inhibitors, 2  $\mu$ g/ml aprotinin, 2  $\mu$ g/ml leupeptin, 1.0 mM PMSF, 1.0 mM DTT. After addition of 25  $\mu$ l 10% Nonidet P-40 and incubation for 5 min on ice, the nuclear fractions were collected by centrifugation at 12,000 x g for 5 min at 4°C. The nuclear pellets were dissolved in a hypertonic buffer and gently shaken for 1 hr at 4°C. After centrifugation at 12,000 rpm for 15 min, the supernatants were collected and stored at -70°C, the synthetic oligonucleotide containing NF $\kappa$ B binding sequence (Promega), 5'GATTGAGGGGACTTTCCAGGC-2' was end-labeled with [ $\gamma$ -<sup>32</sup>P]ATP by T4 polynucleotide kinase (Promega). The labeled oligonucleotide (20,000 cpm) was incubated with 10  $\mu$ g nuclear protein in 20  $\mu$ l binding buffer containing 2 mM HEPES (pH 7.5), 50 mM NaCl, 0.5 mM EDTA, 1 mM MgCl<sub>2</sub>, 0.5 mM dithiothreitol (DTT), 2% glycerol, 1  $\mu$ g poly(dI-dC), and 2  $\mu$ g bovine serum albumin. After the binding reaction was carried out at room temperature for

30 min, the DNA protein complex was subjected to electrophoresis on a 6% native polyacrylamide gel at 100 V for 1 hr.

Maximum levels of NF $\kappa$ B were noted 12 hr after PB exposure (Fig. 19). It appears that the activation process is slow since ROS are generated in the first 10 min of PB treatment. However, subsequent mitochondrial damage may further increase ROS levels and contribute to the NF $\kappa$ B activation process.

Figure 20 shows that activation of NF $\kappa$ B by PB is dose related with the maximum effect at 250  $\mu$ M. Significantly, a decrease in NF $\kappa$ B was seen at 500  $\mu$ M PB, a dose which caused less apoptosis suggesting that NF $\kappa$ B activation and apoptosis are related.

If NF $\kappa$ B is activated by ROS, then antioxidants should block the effect. Accordingly we tested the action of PBN, SOD and CAT on NF $\kappa$ B activation. Figure 21 shows that these antioxidants markedly inhibit NF $\kappa$ B activation. These data further link the toxic effect of PB with NF $\kappa$ B activation.

Atropine and MK-801 block both ROS generation and apoptosis produced by PB. If activation of NF $\kappa$ B is related to these actions of PB, then atropine and MK-801 should diminish the activation. Figure 22 shows that these receptor antagonists decrease activation of NF $\kappa$ B, linking muscarinic and NMDA surface receptors to this redox-sensitive transcription factor.

The data show parallel changes in NF $\kappa$ B activation and toxic effects of PB in rat cerebellar granule cells. Since NF $\kappa$ B can increase both antiapoptotic proteins (Bcl-2, Bcl-X<sub>2</sub>) and apoptosis-induced proteins (Bax, Bad), it remains to be determined whether the increases in NF $\kappa$ B by PB are part of a cell destructive or cell protective mechanism.

## **6) Key Research Accomplishments**

- Pyridostigmine causes apoptotic brain cell death in rats.
- The apoptotic process initiated by pyridostigmine continues for at least 30 days after termination of treatment.
- Apoptosis can also be induced by pyridostigmine in cultured rat brain cells indicating that the action is not indirect through the immune system.
- Muscarinic receptors mediate pyridostigmine-induced apoptosis since atropine can inhibit the effect either in rats or in cultured brain cells.
- Pyridostigmine causes a rapid generation of reactive oxygen species in cultured brain cells and antioxidants decrease both the oxidative stress and the apoptosis caused by this drug.
- NMDA type glutamate receptors are also involved in the oxidative stress and brain cell death due to pyridostigmine since NMDA blockers inhibit these effects.
- Molecular mechanisms in the oxidative stress and brain cell death caused by pyridostigmine involve changes in the redox-sensitive transcription factor NF $\kappa$ B, loss of mitochondrial membrane potential, cytochrome c release into the cytoplasm, and activation of the cysteine protease, caspase-3.

## **7) Reportable outcomes:**

### **A. Manuscripts**

1. Li, L, Gunasekar, P.G., Borowitz, J.L. and Isom, G.E. (2000) Muscarinic receptor-mediated pyridostigmine-induced neuronal apoptosis. *Neurotoxicology* 21: 541-552.
2. Li, L., Borowitz, J.L. and Isom, G.E. (2001) Reactive oxygen species mediation of pyridostigmine-induced apoptosis: Involvement of the muscarinic and NMDA receptors. Submitted for publication.

## **B. Abstracts**

1. Li, L., Borowitz, J.L. and Isom, G.E. (2001) Muscarinic receptor mediated pyridostigmine-induced apoptosis in cerebellar granule cells. *The Toxicologist* (in press).
2. Li, L., Borowitz, J.L. and Isom, G.E. (2000) Pyridostigmine-induced cerebellar granule cell DNA fragmentation via loss of mitochondrial membrane potential and activation of caspase-like proteases. *The Toxicologist*, vol. 54: 117.
3. Li, L., Gunasekar, P.G., Shi, L., Borowitz, J.L. and Isom, G.E. (1999) The role of oxidative stress in pyridostigmine bromide-induced apoptosis in cerebellar granule cells. *Soc. Neurosci.*, vol. 25: 2091.
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## **8) Conclusions:**

Pyridostigmine can cause brain damage in rats, although not all animals are susceptible. The mechanism involves muscarinic and NMDA receptor activation, followed by oxidative stress. Blockade of muscarinic receptors by atropine and treatment with antioxidants protect against pyridostigmine-induced damage of rat brain cells. The enhanced generation of reactive oxygen species initiates a cascade of intracellular reactions that lead to apoptosis. The animal studies show that this cytotoxic process can be delayed up to 30 days following administration.

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## **10) Appendices**

2 manuscripts  
4 abstracts

### **List of Personnel paid by U.S. Army contract DAMD17-97-1-7354**

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Hou, Huiying (Rose)  
Isom, Gary E.  
Kafrawy, Osman  
Li, Li  
Shi, Lingling

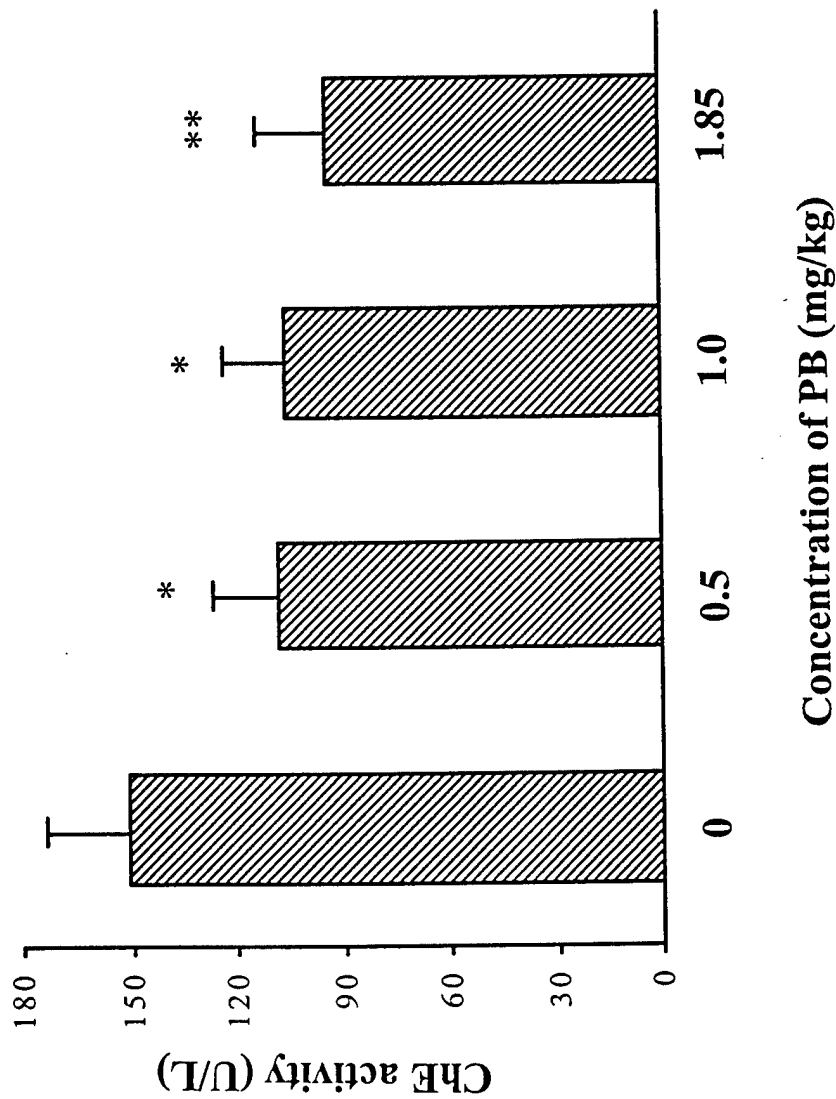


Figure 1. Effect of pyridostigmine on ChE activity of rat blood. Three hours after the last injection of PB, blood was collected and serum was separated for detecting ChE activity. Mean  $\pm$  SD, n=4. \*:  $p < 0.05$ ; \*\*:  $p < 0.01$  compared with control.

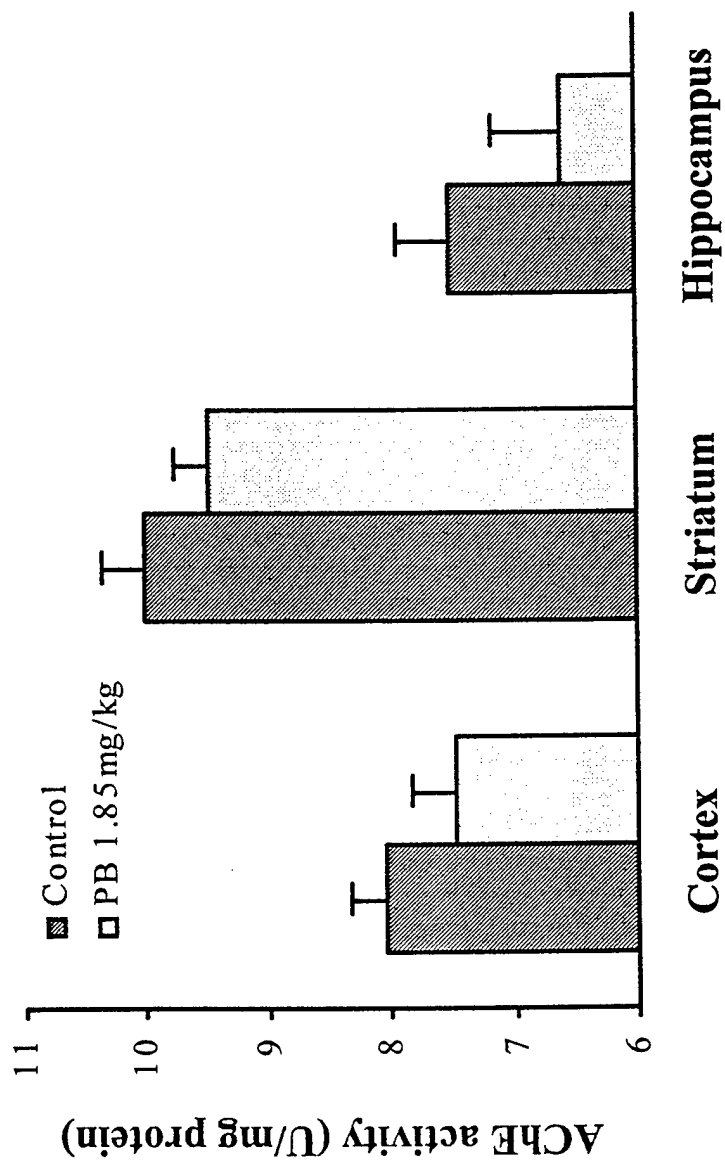


Figure 2. Brain ChE activity after acute treatment with pyridostigmine. Rat brains were harvested 20 min after administration of single dose of PB. Means  $\pm$  SE, n=4.

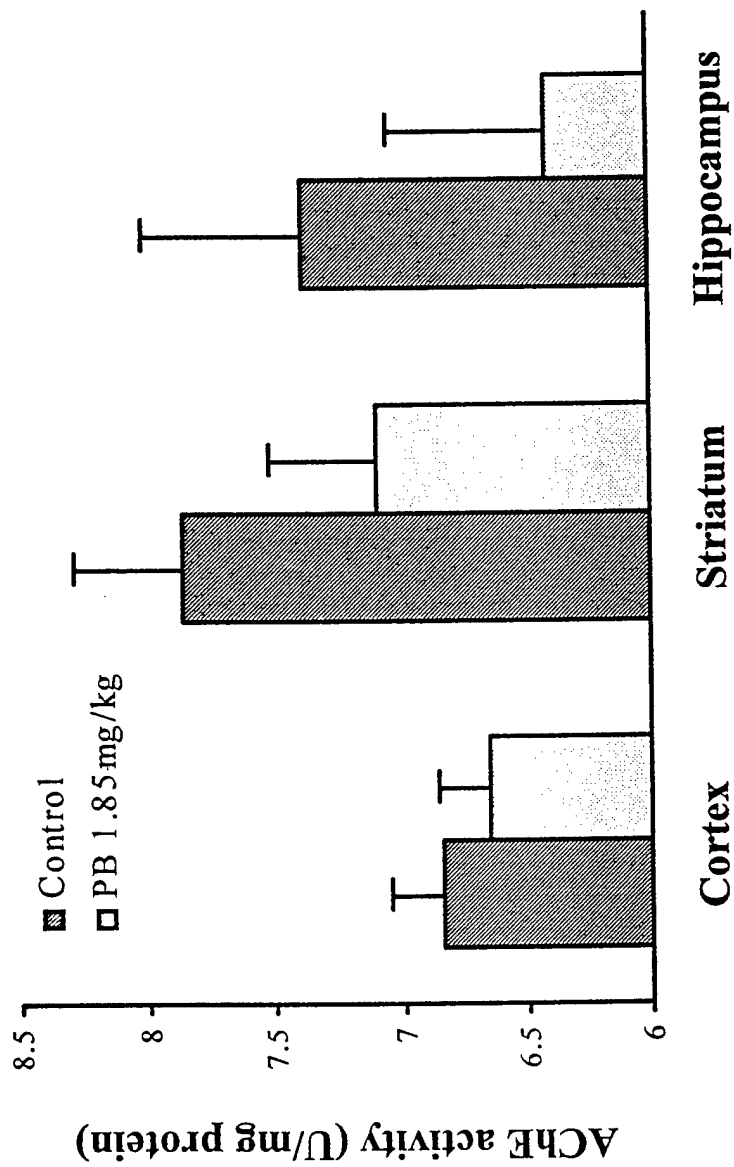


Figure 3. Brain ChE activity after repeated treatment with pyridostigmine. Rat brains were harvested 20 min after the last treatment of PB (1.85 mg/kg twice daily for 4 days. Means  $\pm$  SE, n=4.

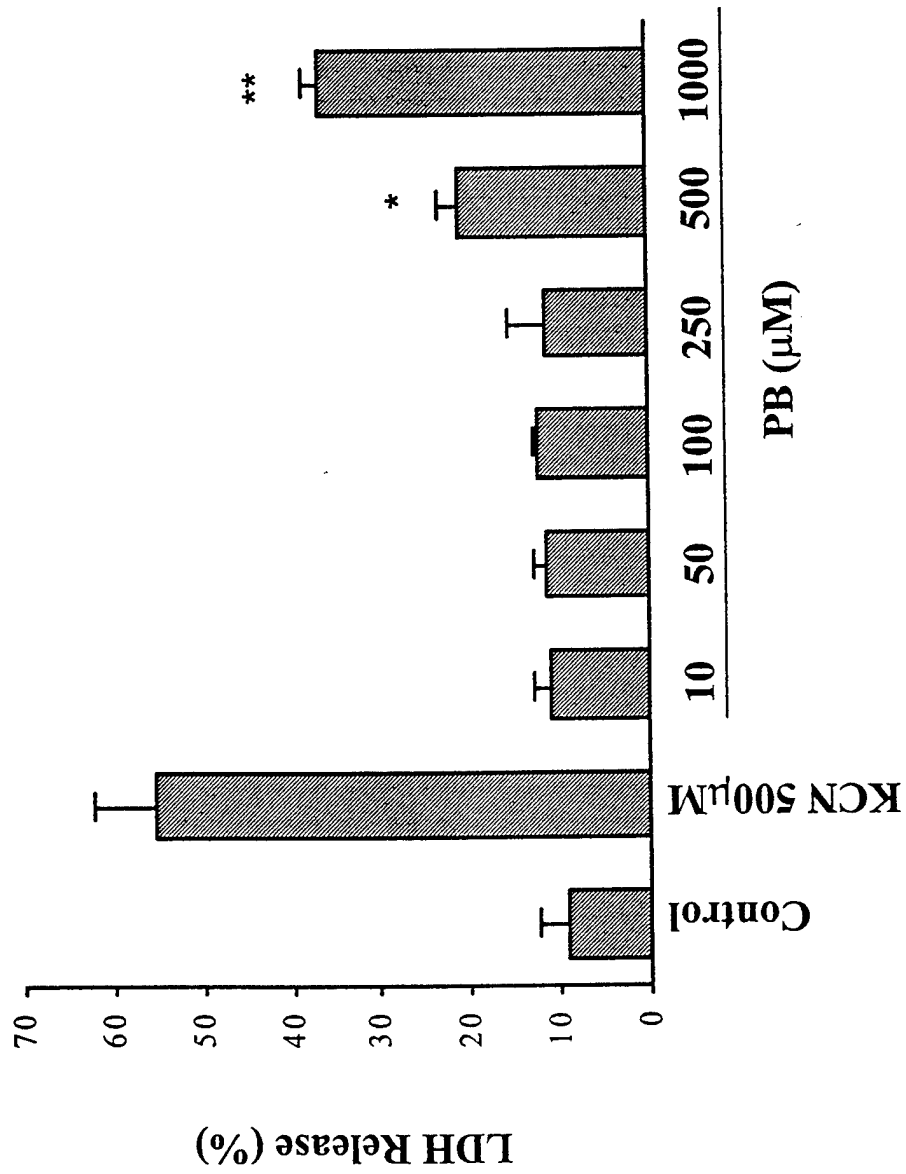


Figure 4. PB-induced cytotoxicity in cultured cerebellar granule cells. Cells were exposed to PB for 24 hr. Efflux of LDH from the cells was expressed as a percentage of total LDH ( extracellular and intracellular). Cyanide (500 µM) served as a positive control. The bars represent means  $\pm$  SD of four experiments and asterisks indicate a significant difference from control at \*:  $p < 0.05$ ; \*\*:  $p < 0.01$ .

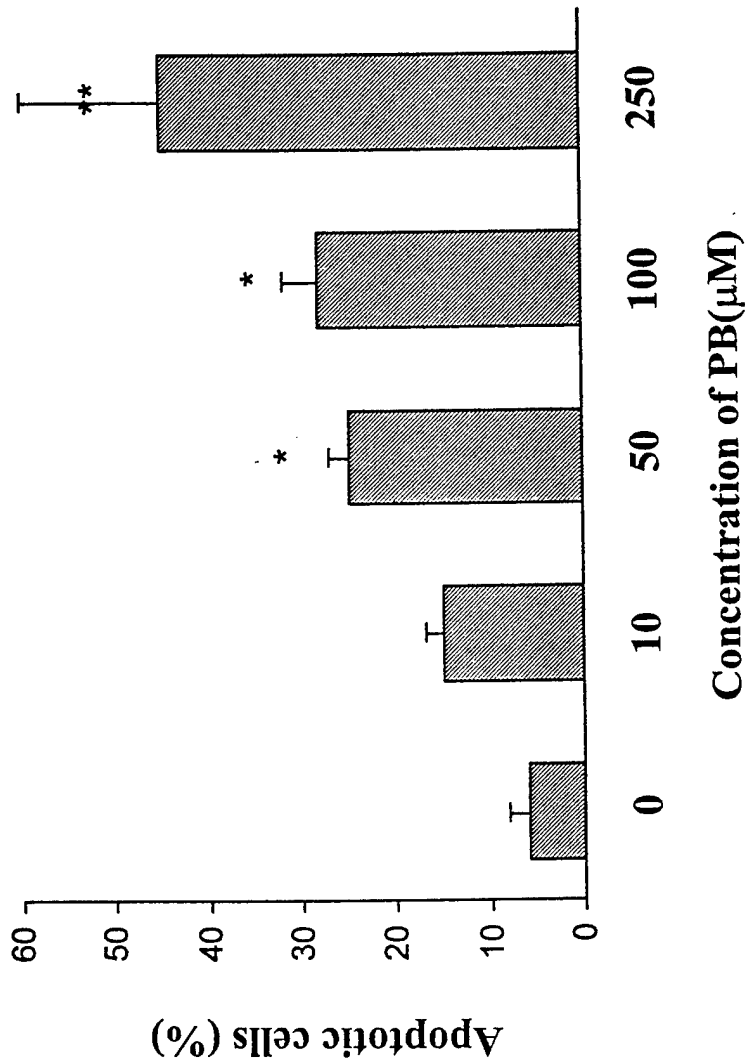


Figure 5. Quantitation of apoptosis after treatment with PB (10-250 µM). The data represent the percentage cells that were TUNEL positive following treatment with PB for 24 hr. Each bar is mean  $\pm$  SD of three experiments and asterisks indicate a significant difference from control at \*:  $p < 0.05$ ; \*\*:  $p < 0.01$

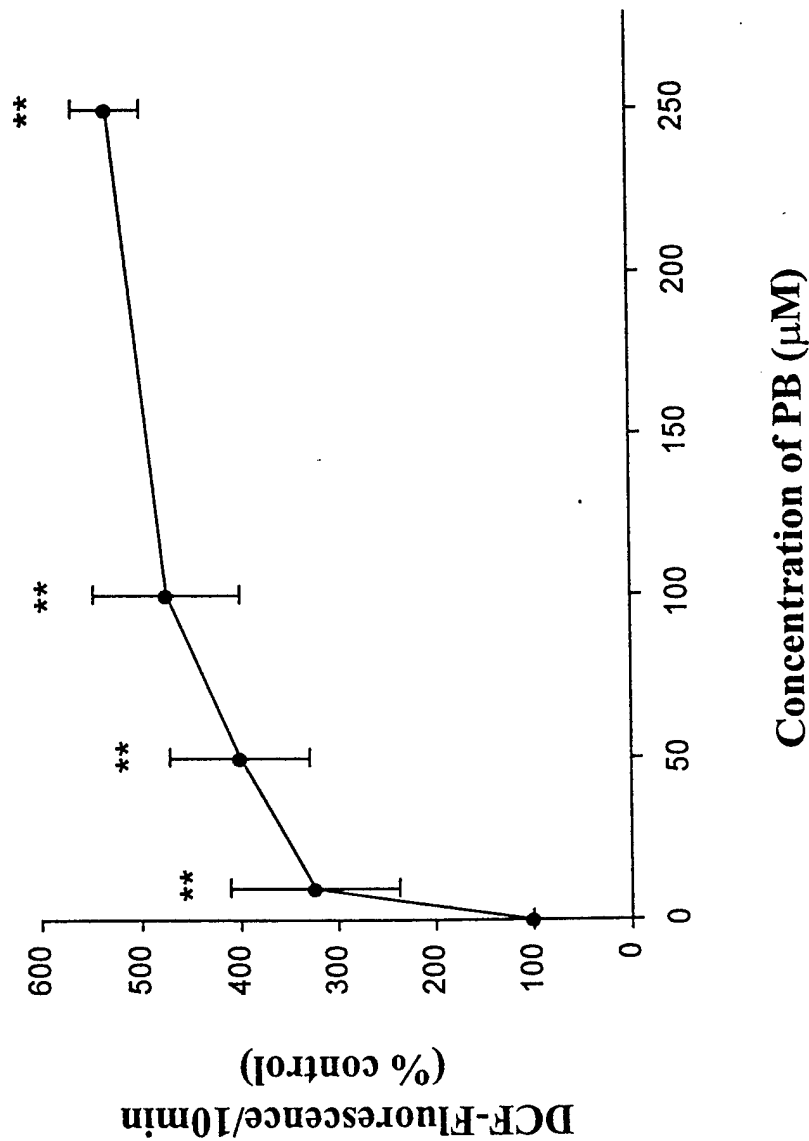


Figure 6. PB-induced ROS generation in cerebellar granule cells. Generation of intracellular ROS after PB exposure was detected using oxidation of non-fluorescent 2',7'-dichlorofluorescein diacetate to fluorescent 2',7'-dichlorofluorescein. Cells were loaded with DCF-DA for 20 min and fluorescence intensity was monitored after treatment with PB over a 10 min period. Data are expressed as means  $\pm$  SD for three or more experiments and the asterisks indicate significant difference from control, \*\*:  $p < 0.01$ .

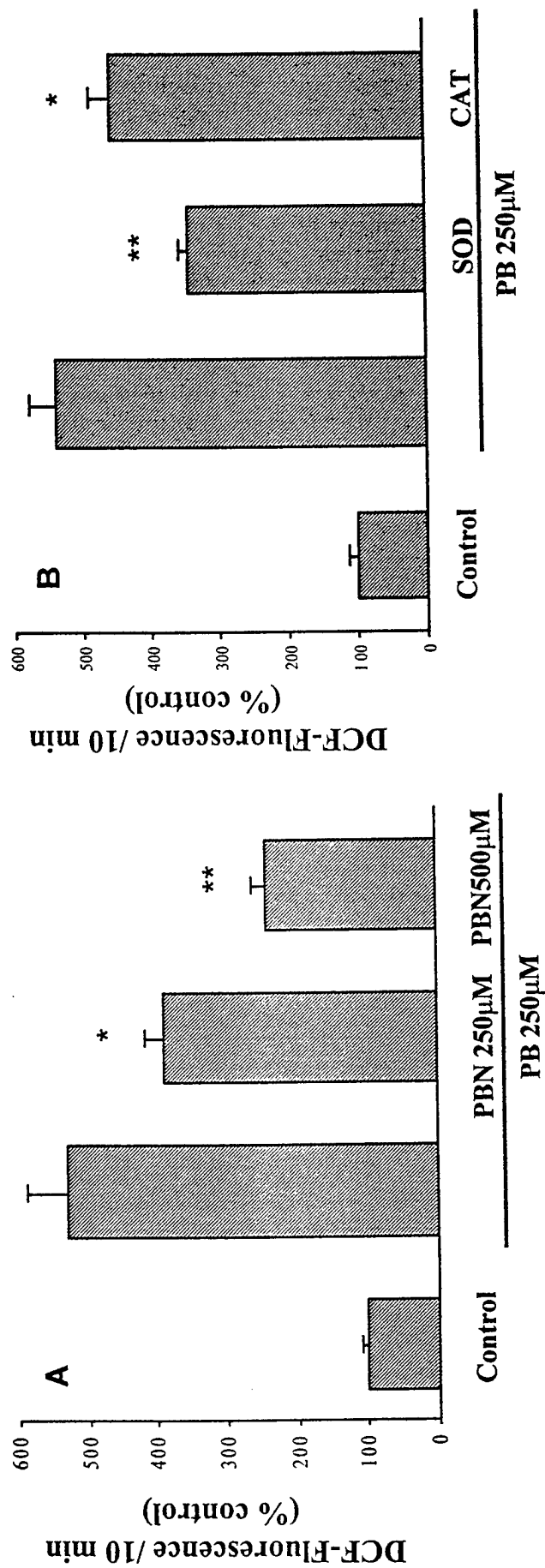


Figure 7. The effects of antioxidants on PB-induced ROS generation in cerebellar granule cells. Cells loaded DCF-DA were pretreated with phenyl-N-tert-butyl nitro (PBN), superoxide dismutase (SOD), catalase (CAT) or N<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME) for 10 min. Fluorescence intensity was monitored after treatment with PB over a 10 min period. A: ROS generation was attenuated by PBN (250 µM); B: Inhibition of ROS generation by SOD (100 U/ml), or CAT (100 U/ml); The bars represent means ± SD of three or more experiments and asterisks indicate significant difference from control, \*: p<0.05; \*\*: p<0.01.

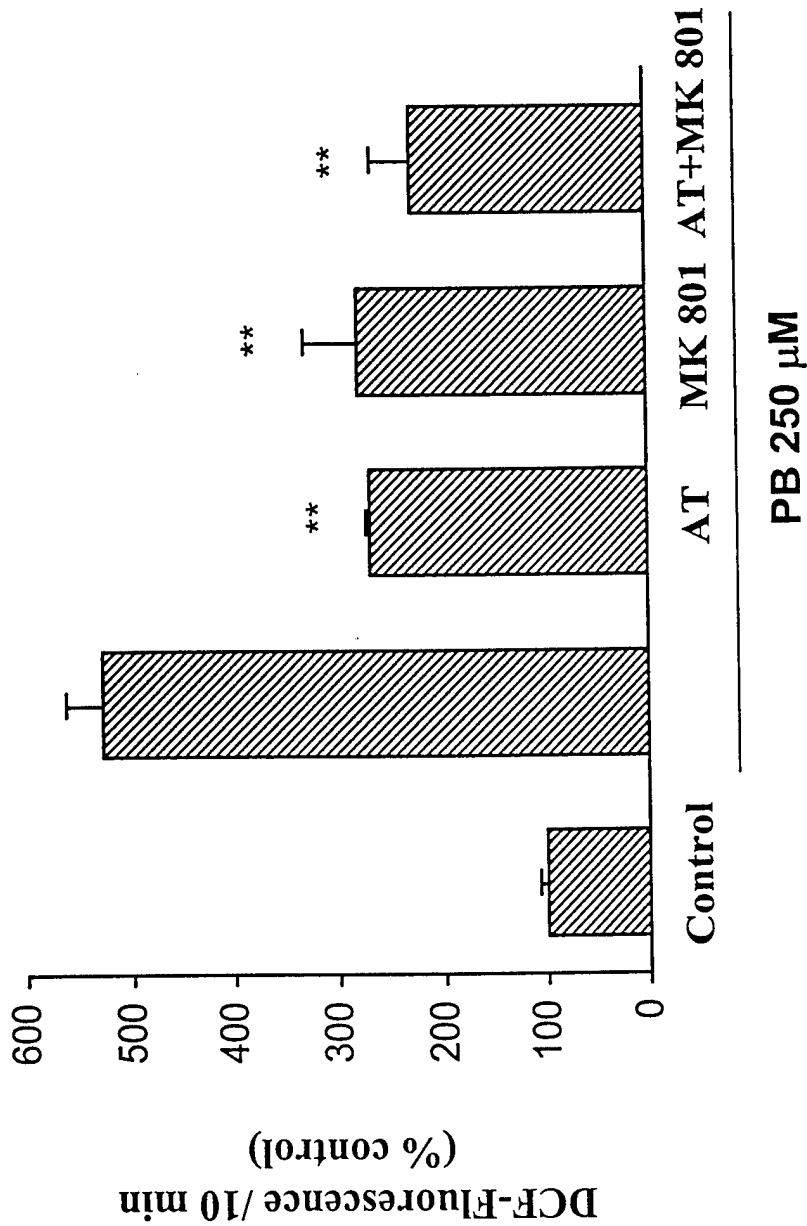


Figure 8. Blockade of ROS generation by atropine and MK 801. Cells loaded DCF-DA were pretreated with atropine or MK 801 for 10 min and fluorescence intensity was monitored after treatment with PB over a 10 min period. Bars represent means  $\pm$  SD of three or more experiments and asterisks indicate significant difference from control, \*\*:  $p < 0.01$ .

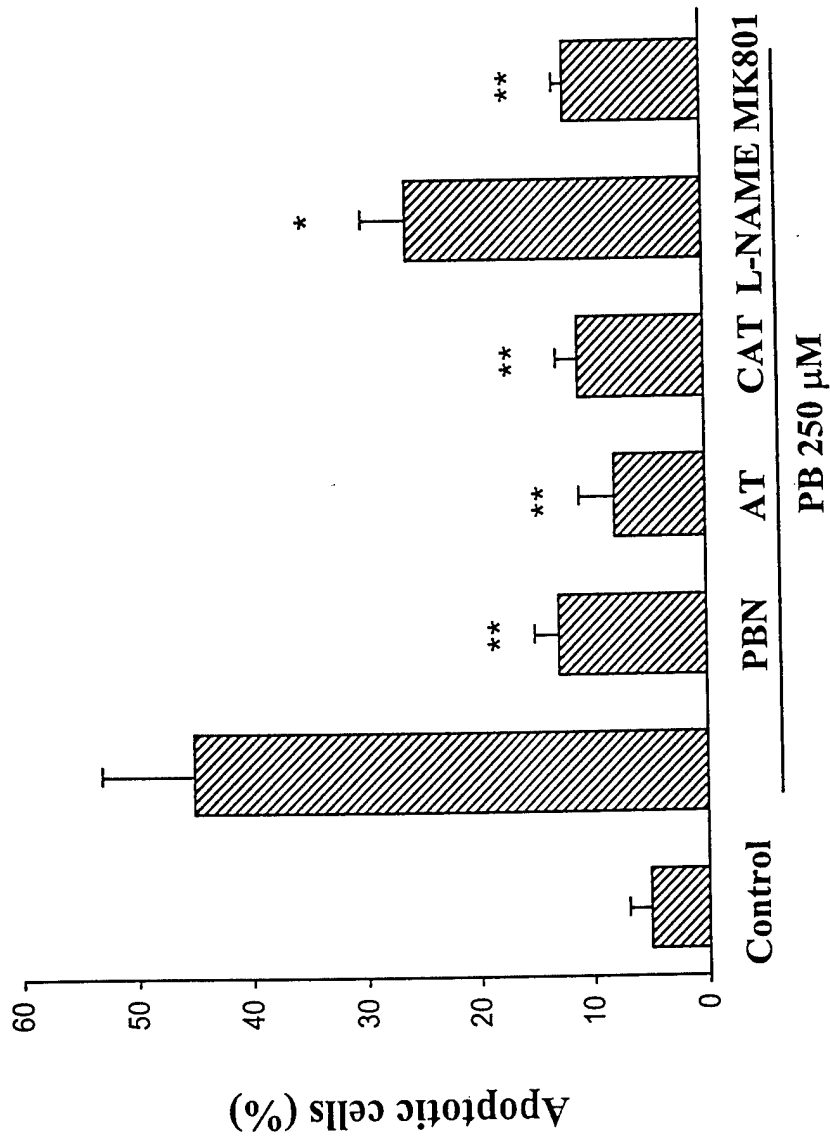


Figure 9. The effect of antioxidants, atropine and MK 801 on PB-induced apoptotic cell death. The data represent the percentage of apoptotic cells and each bar is mean  $\pm$  SD of three experiments and asterisks indicate a significant difference from control at \*\*:  $p < 0.01$ .

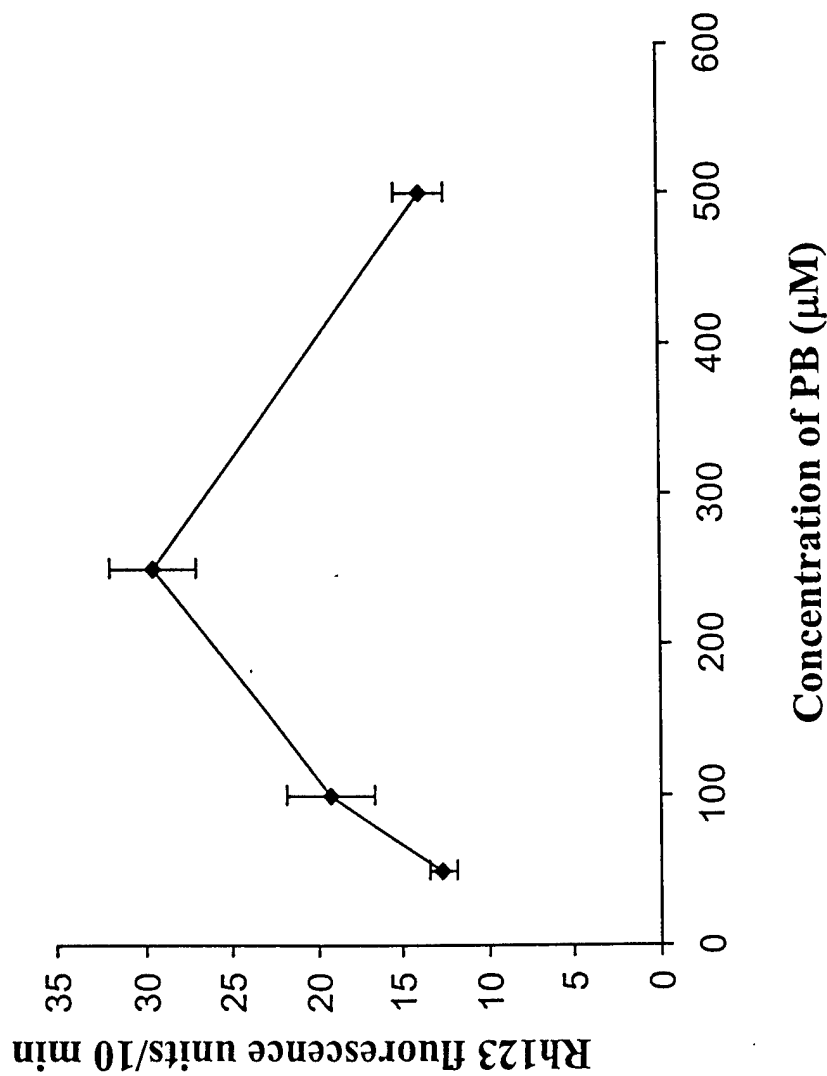


Figure 10. PB-induced loss in mitochondrial membrane potential in cerebellar granule cells. Cells were loaded with rhodamine 123 for 20 min and washed. Fluorescence changes were recorded for 10 min after PB stimulation. The effect of PB (50-250 µM) on fluorescence increases dose-dependently but decreases at the highest doses. Means  $\pm$  SD, n=3

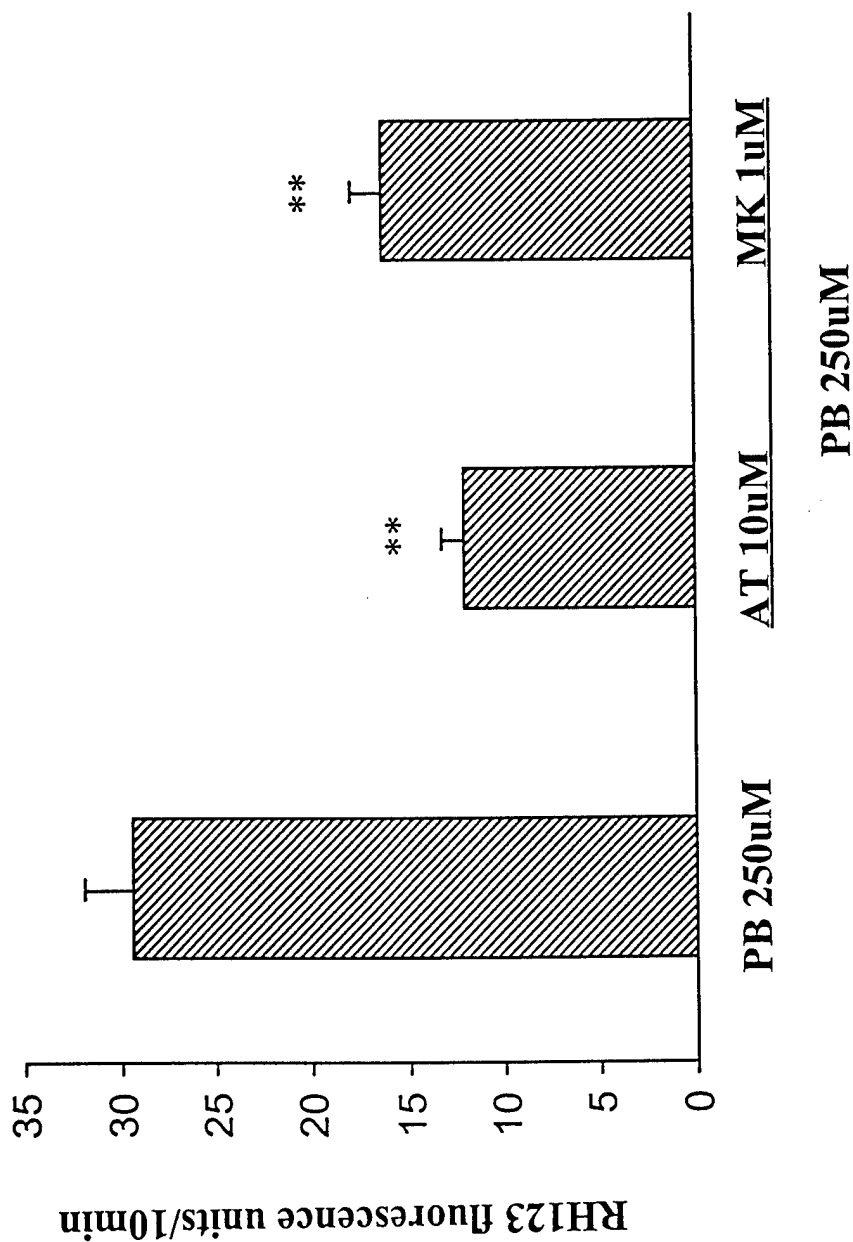


Figure 11. Inhibitory effect of muscarinic receptor antagonist atropine and NMDA receptor blocker MK 801 on PB-induced change of mitochondrial membrane potential in cerebellar granule cells. Cells pretreated with atropine or MK 801 were stimulated by PB for 10 min and fluorescence was recorded. Mean  $\pm$  SD, n=3. \*\*: p<0.01 compared with PB treatment.

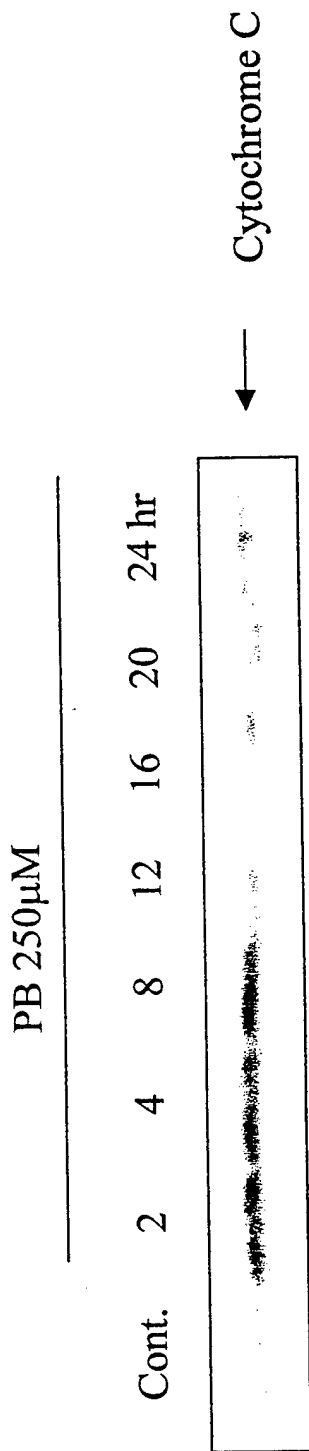


Figure 12. Time-course of PB-induced cytochrome c release. After treatment with PB (250  $\mu$ M), cells were collected at indicated time and mitochondria-free cytosolic extracts were prepared to determine cytochrome c by western blot.

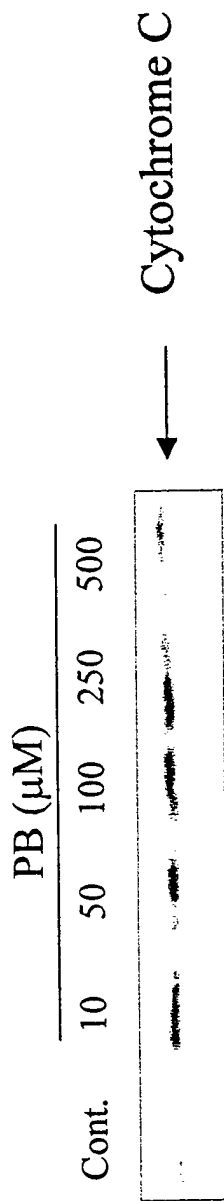


Figure 13. PB-induced release of cytochrome c. Granule cells were incubated with different concentrations of PB for 8 hr and western blots were used to determine cytochrome c released.

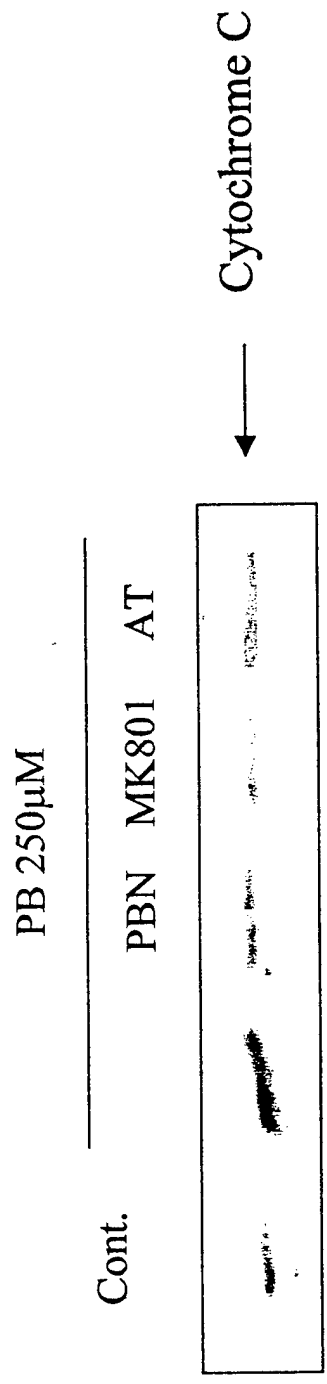


Figure 14. Blockade of PB-induced cytochrome c release by antioxidant (PBN 250  $\mu$ M), a muscarinic receptor atropine (10  $\mu$ M) and an NMDA receptor blocker MK801 (1  $\mu$ M).

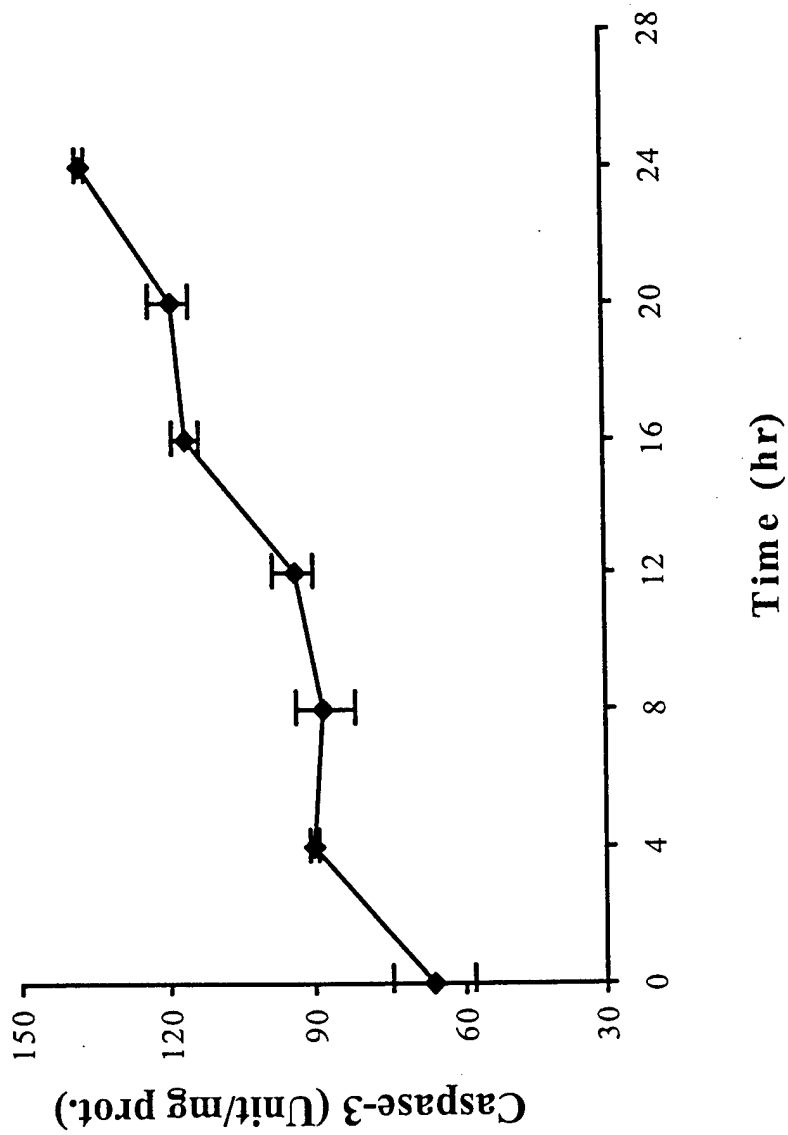


Figure 15. Time-course of caspase-3 like protease activity in cerebellar granule cells after treatment with 250  $\mu$ M PB. Mean  $\pm$  SD, n=3

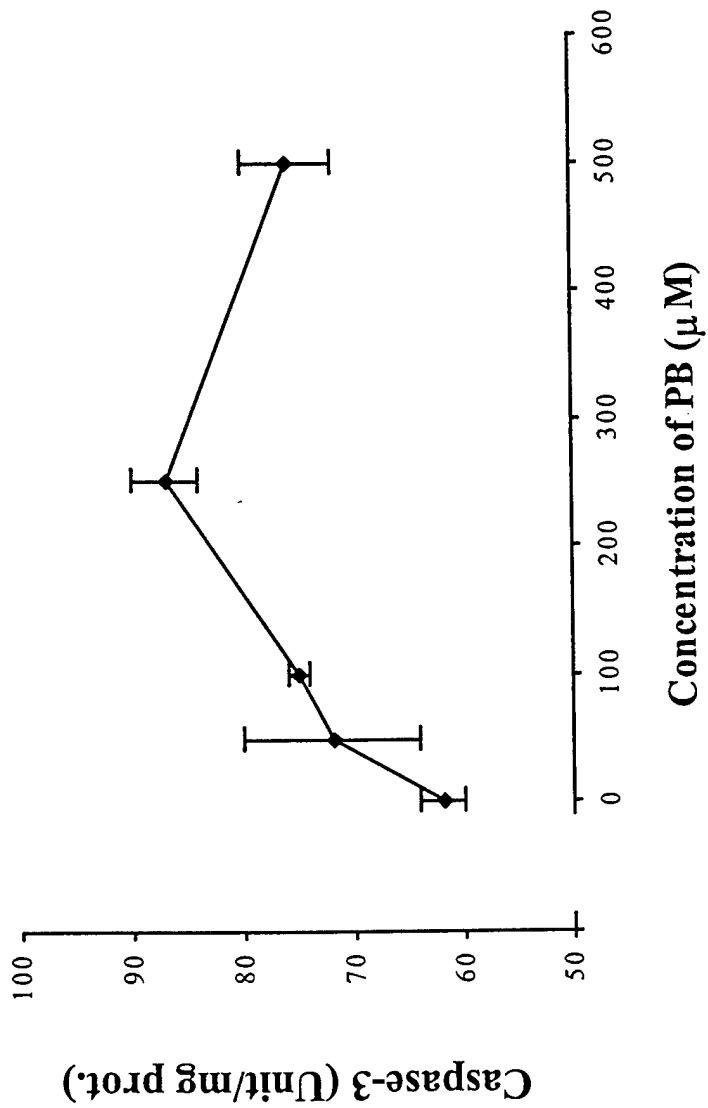


Figure 16. PB-induced caspase-3 like protease activity in cerebellar granule cells. Cells were treated with different concentrations of PB for 24 hr. Mean  $\pm$  SD, n=3

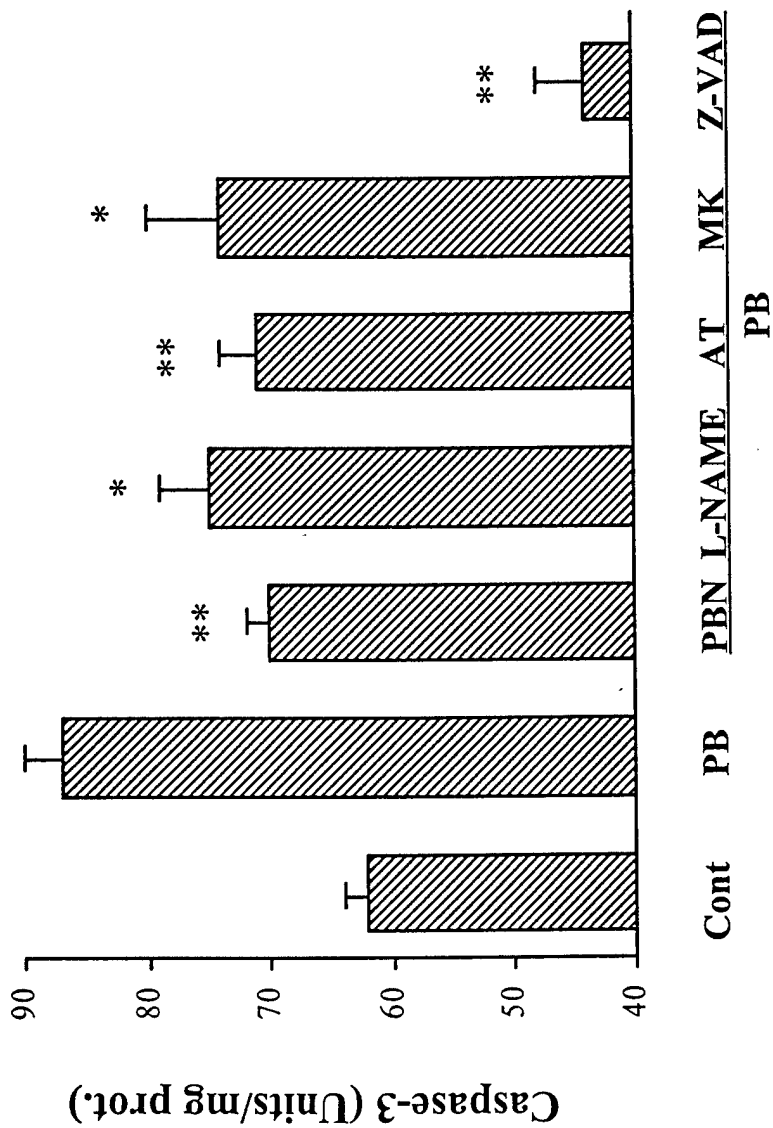


Figure 17. Blockade of 250  $\mu$ M PB-induced caspase-3 like protease activity by antioxidants (PBN and L-NAME); a muscarinic receptor antagonist, atropine; an NMDA receptor blocker, MK 801; and caspase blocker, z-VAD. Mean  $\pm$  SD, n=3. \*:  $p < 0.05$ ; \*\*:  $p < 0.01$ .

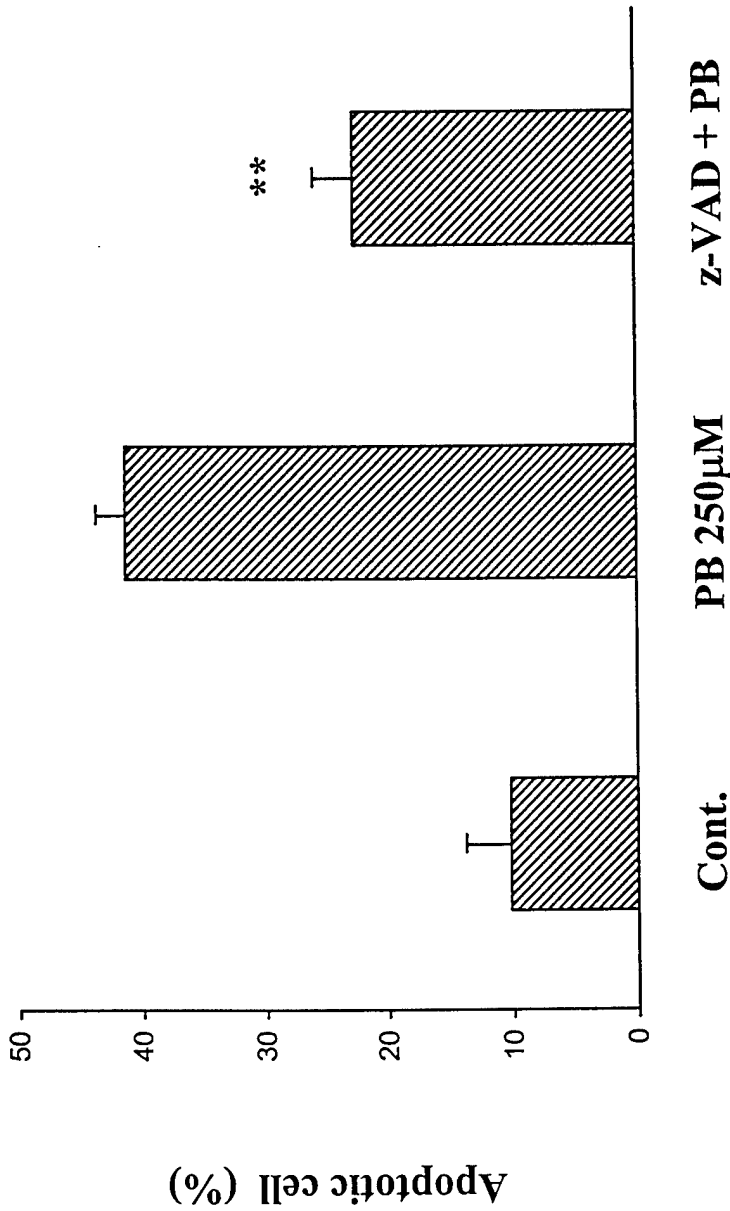


Figure 18. Blockade of PB-induced apoptosis in cerebellar granule cells by caspase blocker z-VAD detected by TUNEL staining. Cells were pretreated with z-VAD (100 µM) for 30 min and then PB was added. Determinations were made 24 hr later. Mean  $\pm$  SD, n=3. \*\*: p<0.01.

PB 250 $\mu$ M

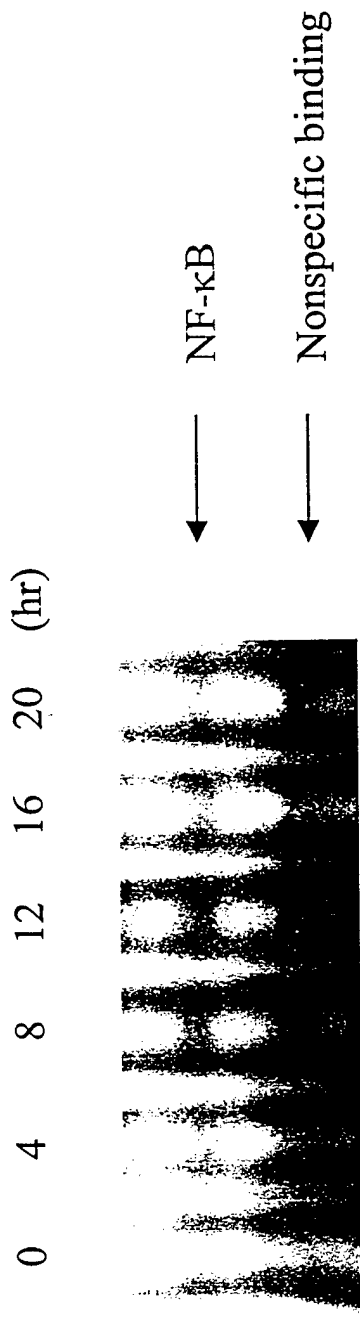


Figure 19. Time-course of PB-induced increase in DNA binding activity of NF- $\kappa$ B. After treatment with PB (250  $\mu$ M), cells were collected at the indicated times and the nuclear was extracted to determine NF- $\kappa$ B by electrophoresis mobility shift assay.

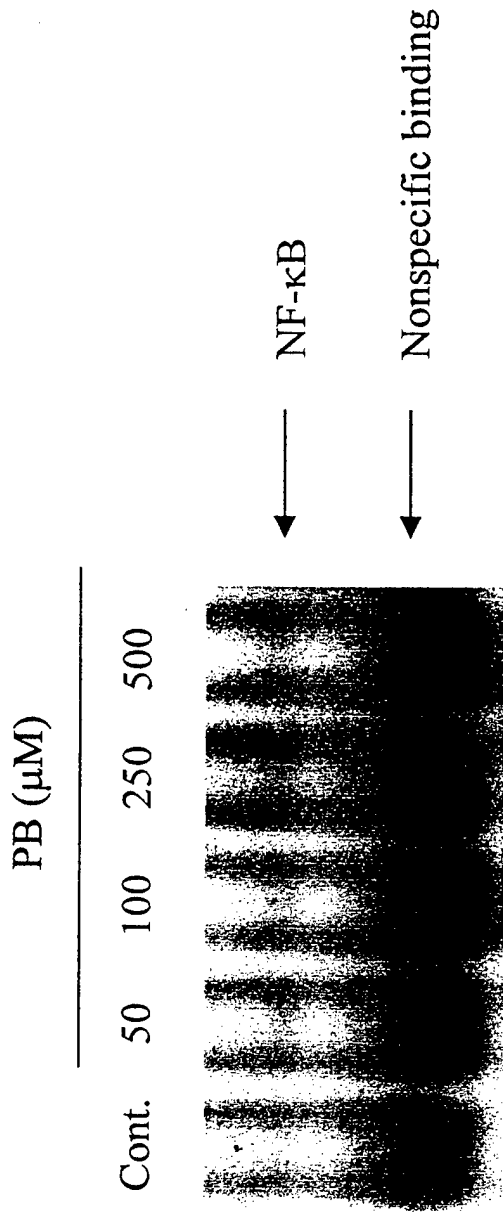


Figure 20. PB-induced increase in DNA binding activity of NF- $\kappa$ B. Granule cells were incubated with different concentrations of PB for 12 hr.

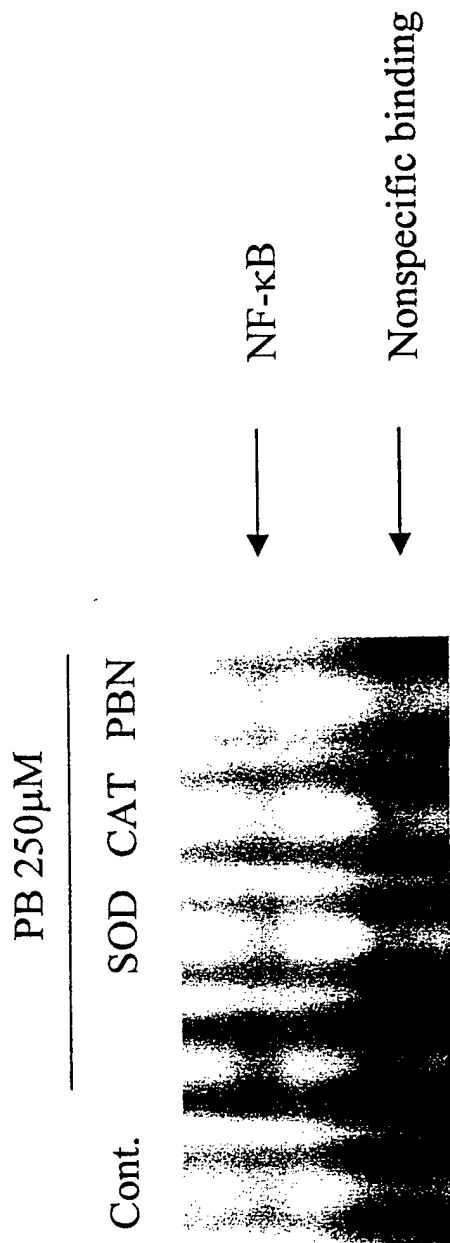


Figure 21. Blockade of PB-induced increase in DNA binding activity of NF- $\kappa$ B by antioxidants SOD (100U/ml), Catalase (100U/ml), and PBN (250 $\mu$ M). Determination was made 12 hr after PB treatment.

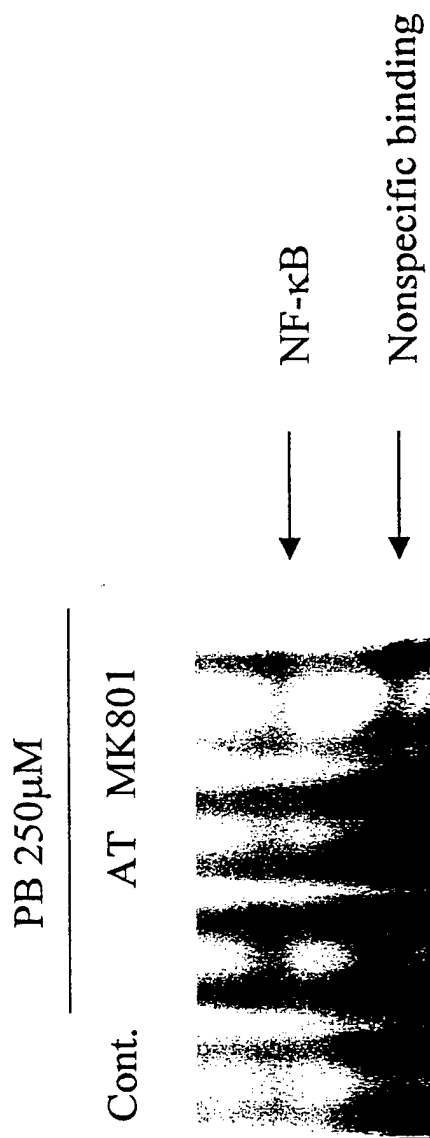


Figure 22. Blockade of PB-induced increase in DNA binding activity of NF- $\kappa$ B by muscarinic receptor antagonist atropine (10  $\mu$ M) and NMDA receptor blocker Mk 801 (1  $\mu$ M).