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## 5. INTRODUCTION

Pyridostigmine causes apoptotic brain cell destruction in rats. Since the apoptosis was observed for at least 30 days after the last pyridostigmine injection, it appears that this drug initiates a process which continues to damage brain cells long after cessation of drug treatment. In year one of this project, it was documented that pyridostigmine produced apoptotic cell death in the brain in rats administered the compound over short periods. The present report summarizes experiments continued in project year 02 designed to clarify the mechanisms by which pyridostigmine can initiate apoptosis in brain cells. Two different receptors are involved since blockade of either of these receptors can attenuate the apoptosis. Furthermore activation of either of these receptors leads to generation of reactive oxygen species and elevation of intracellular calcium. Accumulation of calcium and oxygen radicals in brain cells appears to initiate a persistent programmed cell death. These studies provide insight into apoptotic mechanisms initiated by the drug pyridostigmine.

## 6. BODY

### EXPERIMENTAL METHODS

#### Cell Culture

Cerebellar granule cell cultures were prepared from 7- to 8-day old rat pups as described previously (Gunasekar *et al.*, 1996). Briefly, cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 22 mM glucose, 25 mM KCl and 1 ml penicillin/streptomycin (5000 U/ml)/liter at pH 7.4 on 5 µg/ml poly-L-lysine-coated culture flasks. Cytosine arbinofuranoside (10 µM) was added 20 hr later to prevent proliferation of nonneuronal cells. Mature cells (8-12 days *in vitro*) were used for the experiments and approximately 95% of surviving cells were cerebellar granule cells.

### **Quantitation of Cytotoxicity**

Cytotoxicity was estimated by measurement of LDH efflux from damaged cells into medium over 24 hr exposure. Cerebellar granule cells grown in 24-well culture dishes (10 days *in vitro*) were used for the assays. After incubation, medium was removed and cells were lysed for 10 min in 0.1 M potassium phosphate buffer containing 0.5% v/v Triton X-100, pH 7.4. LDH activity was determined by a spectrophotometric method (Gunasekar *et al.*, 1996) in both the medium and lysis buffer. Percent LDH release = LDH activity in medium/LDH activity both in medium and lysis buffer.

### **Monitoring Generation of Free Radicals**

2,7-Dichlorofluorescein, the fluorescent product of 2,7-dichlorofluorescein diacetate (DCF-DA), was used to monitor generation of free radicals (Gunasekar *et al.*, 1995). Cerebellar granule cells were loaded with DCF-DA and fluorescence monitored with an SLM-8000 spectrofluorometer attached via fiberoptics to a Nikon diaphot TMD microscope. To load cells with DCF-DA, culture medium was replaced with prewarmed Kreb's Ringer solution and 10  $\mu$ l of 30 mM DCF-DA was added and incubated for 15 min at room temperature in the dark. Coverslips containing granule cells loaded with DCF-DA were placed in a cell chamber (Medical System, Inc., Greenvale, NY) mounted on a heated (37°C) microscope stage after double washing with Kreb's Ringer solution. Fluorescence of single cells was monitored over a 10 min period after addition of different concentrations of pyridostigmine in the absence or presence of superoxide dismutase (SOD) (100 U/ml), catalase (CAT) (100 U/ml),  $\alpha$ -phenyl-tert-butyl nitron (PBN) (250-500  $\mu$ M), atropine (10-20  $\mu$ M) at excitation and emission wavelengths of 475 and 525 nm. All the compounds were added 5 min before and fluorescence intensity was recorded over a 10 min period.

### Measurement of Cytosolic Free Ca<sup>2+</sup>

Cytosolic free Ca<sup>2+</sup> levels were determined in granule cells as previously described (Patel *et al.*, 1994). Briefly, granule cells on glass coverslips were loaded with fura-2 by incubating with 5 μM fura-2AM for 45 min at room temperature in Locke's solution containing (in mM) NaCl, 154; KCl, 5.6; MgCl<sub>2</sub>, 2.3; NaHCO<sub>3</sub>, 3.6; HEPES, 5.0 and D-glucose, 5.6; pH 7.4. Dye loading was terminated by replacing the loading solution with fresh Locke's solution. For microfluorescence measurement of [Ca<sup>2+</sup>]<sub>i</sub>, the coverslips were mounted in the cell chamber maintained at 37°C and placed on an inverted stage Nikon diaphot TMD microscope connected to a SLM-8000 spectrofluorometer (SLM-AMINCO, Inc., Urbana, IL). [Ca<sup>2+</sup>]<sub>i</sub> measurements were made on a small group (1-3 cells) of fura-2-loaded granule cells. After basal [Ca<sup>2+</sup>]<sub>i</sub> levels were obtained, test compounds were added via an inlet port connected to syringes for media replacement (the solution equilibrated in the chamber within 10 sec of addition), and fluorescence was monitored at emission wavelength 510 nm while excitation wavelength was alternated between 340 and 380 nm every 15 sec. MgCl<sub>2</sub> was omitted from the Locke's solution during the measurement of [Ca<sup>2+</sup>]<sub>i</sub>. Fluorescence was converted on a real time basis to Ca<sup>2+</sup> concentrations by use of the SLM 8100 software Intracellular Probe measurement system (SLM-AMINCO, Inc., Urbana, IL) according to the fluorescence ratio method (Grynkiewicz *et al.*, 1985).

### TUNEL Staining

To determine if pyridostigmine caused DNA fragmentation, the method of terminal deoxynucleotidyl transferase (TdT dUTP nick-end labeling (TUNEL) was used (*In Situ* Cell Death Detection Kit, Boehringer Mannheim). Control and pyridostigmine-treated cerebellar granule cells on coverslips were washed with PBS twice and fixed in a 4% PBS-buffered

formalin solution (pH 7.4) for 30 min at room temperature. Slides were rinsed with PBS and incubated in permeabilisation solution (0.1% Triton X-100, 0.1% sodium citrate) for 2 min on ice. Then slides were rinsed twice with PBS and dried. 50 µl TUNEL was added to the reaction mixture sample and slides incubated in a humidified chamber for 60 min at 37°C. Slides were rinsed 3 times with PBS and mounted with coverslips. Samples were analyzed using an Olympus BH-2 fluorescence microscope and photographs taken.

### **Extraction and Electrophoresis of DNA**

Intracellular DNA was extracted according to a method described previously (Herrmann *et al.*, 1994; Herget *et al.*, 1998). Untreated or pyridostigmine-treated granule cells ( $2 \times 10^7$ ) were washed with PBS (pH 7.4) twice and collected by centrifugation. The pellets were treated with 0.5 ml lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM EDTA, 0.5% sodium dodecyl sulfate) for 10 min on ice. After treatment with RNase A (final concentration 100 µg/ml) for 1 hr at 37°C, proteinase K (final concentration 100 µg/ml) was added and samples incubated 4 hr at 50°C. DNA was precipitated with an ethanol solution (0.1 volumes of 3 M sodium acetate, pH 5.2, and 2.5 volumes of cool absolute ethanol). Pellets were dissolved in Tris-EDTA. For analysis, 10 or 20 µg of DNA was loaded on a 1.2% agarose gel containing ethidium bromide (10 µg/ml) and electrophoresed in 0.5x Tris-boric acid-EDTA buffer at 70 V for 2 hr. DNA was visualized under ultraviolet light and photographed.

### **Electron Microscopic Analysis of Apoptotic Cells**

For electron microscopy cells were pelleted by centrifugation and fixed in 4% paraformaldehyde in PBS. The pellets were postfixed in 2% OsO overnight and then dehydrated in an ethanol series. Pieces were embedded in Epon 8-10, cut into 60-90 µm sections using a microtome and mounted on grids. Sections were stained for 30 min in 2% uranyl acetate and

lead citrate and examined by transmission electron microscopy (JOEL 100x) at a magnification of 6,500-10,000. Apoptotic cells are characterized by chromatin margination to nuclear membrane, chromatin clumping and shrinkage of cell cytoplasm.

## RESULTS

### **Lactic Acid Dehydrogenase (LDH) Release from Cerebellar Granule Cells by Pyridostigmine**

To determine whether pyridostigmine can cause obvious neuronal cell death *in vitro*, cerebellar granule cells were incubated with increasing concentrations of the drug for 24 hrs. Figure 1 shows that cell disruption and release of the cytoplasmic enzyme, LDH could not be detected at the concentrations employed (10 to 250  $\mu$ M).

### **Apoptosis of Cerebellar Granule Cells Induced by Pyridostigmine**

Since apoptotic cell death does not involve release of cellular contents (LDH), it is possible that apoptosis may occur at low concentrations of pyridostigmine. Accordingly, cerebellar granule cells were incubated with pyridostigmine at 10, 50, 100 and 250  $\mu$ M for 24 hrs. A marked increase in apoptotic cell death, as detected by TUNEL staining, was noted even at the 10  $\mu$ M level (Fig. 2 & 3). It is clear that even small amounts of pyridostigmine are capable of initiating programmed cell death in primary neuronal cultures.

### **DNA Laddering Caused by Pyridostigmine**

Since TUNEL staining is not conclusive evidence that pyridostigmine causes apoptotic cell death in cultured neuronal cells, it was necessary to demonstrate laddering of DNA fragments. When apoptosis occurs, endonucleases cleave DNA at regular intervals so that DNA strands of increasing lengths appear as a ladder on electrophoresis. Accordingly, cells were incubated for 24 hrs with pyridostigmine and the DNA from the cells extracted and

electrophoresed. Figure 4 demonstrates laddering at several concentrations of pyridostigmine. DNA from untreated cells does not show laddering. These results support the TUNEL staining data and indicate that pyridostigmine does indeed cause apoptosis of neuronal cells in culture.

### **Electron Microscopic Evidence of Apoptosis**

An attempt was made to obtain morphological evidence of apoptosis in cells treated with pyridostigmine. However clear examples of chromatin condensation and margination to the nuclear membrane with cytoplasmic shrinkage were not readily apparent. Figure 5B however, shows a pyridostigmine-treated cerebellar granule cell which may be in the beginning stages of apoptosis.

### **Pyridostigmine-induced Formation of Oxidative Species**

Reactive oxygen species (ROS) have been implicated as an initiation factor of apoptosis (Mills *et al.*, 1999). To determine if ROS play a role in the response to pyridostigmine, granule cells were loaded with 2,6-dichlorofluorescein and treated with pyridostigmine. Even low concentrations of the drug (10  $\mu$ M) caused a marked increase in ROS over a 10 min period, and the response was dose-related (Fig. 6). It appeared that generation of ROS may be important in pyridostigmine-triggered apoptosis.

### **Blockade of ROS Generation and Apoptosis Using Antioxidants**

If formation of ROS initiates apoptosis by pyridostigmine, then antioxidants should alter ROS generation and alter the apoptotic response. Thus Figures 7 and 8 show that the antioxidants PBN, superoxide dismutase and catalase all reduce the detectable levels of ROS generated by pyridostigmine. Thus antioxidants block ROS formation by pyridostigmine in cerebellar granule cells and results suggest that multiple types of ROS including superoxide and hydrogen peroxide are involved.

In addition the antioxidant PBN decreases TUNEL staining in pyridostigmine-treated cerebellar granule cells (Fig. 9) and also blocks pyridostigmine-induced laddering in these cells (Fig. 10). These data suggest a cause-effect relationship between ROS formation and apoptosis induced by pyridostigmine in neuronal cultures.

### **Receptors Mediating Pyridostigmine-induced Apoptosis in Neuronal Cultures**

Being a cholinesterase inhibitor, it was likely that pyridostigmine caused accumulation of acetylcholine and activation of cholinergic receptors. Thus atropine was used to evaluate the role of muscarinic receptors in free radical generation and apoptosis caused by pyridostigmine in cerebellar granule cells. Atropine markedly blocked ROS generation (Fig. 11), TUNEL staining (Fig. 12) and DNA laddering (Fig. 13) caused by pyridostigmine. Apparently, muscarinic receptors play an important role in the oxidative stress-induced apoptosis caused by pyridostigmine.

Since toxic actions of a variety of chemicals involves NMDA type glutamate receptors (Crews *et al.*, 1998; Gunasekar *et al.*, 1997), the NMDA receptor antagonist MK-801 was tested against the actions of pyridostigmine. Fluorescence (Fig. 14) TUNEL staining (Fig. 15) and laddering (Fig. 16) induced by pyridostigmine were all inhibited by MK-801 in cerebellar granule cells. It appears that both muscarinic and NMDA receptors are involved in mediating the toxic effect of pyridostigmine in neuronal cells.

### **Influence of Calcium on ROS Generation Induced by Pyridostigmine**

ROS generation has been linked to increases in intracellular calcium levels (Ardelt *et al.*, 1994). To test whether the increases in ROS caused by pyridostigmine were also related to elevated  $[Ca^{++}]_i$ , BAPTA, the calcium chelator and calcium free media were employed. Figure 17 shows that about half of the ROS formation induced by pyridostigmine is mediated by

calcium. Calcium may make an important contribution to the oxidative stress induced by pyridostigmine and may also mediate other toxic effects of this drug.

### **Effect of Pyridostigmine on Free Intracellular Calcium in Cerebellar Granule Cells: Involvement of Different Receptors**

Since previous studies showed calcium was involved in ROS generation by pyridostigmine, the extent of the effect and receptors mediating this action were examined. Figure 18 shows a dose-related increase in  $[Ca^{++}]_i$  in granule cells exposed to pyridostigmine. This effect was markedly inhibited by MK-801 and also inhibited by atropine to a lesser extent (Fig. 19). NMDA receptors appear to be more potent than muscarinic receptors in increasing  $[Ca^{++}]_i$  in cerebellar granule cells after exposure to pyridostigmine.

## **DISCUSSION**

Pyridostigmine injected into rats causes apoptotic brain cell death mainly in cerebral cortex (Li *et al.*, 2000). Present studies extend this important observation to show that neurons in culture also undergo apoptosis when exposed to pyridostigmine and that oxidative stress is a critical factor in this process. Both muscarinic and glutamate (NMDA) receptors are involved since blockade of these receptors attenuates the response. Elevation of cell calcium subsequent to activation of muscarinic and NMDA receptors is a key step and contributes to formation of oxidative species. These data provide insight into the mechanisms by which pyridostigmine causes neuronal degeneration in rats

Whether or not pyridostigmine can act directly with its quaternary structure on the CNS is controversial. Chaney *et al.* (1999) maintain that the convulsions and lethal action of pyridostigmine in mice are peripheral in origin since they can be blocked with either atropine or methyl atropine. They further show that even lethal doses of pyridostigmine do not cause

inhibition of brain ChE in rats. Death after a high dose of pyridostigmine involves peripheral effects such as bronchoconstriction or increased lung mucus secretion so that it is not surprising that atropine or methylatropine can prevent death by pyridostigmine. The significant though not complete protection against pyridostigmine-induced convulsions by atropine or methyl atropine may also be mediated by actions on the lung to prevent any possible anoxic contribution to the convulsive seizures. Evidence that the seizures were at least partly central in origin was the fact that they were significantly inhibited by the anticonvulsant fosphenytoin (Chaney *et al.*, 1999).

Servatius *et al.* (1998) compare behavioral effects of pyridostigmine on Sprague-Dawley and Wistar rats. Pyridostigmine was given in drinking water for 7 days. Plasma ChE activity decreased about 20% in all rats. After pyridostigmine administration rats were tested for noise-induced startle responses. No change was seen in Sprague-Dawley rats compared to control but 15 and 22 days after termination of pyridostigmine dosing, exaggerated startle responses were noted in the Wistar-Kyoto animals. No effects of pyridostigmine were seen on analgesia or open field activity. The data suggest that persistent exaggerated startle responses produced by pyridostigmine in Wistar-Kyoto rats result from pyridostigmine-induced alterations in CNS activity.

Servatius *et al.* (1998) suggest that the influence of pyridostigmine on brain function may be related to plasma butyryl ChE. Lower butyryl ChE either due to stress or to an inherited deficit may allow circulating pyridostigmine to more readily enter the brain. This may explain the increased susceptibility of Wistar-Kyoto rats to pyridostigmine since these animals have low butyryl ChE levels.

Based on the report by Friedman *et al.* (1996) that pyridostigmine markedly inhibited brain ChE in mice exposed to a swim stress protocol, we suggest that repeated injection of

pyridostigmine is a form of stress and may allow for increased penetration of the drug through the blood brain barrier of certain animals. Hence some of the rats treated with pyridostigmine in our lab showed apoptotic brain damage most likely related to excess cholinergic activity.

A basic assumption in these experiments is that muscarinic receptors are able to release glutamate. This assumption is supported by a report that carbachol releases glutamate and glycine from dorsal cochlear nucleus brain slices (Chen *et al.*, 1999). No other amino acids were released from brain slices by this muscarinic agonist. Furthermore, other neurotoxic chemicals are capable of releasing glutamate and initiating an oxidative stress-mediated apoptotic neuronal death. For example cyanide releases glutamate (Patel *et al.*, 1991) and NMDA receptor activation is a critical aspect of the neurotoxic action of this compound (Patel *et al.*, 1992; Patel *et al.*, 1994). Cyanide injected into mice (6 mg/kg ip, twice daily for 3-12 days) causes cortical apoptosis but in these same animals necrosis occurred in the substantia nigra (Mills *et al.*, 1999). Pretreatment of the mice with the antioxidant PBN, reduced the apoptosis but did not prevent necrosis in the substantia nigra. Excitatory amino acids may be involved in the cell death mechanisms of a variety of neurotoxic chemicals.

Pyridostigmine is not the only ChE inhibitor known to produce apoptotic cell death. Akbarsha and Sivasamy (1997) reported that the anticholinesterase, phosphamidon, caused apoptosis in spermatogenic line cells. Also, muscarinic agonists, carbachol and oxotremorine, induce apoptosis in rat thymocytes (Yamada *et al.*, 1997). Furthermore Bagchi *et al.* (1995) suggested that oxidative species may “serve as common mediators of apoptosis in response to many toxicants” including organophosphate ChE inhibitors. Our findings with pyridostigmine appear to be in agreement with reports in the literature.

Both muscarinic and NMDA receptors contribute to the toxic insult by pyridostigmine. Presumably muscarinic receptor activation occurs initially and subsequently glutamate is released to activate NMDA receptors. Continued actions on these two receptors combine to increase ROS with subsequent apoptotic cell death. Pyridostigmine-induced calcium influx into granule cells is nearly abolished by MK-801 indicating that this receptor is important for calcium entry. MK-801 was only moderately effective against the increase in ROS induced by pyridostigmine suggesting that factors other than calcium may contribute to the increase in ROS mediated by the NMDA receptor. By contrast the extent of the reduction by atropine (10  $\mu$ M) of the pyridostigmine-induced increases in calcium and ROS were equivalent. Apparently ROS generated by muscarinic receptor activation are directly linked to calcium.

If the present data are to be relevant, the amounts of pyridostigmine employed should not be excessive. Pyridostigmine has a volume of distribution of 1.1 l/kg and effective concentrations in the plasma are 50-100 ng/ml (Hardman *et al.*, 1996). Assuming all the drug distributes without loss to all body tissues, plasma concentration in rats given 0.5 mg/kg is about 330 ng/ml. The true plasma level is very likely less than that considering the drug is rapidly excreted in the urine. Our lowest concentration *in vitro* (10  $\mu$ M) is roughly equivalent to our highest dose *in vivo*. Thus the amounts of pyridostigmine employed in this contract are near the upper limit of the therapeutic range, and are reasonable for evaluating toxic effects of this drug.

#### ADDENDUM

In the course of these studies it was clear that brain cholinesterase activity should be determined in pyridostigmine-treated rats. Animals were sacrificed 20 min after treatment with 1.85 mg/kg ip. The procedure used (Ellman *et al.*, 1961) was the same as that employed for measurement of serum cholinesterase activity in our previous annual report. Figure 20 shows

that pyridostigmine does not significantly decrease cholinesterase activity in rat cerebral cortex, striatum or hippocampus.

Thinking that the dose schedule of our original experiments may allow more pyridostigmine to enter the brain, we repeated these experiments injecting 1.85 mg/kg ip twice daily for 4 days and sacrificing the rats 20 min after the last dose. Again no significant inhibition of brain cholinesterase was evident (Fig. 21). Some inhibition of cholinesterase may occur however, since on the average all groups of brain samples from pyridostigmine-treated animals show less cholinesterase activity than controls. It is possible therefore that in some of the rats inhibition of brain cholinesterase occurred. Comparison of means indicated no significant difference but this may obscure what happens in individuals. Interestingly, pyridostigmine has been reported to lower brain cholinesterase activity in mice (Deyi *et al.*, 1981).

To document inhibition of cholinesterase in cerebellar granule cells treated with pyridostigmine, assays were done both in lysed cells and in the medium after a 24 hr incubation. Marked decreases in activity of cholinesterase in the medium were noted (Fig. 22), but granule cells per se did not show any enzyme activity. These data confirm that pyridostigmine inhibits cholinesterase *in vitro* in the amounts employed in these studies.

## 7. KEY RESEARCH ACCOMPLISHMENTS

- Obtained clear evidence of pyridostigmine-induced apoptosis in cultured neurons using TUNEL staining and DNA electrophoresis.
- Showed that omission of calcium from the medium and inclusion of the calcium chelator, BAPTA, inhibited oxygen radical generation by pyridostigmine.
- Determined the extent of calcium influx caused by activation of muscarinic and NMDA receptors by pyridostigmine in cerebellar granule cells.
- Directly linked pyridostigmine-induced brain cell death with oxidative stress by preventing cell death with an antioxidant,  $\alpha$ -phenyl-tert-butyl nitron (PBN).

## 8. REPORTABLE OUTCOMES

A manuscript entitled "Muscarinic Receptor-Mediated Pyridostigmine-Induced Neuronal Apoptosis" by L. Li, P.G. Gunasekar, J.L. Borowitz and G.E. Isom, has been accepted for publication in Neurotoxicology and is in revision. The work in this manuscript was supported by U.S. Army contract DAMD17-97-1-7354.

## 9. CONCLUSIONS

1. Pyridostigmine-induced apoptosis in rat brain also occurs in cultured cerebellar granule cells showing that the effect is exerted directly on neurons.
2. Parallel activation of two different receptor types (muscarinic and NMDA glutamate receptors) mediates pyridostigmine-induced neuronal apoptosis.
3. Increases in cell calcium and generation of reactive oxygen species accompany the apoptotic action of pyridostigmine.
4. Antioxidants block the toxic effects of pyridostigmine on brain cells.

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## FIGURE LEGENDS

Figure 1. Lactate Dehydrogenase (LDH) release by Pyridostigmine Bromide (PB) from Rat Cerebellar Granule Cells. Duration of drug treatment, 24 hr. Cyanide served as a positive control. Means  $\pm$  SD are shown for 4-7 experiments. Statistical evaluation was done by ANOVA in this and subsequent figures. No significant differences in LDH release were detected.

Figure 2. Apoptotic Cell Death Caused by Pyridostigmine (PB). Fluorescent TUNEL staining is shown in cerebellar granule cells after 24 hr exposure to PB.

Figure 3. Quantitative Estimate of Apoptotic Cell Death Caused by Pyridostigmine (PB) as Indicated by TUNEL Staining. Data from Figure 2 are included here. Means  $\pm$  SD are shown for n=3-4. Asterisks indicate significant difference \*  $p < 0.05$ ; \*\*  $p < 0.01$ .

Figure 4. DNA Laddering Induced by Pyridostigmine (PB) in Rat Cerebellar Granule Cells (24 hr exposure). Presence of laddering is additional evidence that PB causes apoptosis.

Figure 5. Electron Micrograph of Cerebellar Granule Cells Treated with Pyridostigmine (250  $\mu$ M for 24 hrs). "A" is control and "B" is treated. Beginning stages of apoptosis may be illustrated by the cell on the left in B. Magnification 6500X.

Figure 6. Free Radical Generation Induced by Pyridostigmine (PB) in Granule Cells. Asterisks indicate significant increases in oxygen radical formation (\*  $p < 0.05$ ; \*\*  $p < 0.01$ ). Means  $\pm$  SD are shown for n=3-4.

Figure 7. Inhibition of Pyridostigmine (PB)-induced Free Radical Generation by the Antioxidant PBN. PBN ( $\alpha$ -phenyl-tert-butyl nitron) was added to the granule cell media 5 min prior to PB. Means  $\pm$  SD are shown for an n of 3. Differences are highly significant by ANOVA ( $p < 0.01$ ).

Figure 8. Inhibition of Pyridostigmine (PB)-induced Free Radical Generation by Superoxide Dismutase (SOD) and Catalase (CAT). Enzymes (100 Units/ml) were added to the cell media 5 min prior to pyridostigmine. Means  $\pm$  SE are shown for 3 experiments. The difference for SOD is significant ( $p < 0.01$ ) and for CAT  $p < 0.05$ .

Figure 9. Blockade of Pyridostigmine (PB)-induced TUNEL Staining by the Antioxidant PBN. PBN was added to the cell media 15 min prior to PB and the incubation continued for 24 hrs. Decreases in the incidence of apoptosis are evident in the PBN-treated cells.

Figure 10. Blockade of Pyridostigmine (PB)-induced Laddering in Cerebellar Granule Cells (24 hr exposure) by the Antioxidant PBN.

Figure 11. Inhibition of Pyridostigmine (PB)-induced Oxygen Radical Formation by Atropine (AT). Means  $\pm$  SD for 3 experiments are shown.

Figure 12. Atropine (AT) blocks TUNEL staining (Apoptosis) in Cerebellar Granule Cells After Pyridostigmine (PB) Exposure. Atropine 10  $\mu$ M, was added 15 min before PB and incubation continued for 24 hrs.

Figure 13. Atropine (AT) Blocks DNA Laddering in Cerebellar Granule Cells Caused by Pyridostigmine (PB). AT was added 15 min prior to pyridostigmine and the incubation continued for 24 hrs.

Figure 14. Atropine (AT) and MK-801 (NMDA Receptor Inhibitor) Block Pyridostigmine (PB)-induced Generation of Oxygen Radicals in Cerebellar Granule Cells. Means  $\pm$  SD of 3 experiments. Asterisks indicate significant differences at  $p < 0.01$ .

Figure 15. Blockade of Pyridostigmine (PB)-induced TUNEL Staining (Apoptosis) by MK-801 (NMDA Receptor Inhibitor). MK-801 ( $\mu$ M) was added 15 min prior to PB and incubated for 24 hrs.

Figure 16. Blockade of Pyridostigmine (PB)-induced DNA Laddering by MK-801 (NMDA Receptor Inhibitor). MK-801 was added 15 min before PB and incubation continued for 24 hr.

Figure 17. Effect of Calcium-free Media and the Calcium Chelator (BAPTA) on Pyridostigmine-induced Oxygen Radical Formation. Differences are significant at  $p < 0.001$ .

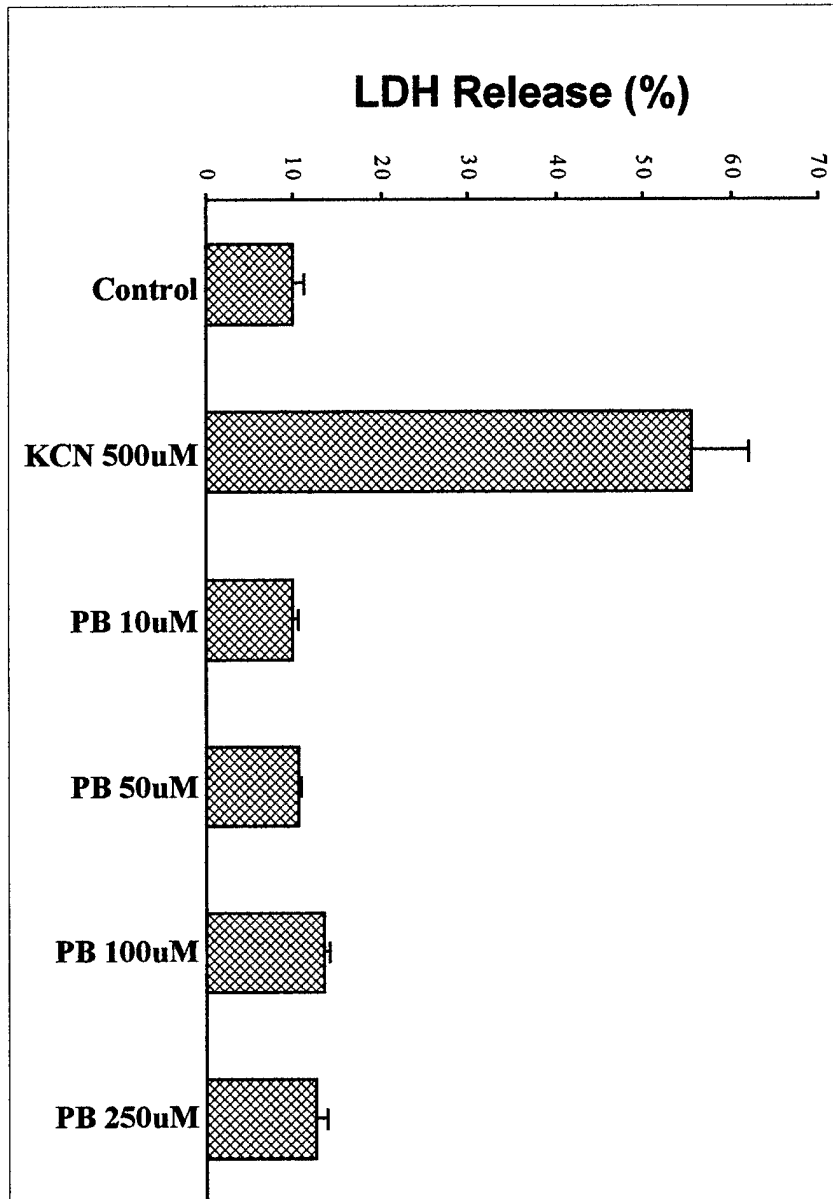
Figure 18. Effect of Pyridostigmine (PB) on Free Intracellular Calcium in Cerebellar Granule Cells. Calcium levels were monitored for a period of 20 min. Means + SD are shown for 3 experiments. Asterisks indicate significance from control,  $p < 0.01$ .

Figure 19. Inhibition of Pyridostigmine (PB)-induced Increases in Intracellular Calcium by Atropine and MK-801 in Cerebellar Granule Cells. Atropine or MK-801 (NMDA receptor inhibitor) was added 5 min prior to PB and calcium levels monitored over a 20 min. Means  $\pm$  SD are shown for  $n = 3$ . Asterisks indicate significant difference from PB alone,  $p < 0.01$ . Inhibition by atropine was significantly less than that by MK-801,  $p < 0.01$ .

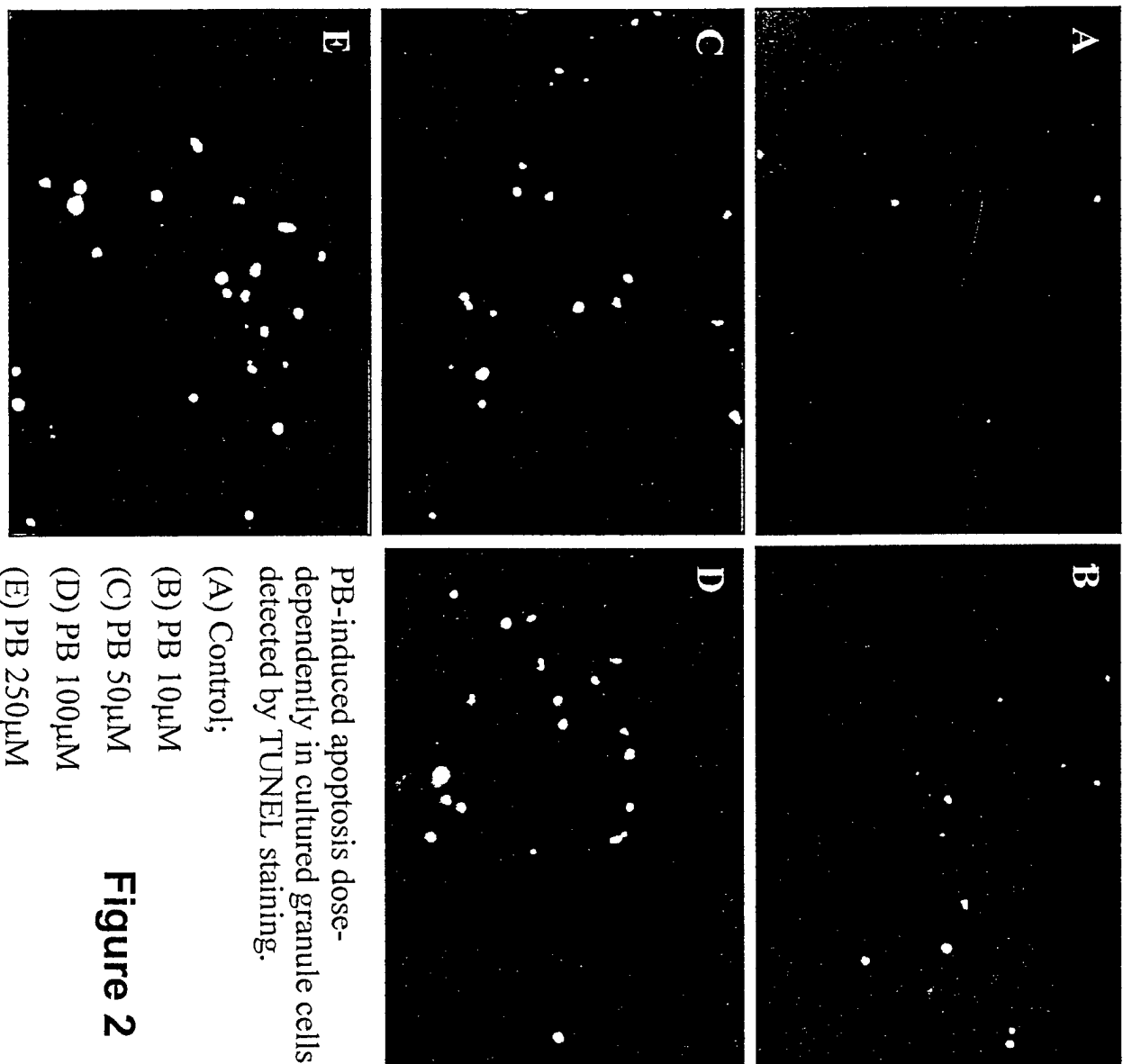
Figure 20. Brain ChE activity after acute treatment with pyridostigmine. Rat brains were harvested 20 min after administration of pyridostigmine (1.85 mg/kg, ip). Means  $\pm$  SE,  $n=4$ .

Figure 21. Brain ChE activity after repeated treatment with pyridostigmine. Rat brains were harvested 20 min after the last treatment of pyridostigmine (1.85 mg/kg twice daily for 4 days, ip). Means  $\pm$  SE,  $n=4$ .

Figure 22. Inhibition of Cholinesterase by Pyridostigmine (PB) in Cerebellar Granule Cells. Cell media was collected 20 min after addition of PB and analyzed for cholinesterase activity. Means  $\pm$  SD are shown for  $n = 4$ . Asterisks indicate significance, \*\*\*  $p < 0.001$  compared to control.



**Figure 1**



**Figure 2**

PB-induced apoptosis dose-dependently in cultured granule cells detected by TUNEL staining.

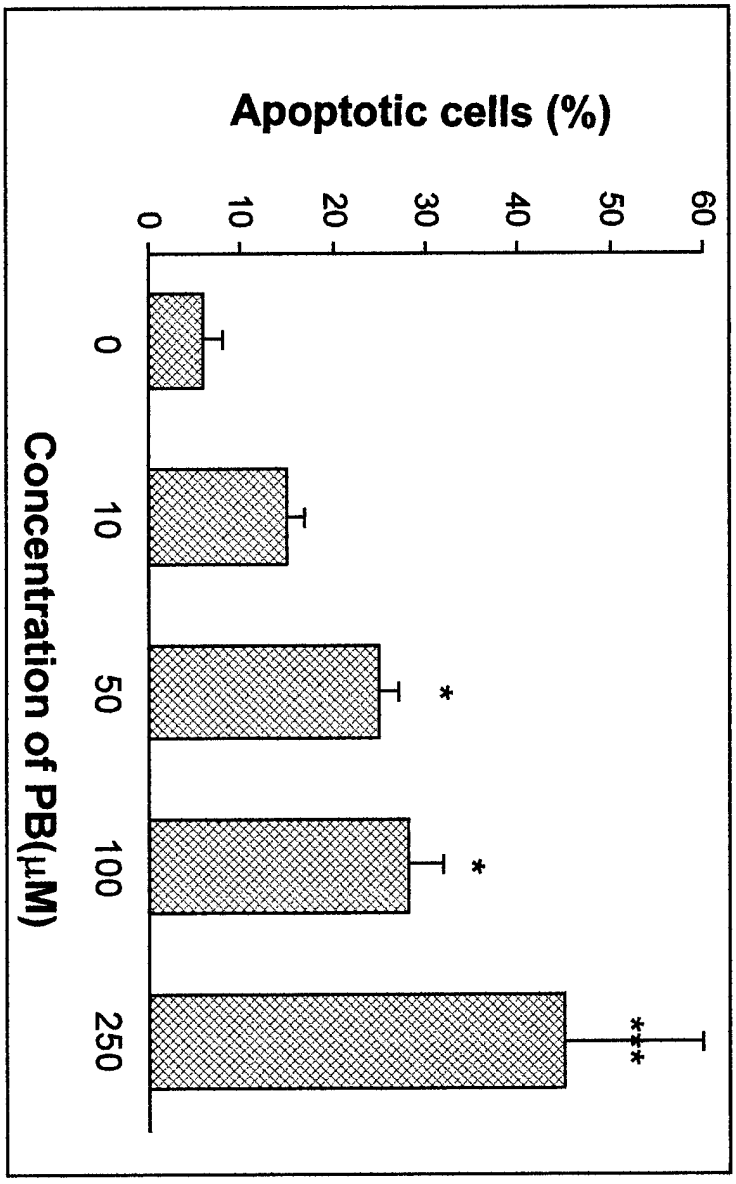
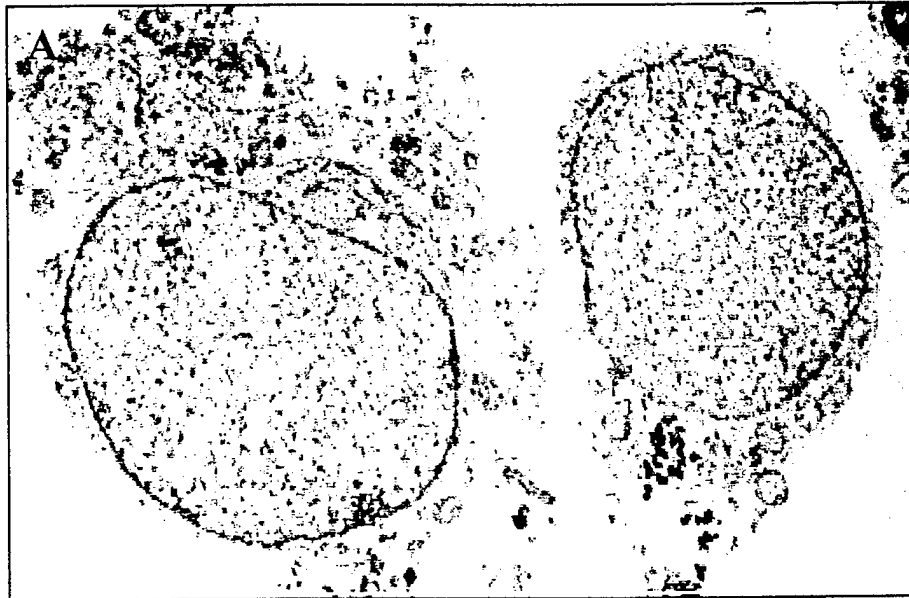


Figure 3

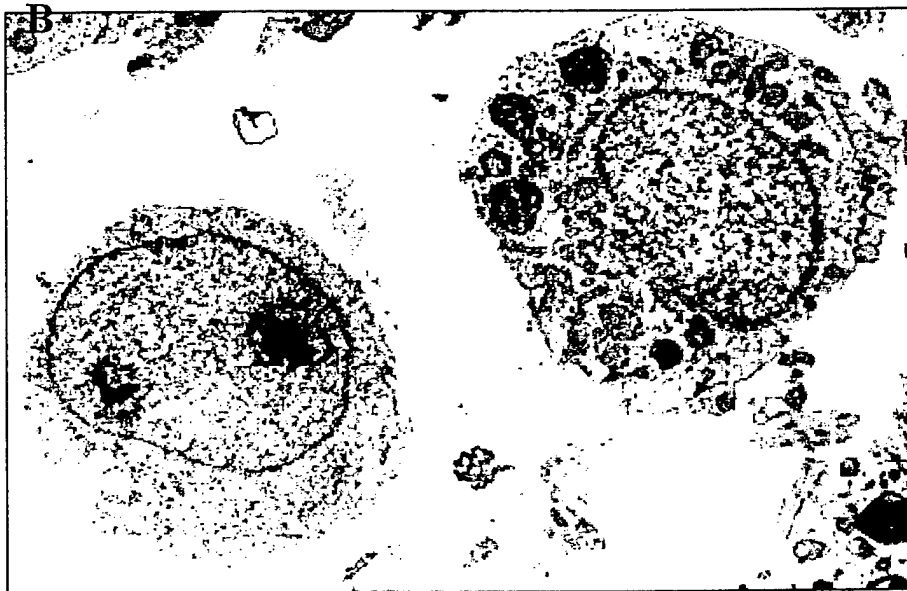
Control  
DNA marker  
PB 500 $\mu$ M  
PB 250 $\mu$ M  
PB 100 $\mu$ M  
PB 50 $\mu$ M  
Control



Figure 4



Control



PB 250uM

Figure 5

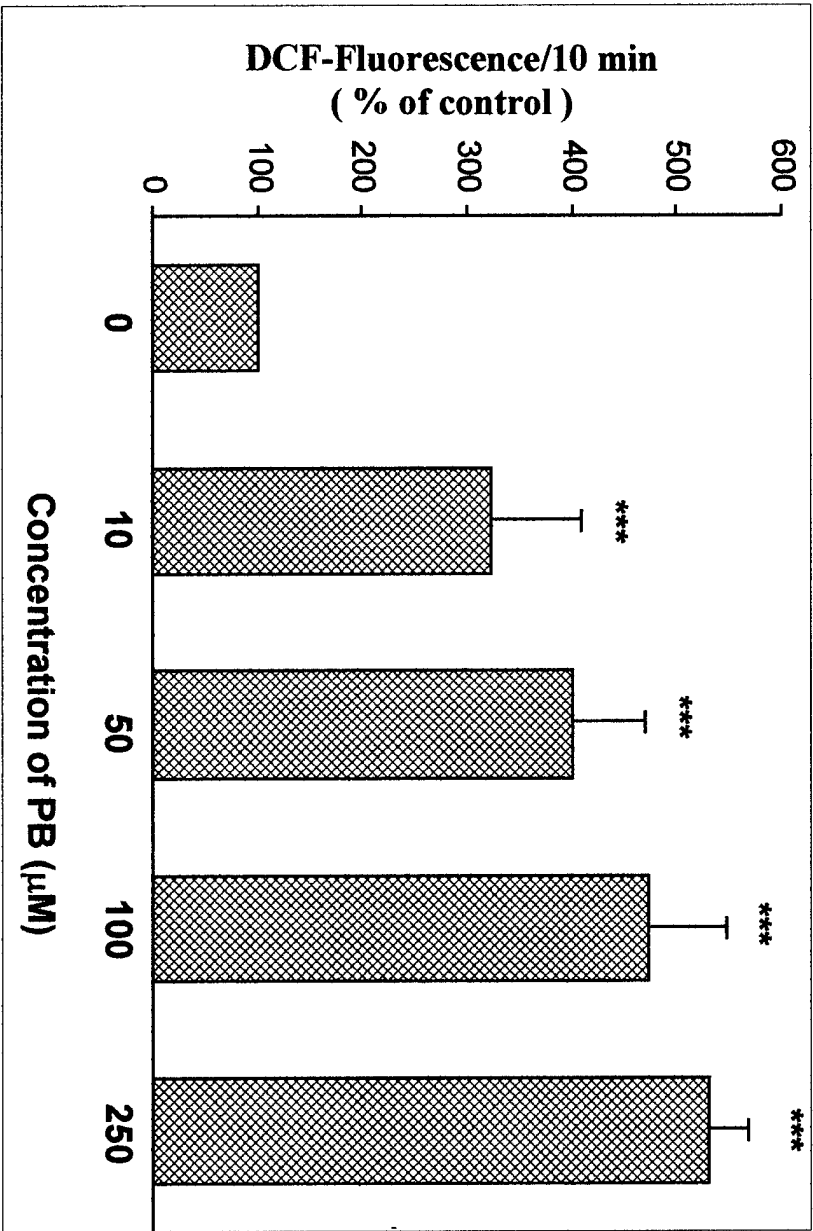


Figure 6

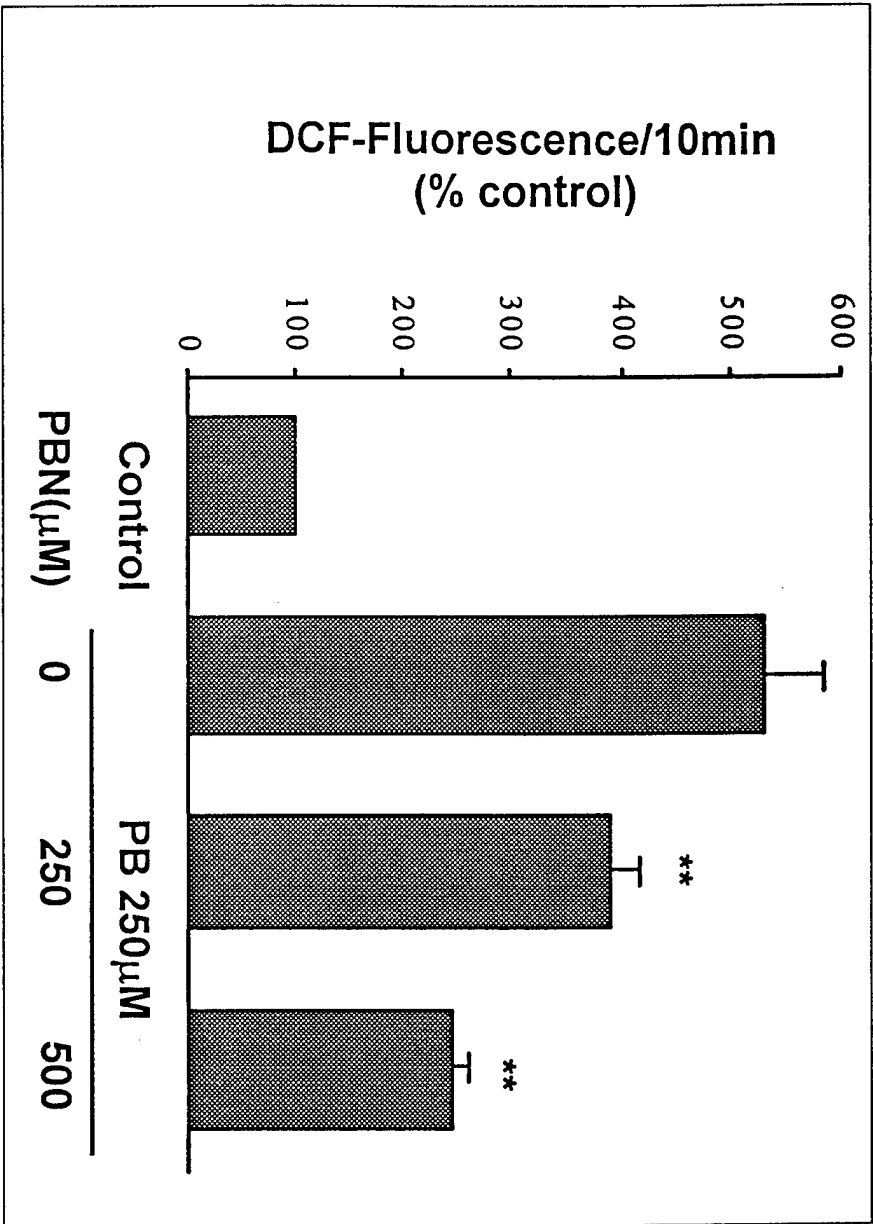


Figure 7

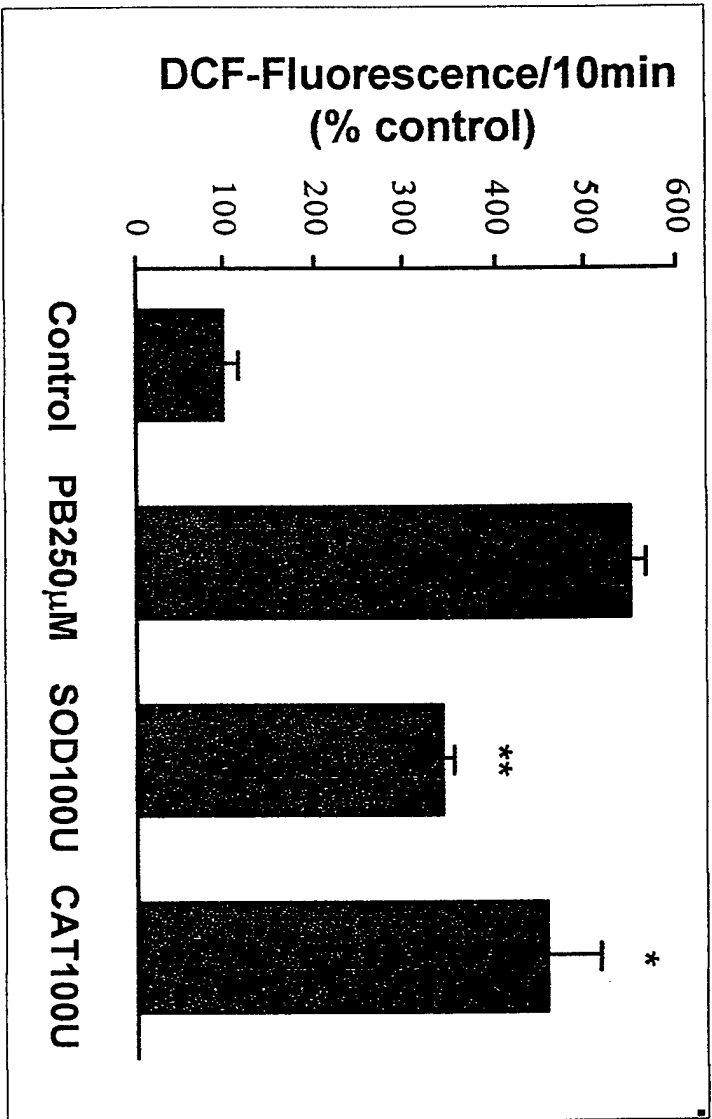
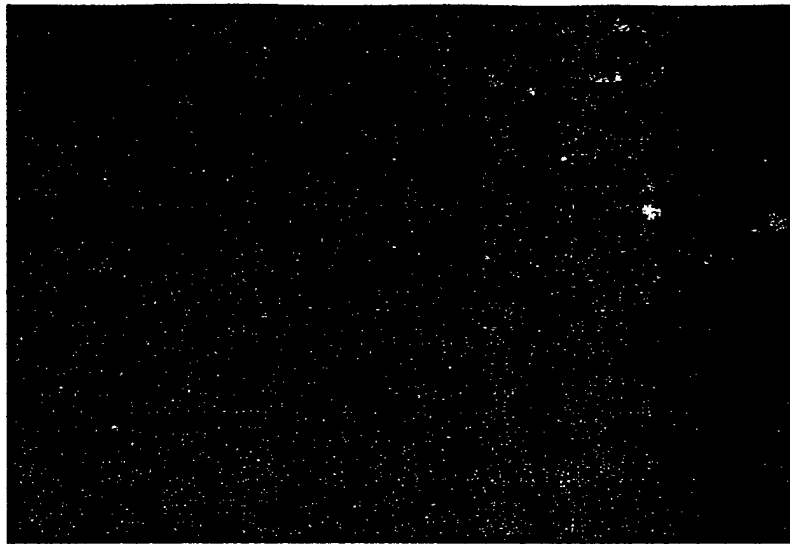
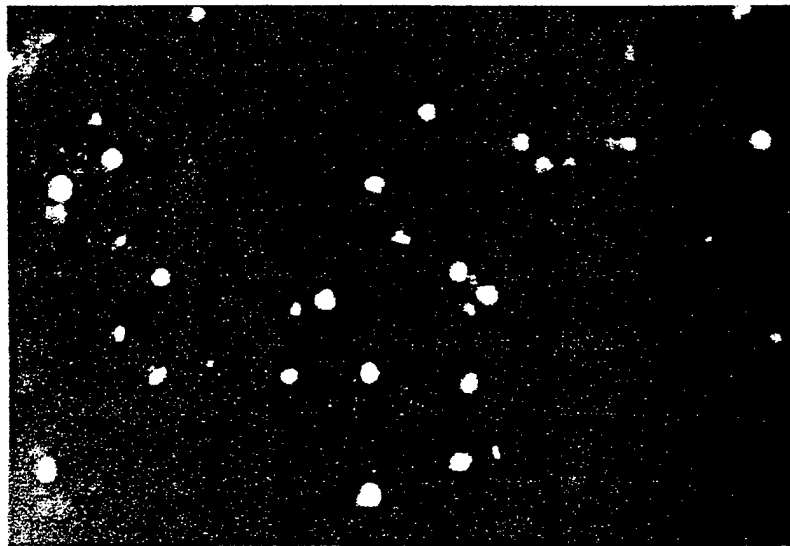


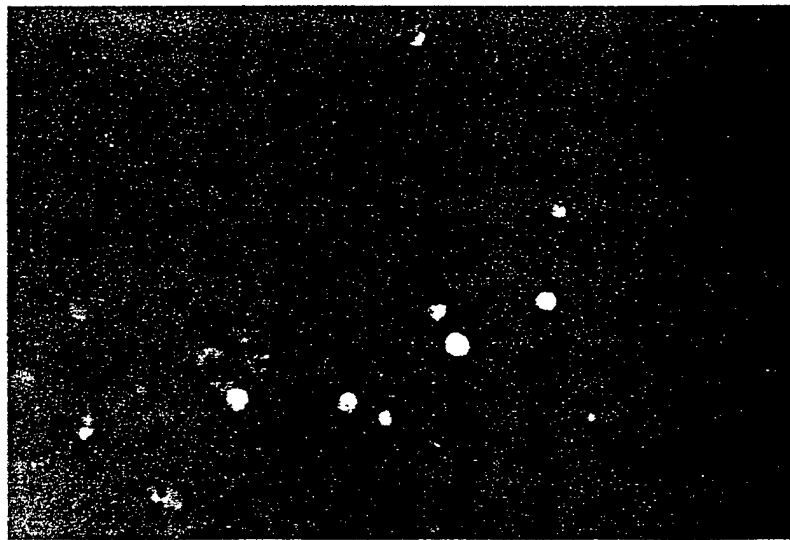
Figure 8



Normal



PB 250 $\mu$ M



PBN+PB

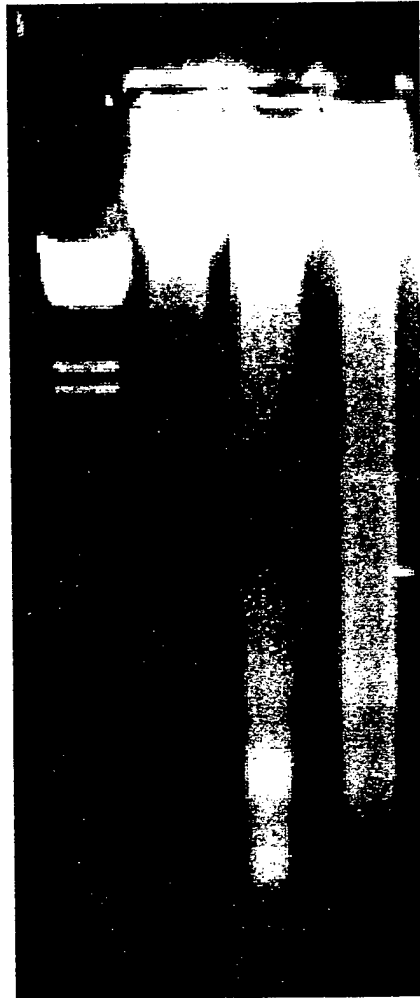
Figure 9

**DNA Marker**

**Normal**

**PB 250 $\mu$ M**

**PBN250 $\mu$ M+PB**



**Figure 10**

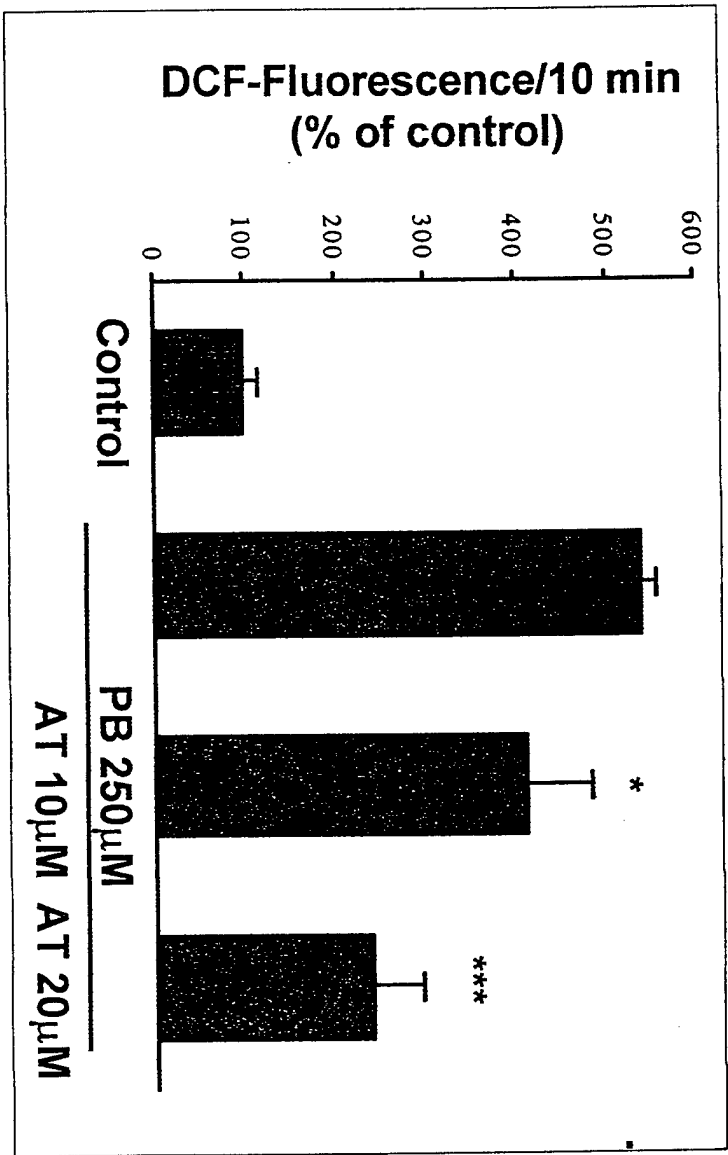
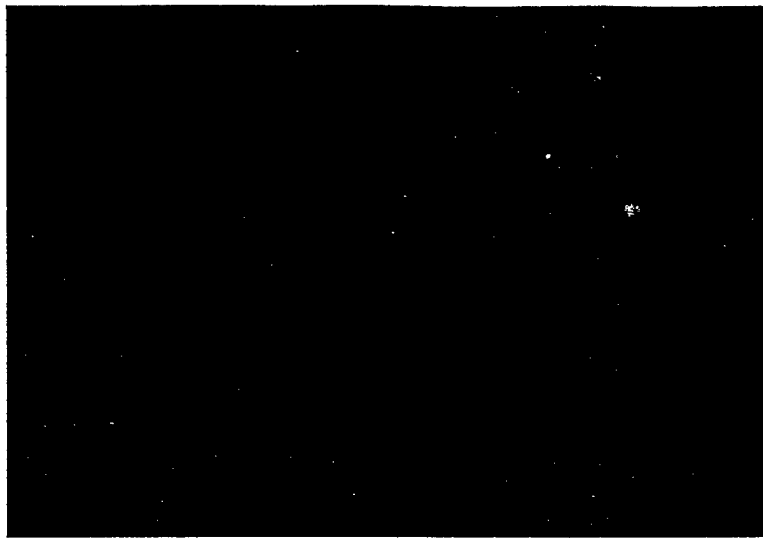
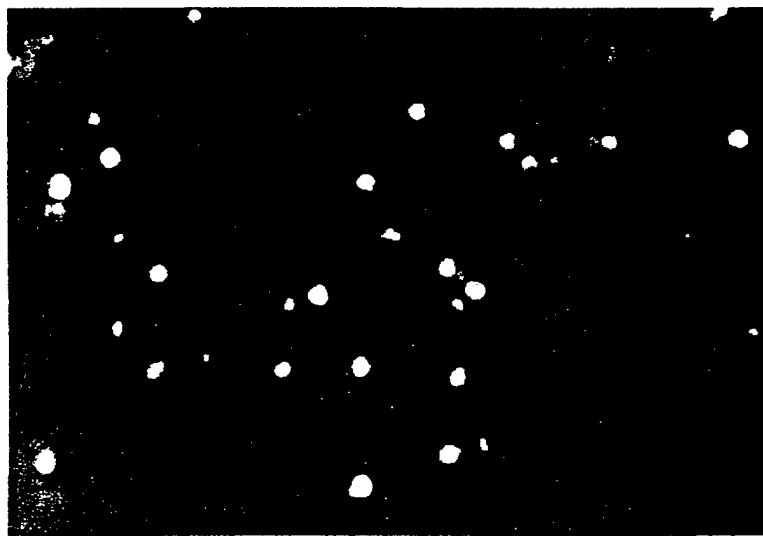


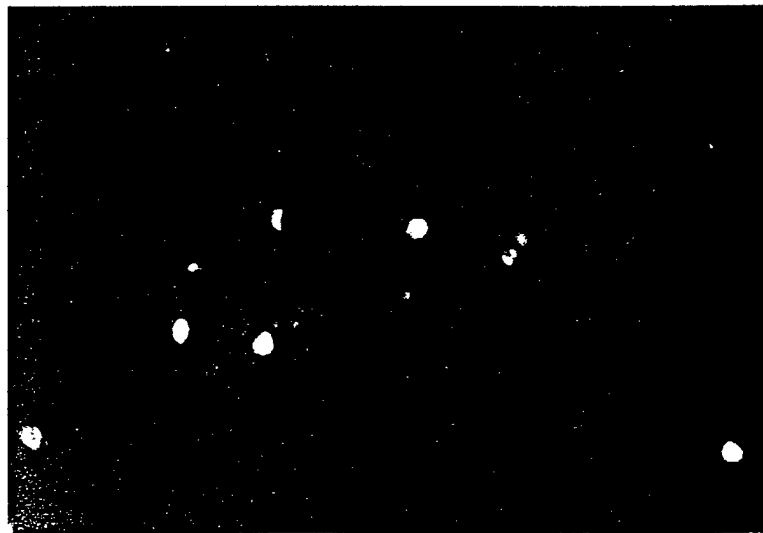
Figure 11



Normal



PB 250 $\mu$ M



AT+PB

Figure 12



**Figure 13**

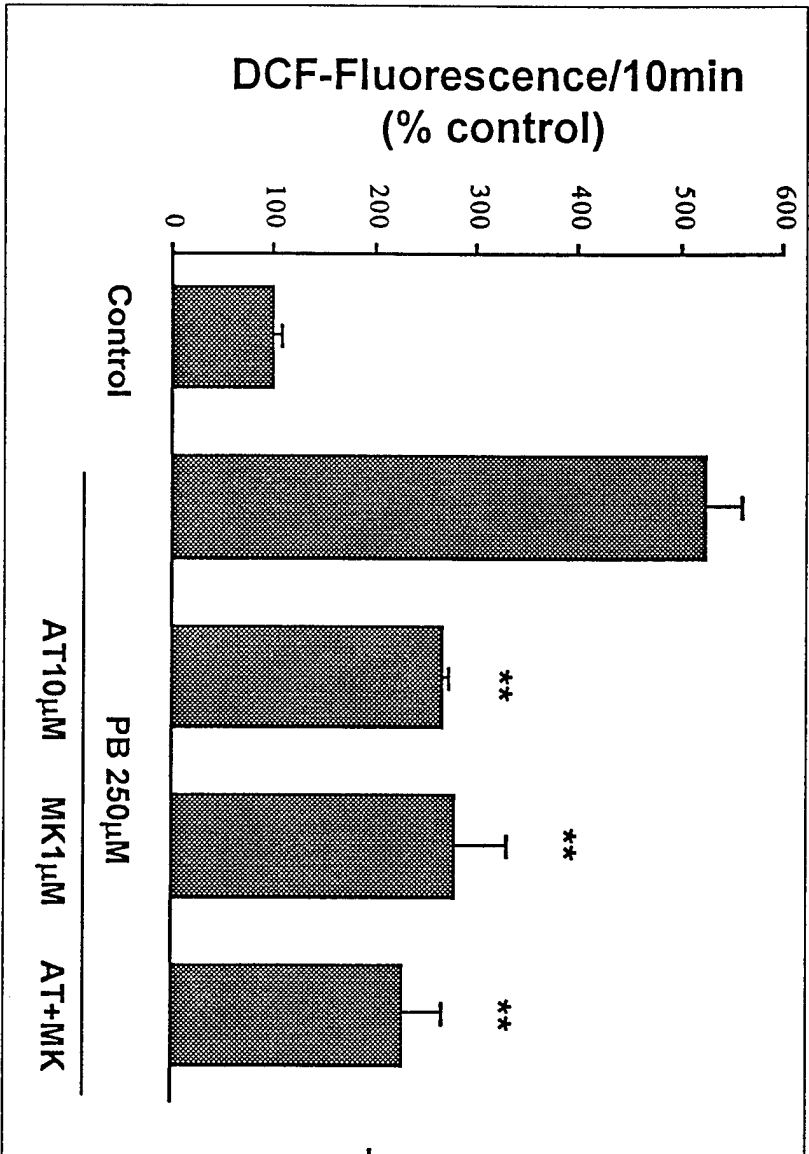
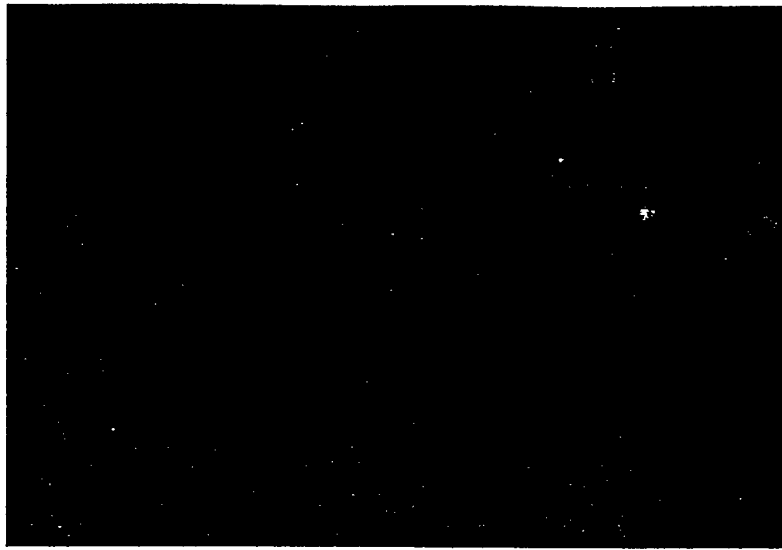
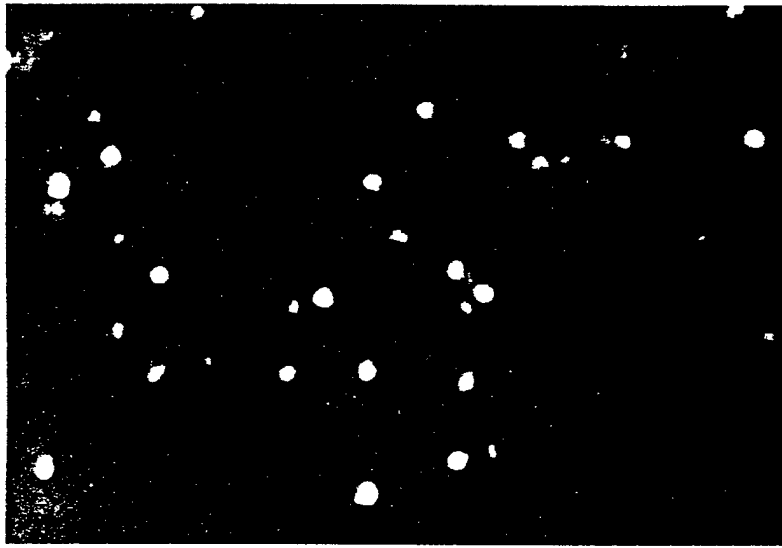


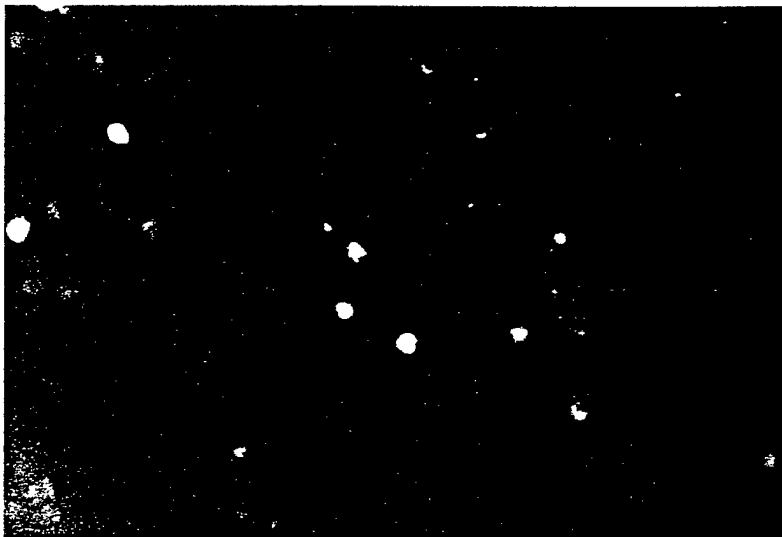
Figure 14



Normal



PB 250 $\mu$ M



MK801+PB

Figure 15



MK 801 block PB induced  
DNA fragment in cultured  
granule cells.

1. DNA standard.
2. Normal
3. PB 250uM.
4. MK 801 1uM+PB250uM

**Figure 16**

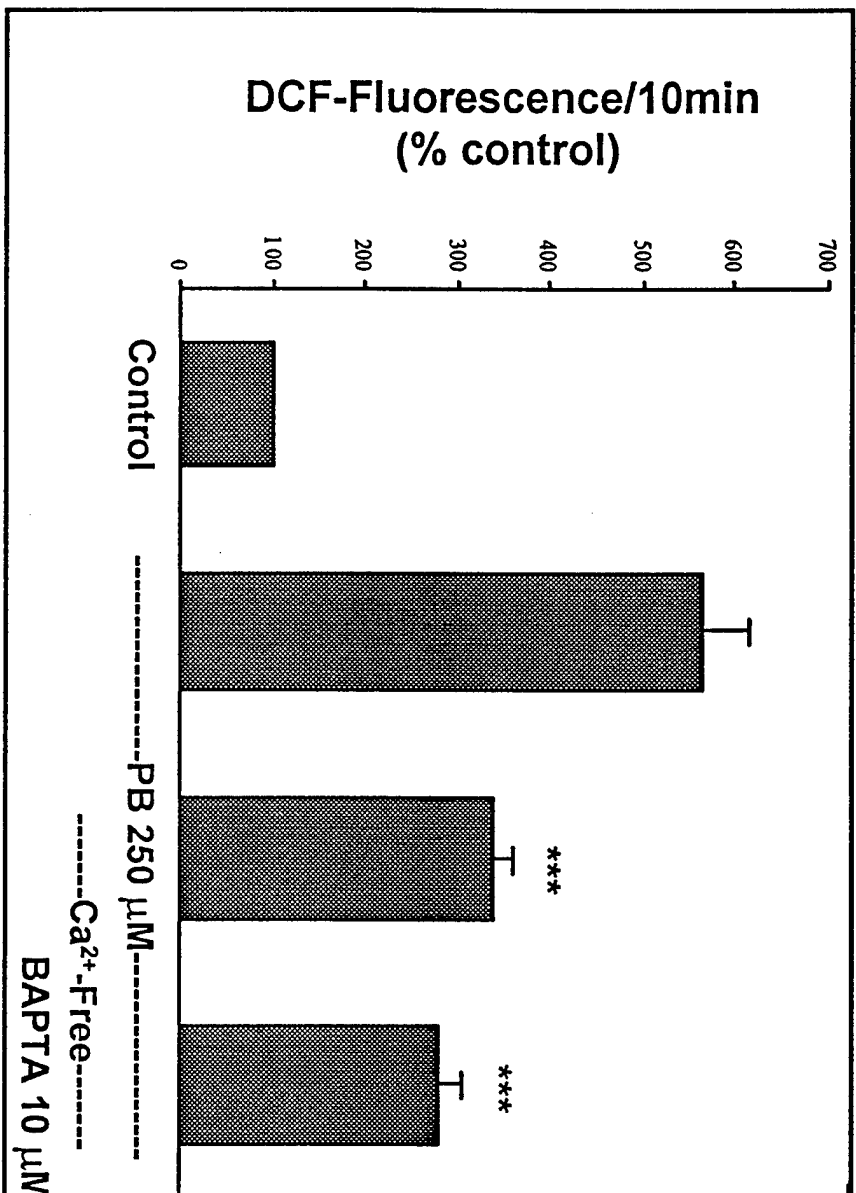


Figure 17

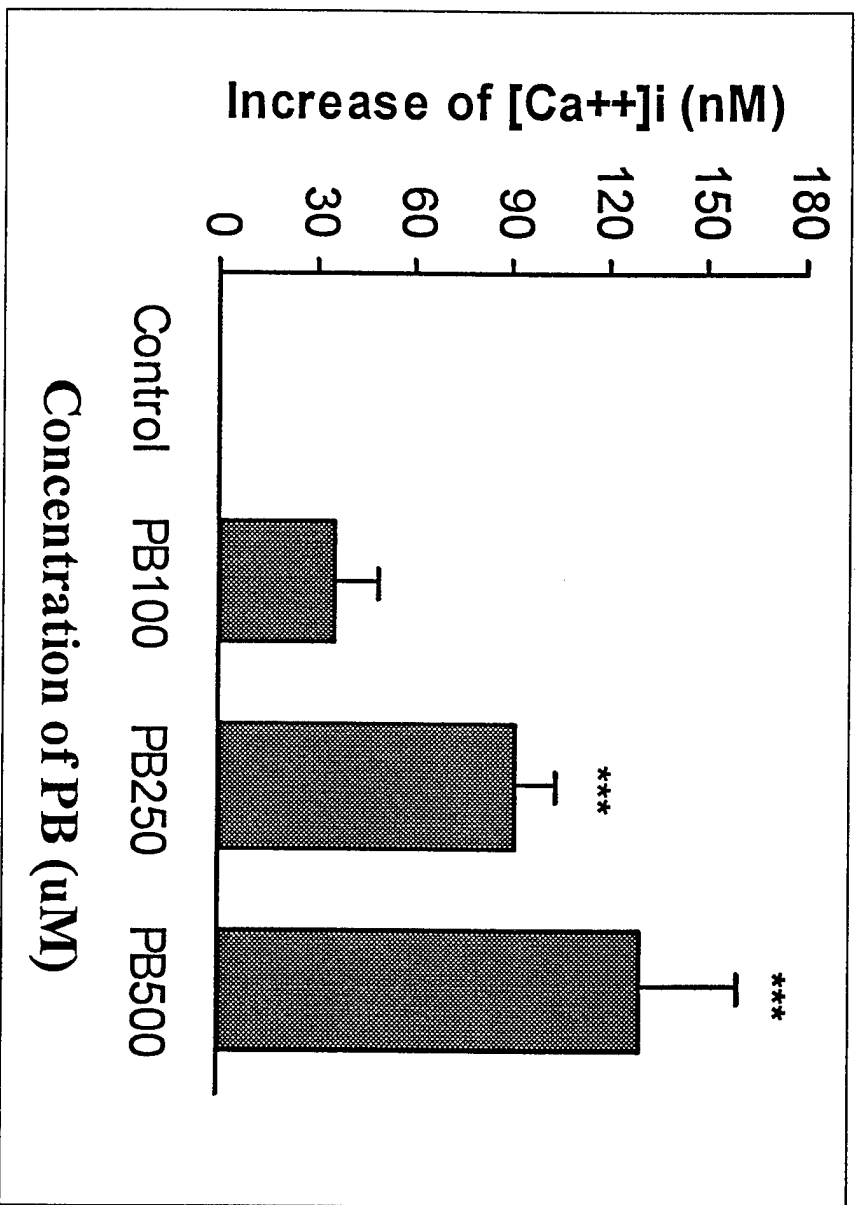


Figure 18

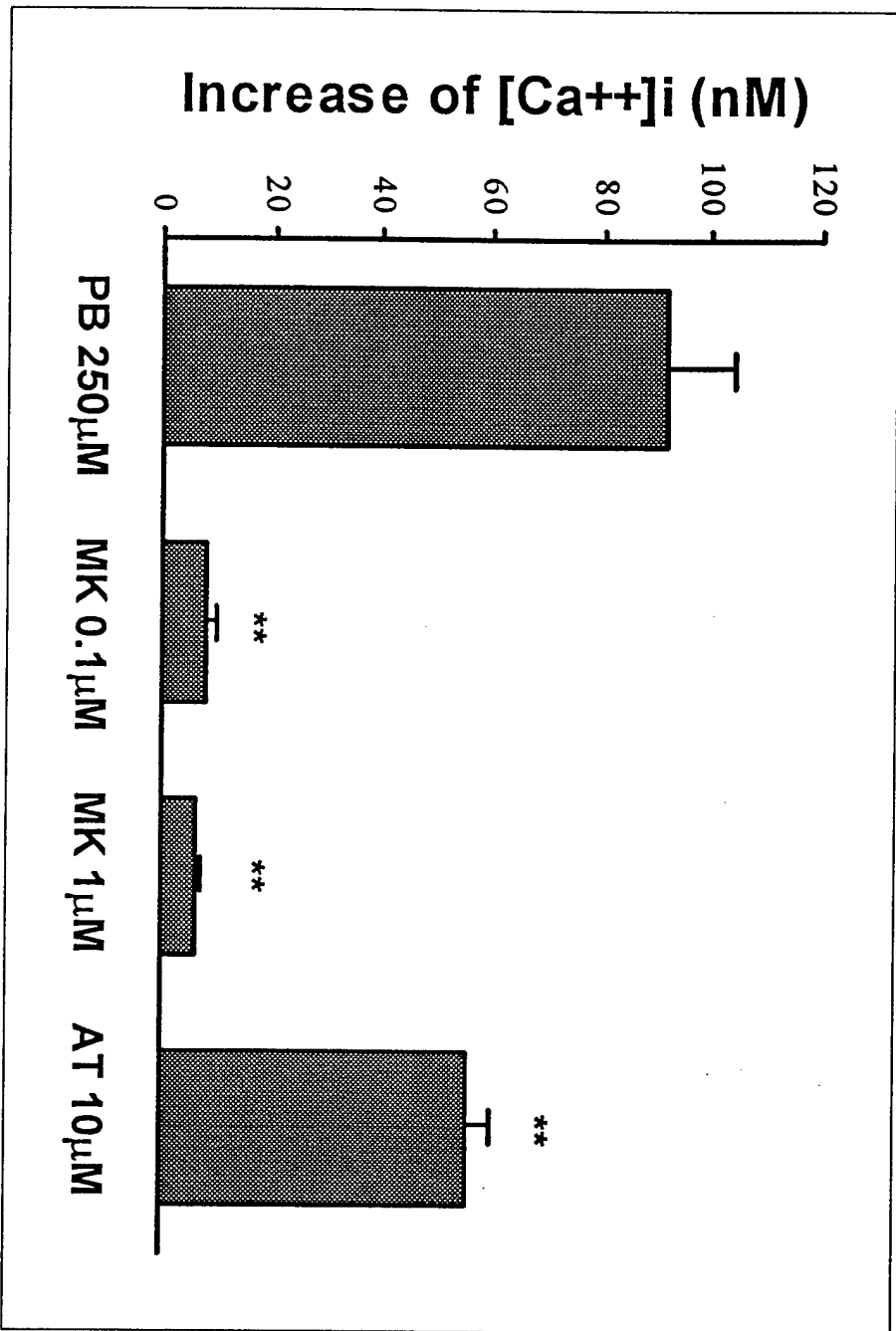


Figure 19

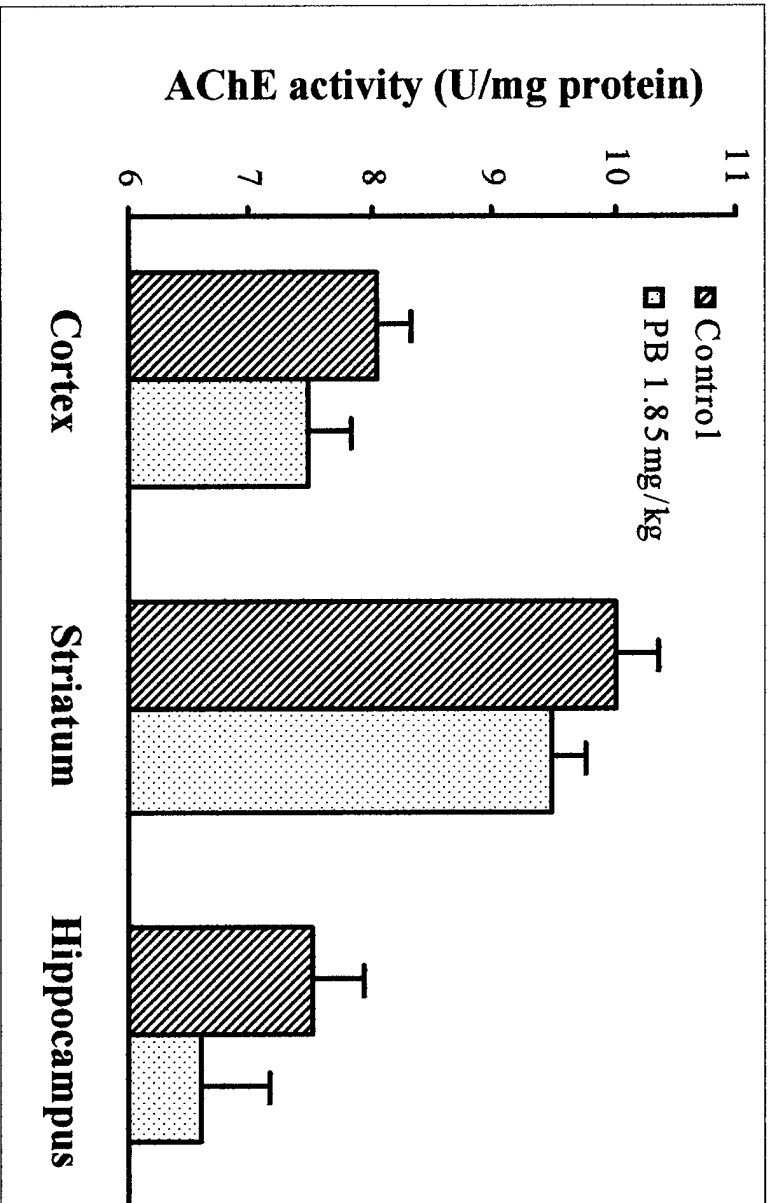


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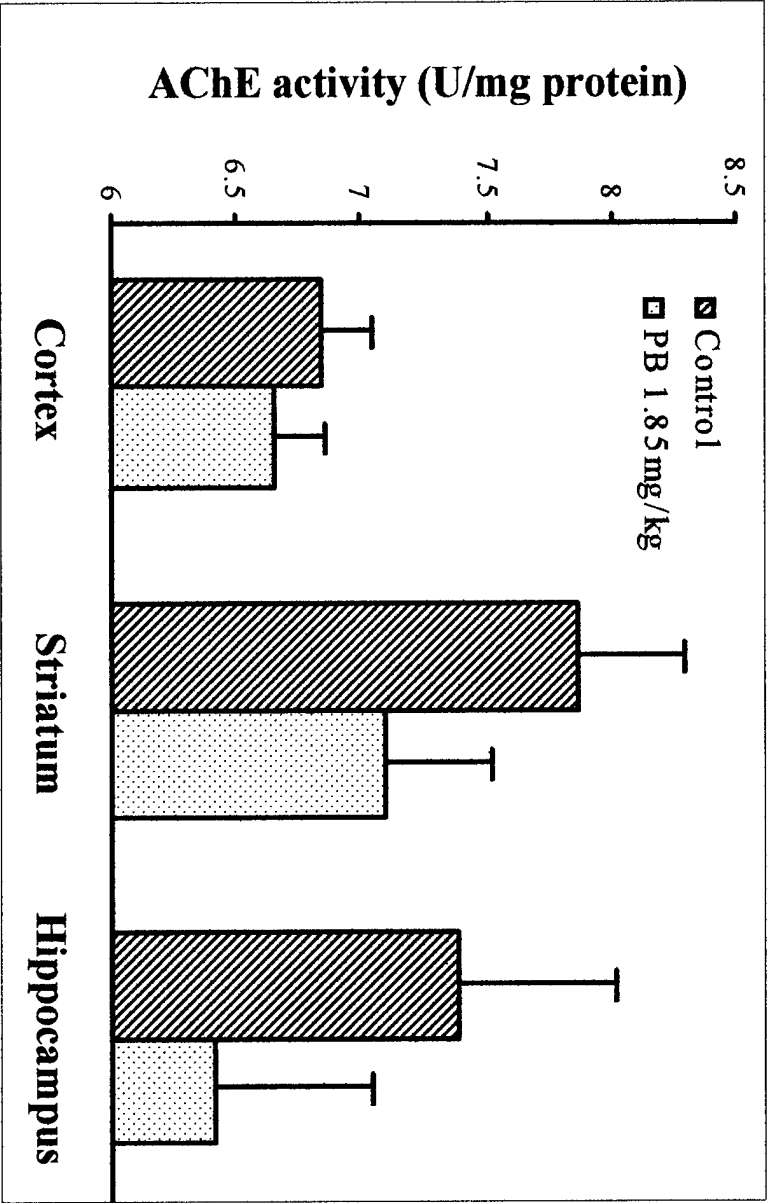


Figure 21

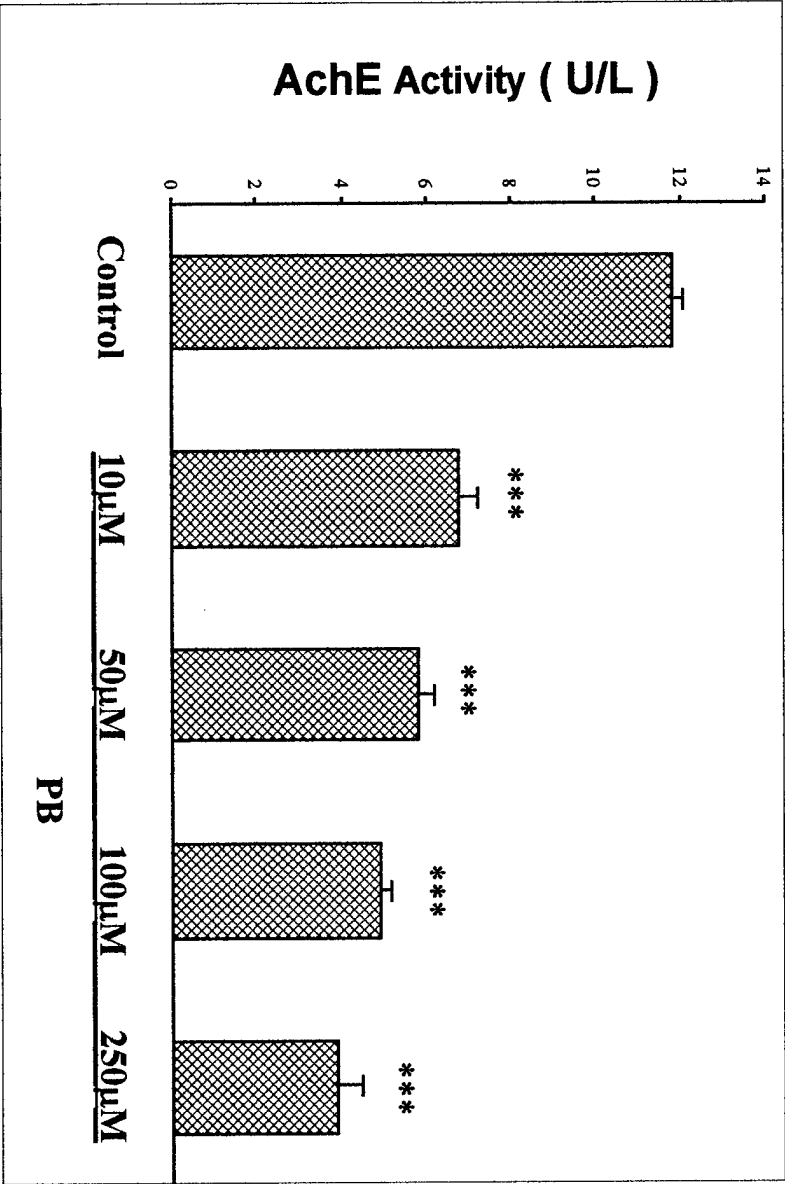


Figure 22