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DIFFERENTIAL EFFECTS OF TRIPHENYLPHOSPHITE AND DIISOPROPYL PHOSPHOROFLUORIDATE ON CATECHOLAMINE SECRETION FROM BOVINE ADRENOMEDULLARY CHROMAFFIN CELLS

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Types I and II organophosphorus compound-induced delayed neurotoxicity (OPIDN) is characterized by axonal degeneration. Type II compounds, however, uniquely cause cell body damage. Primary cultures of bovine adrenomedullary chromaffin cells were used to investigate and assess biochemically the cell body effects of the Type II compound triphenyl phosphite (TPP). Exocytotic secretion of neurotransmitter was measured to determine whether the cytotoxic action of TPP compromised synaptic events. TPP inhibited catecholamine secretion in both a time- and dose-dependent manner. By 4 h, TPP had inhibited nicotine-induced secretion by about 85%. TPP inhibited catecholamine secretion by about 35% as early as 15 min. The IC50 for TPP was about 45 μM. TPP inhibited secretion regardless of the secretagogue used, although nicotine-induced secretion was inhibited to the greatest extent. The Type I OPIDN diisopropyl phosphorofluoridate (DFP) and the nondelayed-type neurotoxic organophosphorus compound O,O-diethyl-O-4-nitrophenyl phosphate (paraoxon) did not inhibit catecholamine secretion from these cells. In contrast, when high potassium was used to induce secretion, significant stimulation was observed in the presence of DFP and paraoxon. Since Ca^{2+} homeostasis plays a key role in both exocytosis and neuronal necrosis, its uptake into the cells was measured radiometrically in the presence of TPP or DFP. Incubation with 100 μM TPP for 4 h resulted in the inhibition of $^{45}Ca^{2+}$ uptake evoked either by nicotine or K^+. No significant inhibition of $^{45}Ca^{2+}$ uptake was observed in the presence of DFP. TPP and DFP produced 95% and 88% inhibition, respectively, of the activity of the neurotoxic esterase enzyme (NTE), a putative target for OPIDN. Results suggest that these changes in the secretory mechanisms of the cell may be involved in the TPP-induced pathological alterations in chromaffin cells.

INTRODUCTION

The organophosphorus acid ester triphenyl phosphite (TPP) has long been recognized for producing delayed neurotoxicity. As early as the

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1930s, Smith and colleagues (Smith et al., 1930, 1932, 1933) described both the acute and delayed neurotoxic effects of TPP in rats and cats. More recently, TPP has been shown to be neurotoxic to hens (Carrington and Abou-Donia, 1988; Carrington et al., 1988). The primary clinical sign for all susceptible species is ataxia followed by hind limb paralysis. Pathological lesions occur in both the central and peripheral nervous systems and are species dependent (Carrington and Abou-Donia, 1988; Carrington et al., 1988; Veronesi and Dvergsten, 1987). Delayed neurotoxicity produced by TPP has been characterized as Type II organophosphorus ester-induced delayed neurotoxicity (OPIDN) (Abou-Donia and Lapadula, 1990; Abou-Donia, 1992). OPIDN is characterized by neurologic dysfunction to the central and peripheral nervous systems following a delay period of 1–2 wk. Segregation of organophosphorus compounds into two classes is based on a variety of parameters including their chemical structure, length of latency period, clinical signs, morphological changes, distribution of pathologic lesions, age and species sensitivity, and inhibition of neurotoxic esterase. Diisopropyl phosphorofluoridate (DFP) is an example of a direct-acting Type I compound, whereas TPP is exemplary of Type II compounds. Triphenyl phosphite is of particular interest because in addition to axonal degeneration, cell body degeneration occurs. Moreover, TPP produces lesions in high brain areas as well as in spinal cord (Tanaka et al., 1992). The pathological lesions produced by TPP are characterized by neuronal necrosis in the spinal grey matter and axonal swelling and degeneration in the brain stem.

Little is known about the mode of action of TPP. It inhibits hen brain neurotoxic esterase (NTE), which has been implicated as a putative target for delayed neurotoxic organophosphorus compounds (Johnson, 1982). TPP significantly inhibits NTE in hen (Carrington and Abou-Donia, 1988); however, only minimal inhibition of NTE was evident in rat (Veronesi et al., 1986). More recently, Konno et al. (1989) found that the activity of creatine kinase and succinate dehydrogenase from skeletal muscle tissue was significantly inhibited 24–48 h after treatment. Consistent with their finding, we found that TPP adversely affected the mitochondria by inducing ultrastructural changes (Knoth-Anderson et al., 1992). Pathologically TPP produced mitochondrial swelling, and biochemically it inhibited adenosine triphosphate synthesis. Neither DFP nor paraoxon had any effect on the cells.

Here we report the effects of Type I and Type II OPIDN-producing organophosphorus compounds on catecholamine secretion from primary cultures of bovine adrenomedullary chromaffin cells. The purpose of this study was to discern whether or not Type II compounds adversely affect the neuronal cell body by compromising their function, that is, synaptic transmission. Chromaffin cells serve as an excellent model for studying presynaptic events, because they develop from the neural crest stem cell. Since they lack axonal-like projections, chromaffin cells are

referred to as truncated sympathetic neurons (Knoth-Anderson and Abou-Donia, 1992). Preliminary accounts of this study have been presented (Abou-Donia and Knoth, 1989; Abou-Donia et al., 1990).

METHODS

Cell Culturing

Chromaffin cells were isolated from bovine adrenal medullae and maintained in primary cultures according to the method of Wilson and Kirshner (1983). Cells were plated using 24-well multiwell plates at a density of 5.2×10^5 cells/cm^2. Cultures were maintained at 37°C in a 5% CO_2/95% air mixture, and experiments were performed 3–7 d after plating. Serum levels were reduced to 1% on d 2 after plating to minimize fibroblast growth and to maximize drug treatment efficacy. Cells were treated with the various compounds as indicated.

Secretion Experiments

For secretion experiments, cells first were washed three times at room temperature with a balanced salt solution (BSS: 50 m*M* NaCl, 4.2 m*M* KCl, 1.0 m*M* NaH_2PO_4, 11.2 m*M* glucose, 10 m*M* HEPES, 2.0 m*M* $CaCl_2$, 0.7 m*M* $MgCl_2$, pH 7.4; Kilpatrick et al., 1982). Any remaining solution was aspirated and the cells incubated 10 min at 25°C in BSS. The BSS was aspirated and experiments were initiated by adding 0.45 ml buffer containing 10 μM nicotine, 100 μM veratridine, or 56 m*M* potassium chloride (equimolar substitution of KCl for NaCl) as the secretagogue. Cells were incubated at either 25°C (nicotine) or 37°C (K^+ or veratridine) for 15 min. Secretion was terminated by cooling the plates on ice and rapidly withdrawing the release medium from each well and transferring it to tubes containing 0.05 ml 0.5 *M* perchloric acid. Any remaining solution left in the wells was aspirated, and the soluble content was extracted by adding 0.4 ml 50 m*M* perchloric acid followed by a freeze-thaw cycle. Catecholamine content from both the media and cell extracts was determined fluorometrically (Merrills, 1963):

$$\text{Percent secretion} = \frac{\text{catecholamine released into the media}}{\text{total catecholamine}} \times 100$$

Total catecholamine includes both that released into the media and that in the cell extract.

NTE Assays

NTE was assayed according to the method of Johnson (1977). Cells were harvested by gently scraping the plates and then centrifuging them at 100 × g for 10 min. The pellet was resuspended in 1 ml 50 m*M* Tris, 1 m*M* EDTA, pH 8.0, and protein was determined by the Bradford method

(Bradford, 1976). NTE was assayed in cells that had been treated with 100 μM TPP, 100 μM DFP, or 1% vehicle (ethanol) for 24 h.

$^{45}Ca^{2+}$ Uptake

For $^{45}Ca^{2+}$ uptake studies, cells plated in multiwell plates were preincubated with 100 μM TPP, 100 μM DFP, or vehicle for 4 h at 37°C. Cells were washed, and $^{45}Ca^{2+}$ (2 μCi/million cells) was added with the appropriate buffer (10 μM nicotine in BSS or K^+ buffer) to the cells. Cells were incubated at room temperature for 1–5 min and then rapidly transferred to ice, the buffer aspirated, and the cells washed 3 × with ice-cold buffer. Cells were then covered with 400 μl 50 mM PCA and subjected to a freeze-thaw cycle. Aliquots of 100 μl were assayed by liquid scintillation counting.

Statistical Analysis

For all unpaired data, groups of two or more were analyzed for overall treatment effect using analysis of variance (ANOVA). If an overall treatment effect was observed, then differences between groups were assessed using Fisher's least significant difference test. For paired data, Student's *t*-test was used to assess differences between groups.

Materials

Triphenylphosphite and *O,O*-diethyl *O*-4-nitrophenyl phosphate were purchased from Aldrich (Milwaukee, Wis.). Diisopropyl phosphorofluoridate, veratridine, and nicotine were from Sigma (St. Louis, Mo.). All other chemicals were reagent grade.

RESULTS

TPP inhibited the exocytotic release of endogenous catecholamines in both a time- and dose-dependent manner. Figure 1 illustrates that nicotine-induced catecholamine secretion was inhibited with increasing concentrations of TPP. The IC50 was approximately 45 μM. For clarity, values have been normalized to a 100% scale. Nicotine-induced catecholamine secretion was inhibited by TPP in a time-dependent manner (Fig. 2) Fifty percent inhibition was achieved by 60 min, and maximal inhibition was reached in 4 h. Exposure to TPP for periods up to 24 h caused no additional inhibition. Incubation for 24 h did not cause cell death, as indicated by the cell's normal expression of catecholamine levels and its ability to exclude trypan blue (data not shown).

Three different secretagogues (nicotine, NIC; veratridine, VER; and high potassium, K^+) were utilized to ascertain whether or not the neurotoxic effect observed upon addition of the organophosphite was strictly a receptor-mediated phenomenon. All three secretagogues induce secretion of catecholamine but initially via separate mechanisms (Fig. 3). Nico-

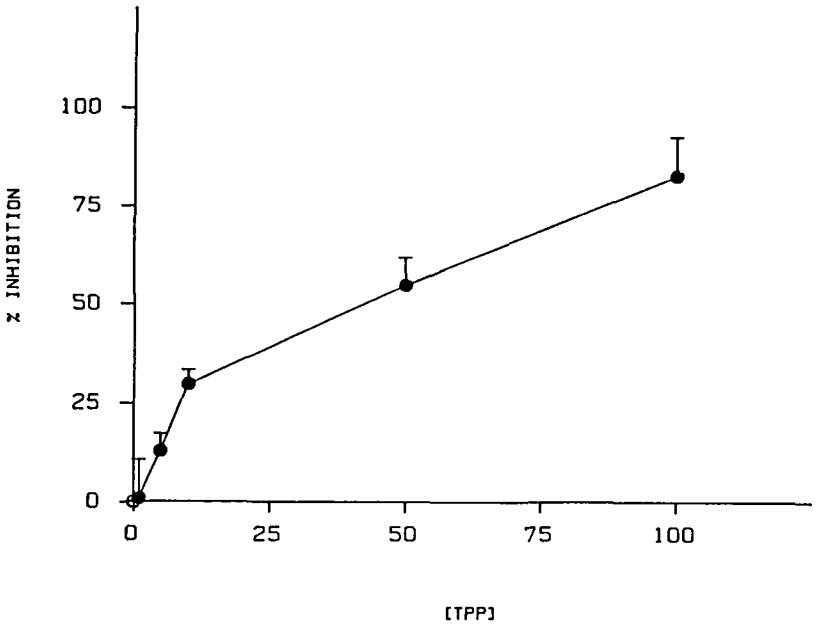

FIGURE 1. Dose-response curve for TPP-induced inhibition of catecholamine secretion from primary cultures of bovine adrenomedullary chromaffin cells. Cells were cultured and secretion experiments carried out as described in the methods. Cells were treated with triphenyl phosphite (TPP) (1, 5, 10, 50, or 100 μM) for 24 h at 37°C in a controlled environment. Vehicle (ethanol) did not exceed 1% of the total volume and had no effect on catecholamine content or catecholamine secretion. In the absence of inhibitor, a 15-min incubation at 25°C with nicotine induced release of 23.56 ± 1.9% endogenous catecholamine. Values were significantly different from control in the presence of 10 μM ($p < .01$), 50 μM ($p < .001$), and 100 μM ($p < .001$) TPP.

tine operates via the acetylcholine receptor, whereas depolarization of the membrane by high K^+ is strictly a non-receptor-mediated event. Veratridine, working via calcium-sensitive sodium channels, is partially receptor-mediated. Regardless of the secretagogue used, however, TPP significantly inhibited catecholamine secretion. DFP and paraoxon did not alter significantly secretion induced by nicotine. In contrast, both compounds significantly increased catecholamine secretion in the presence of high potassium. Veratridine-induced secretion, however, was stimulated only in the presence of paraoxon.

Calcium uptake into the cells was measured radiometrically at three time points under various conditions (Fig. 4). TPP inhibited stimulated uptake (either by nicotine or high potassium) of calcium at all time points compared to paired controls ($p < .05$). DFP did not have any significant effect on stimulated uptake of $^{45}Ca^{2+}$. Both TPP and DFP, however, altered $^{45}Ca^{2+}$ uptake at 5 min under basal conditions ($p < .05$). TPP apparently

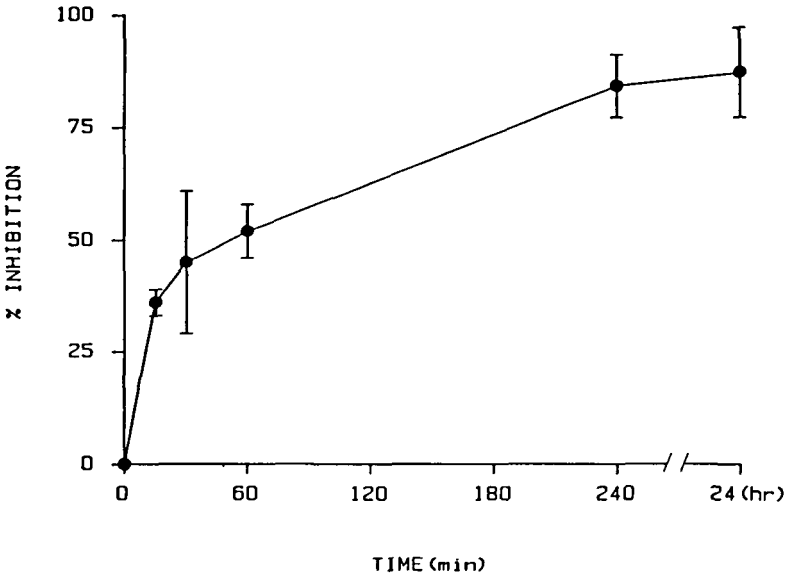

FIGURE 2. Time course for TPP-induced inhibition of catecholamine secretion from primary cultures of chromaffin cells. Cells were cultured and treated with TPP as described in the methods. Cells were incubated with 100 μM TPP for 15 min, 30 min, 60 min, 4 h, or 24 h. Cells were stimulated for 15 min at 25°C in the presence of 10 μM nicotine. Values were normalized to a 100% scale for comparison sake. Values at each time point have an $n = 3 \pm$ SD and are paired with control values at that same time point ($p < .001$ for 60 min and 4 h; $p < .005$ for 15 min and 24 h; $p < .10$ for 30 min).

stimulated calcium uptake approximately 25%, whereas DFP inhibited it about 35%.

TPP and DFP had similar effects on the NTE activity measured in these cells. Incubation with 100 μM TPP or DFP for 4 h significantly inhibited NTE compared to controls. NTE activity in the presence of TPP was inhibited 95% ($p < .001$) in the presence of TPP and 88% ($p < .005$) in the presence of DFP.

DISCUSSION

The Type II organophosphorus compound TPP is used widely as an antioxidant in the plastics and rubber industries (U.S. EPA, 1986). Although TPP was shown in the early 1930s to produce peripheral neuropathy in sensitive animal species, only recently has it been evaluated to determine its underlying mechanism of toxicity. TPP and other OPIDN-producing organophosphorus compounds have been shown to inhibit neurotoxic esterase (NTE; Padilla et al., 1987; Carrington and Abou-Donia, 1988; Veronesi et al., 1986). NTE has been proposed as a putative target for OPIDN (Johnson, 1982). TPP, however, is a much more effective inhibi-

tor of NTE in hens (100% inhibition; Carrington and Abou-Donia, 1988) than in rats (39% inhibition; Veronesi et al., 1986), despite TPP's ability to produce OPIDN in both species. Both TPP and DFP are effective inhibitors of NTE in chromaffin cells; however, only TPP is an effective inhibitor of synaptic transmission. These results indicate a less than primary role for NTE in the development of OPIDN.

Consistent with reports from Konno et al. (1989), we have also shown that the mitochondria are a primary target for TPP neurotoxicity in chromaffin cells (Knoth-Anderson et al., 1992). Only the Type II compound TPP was effective at inducing both morphological and biochemical

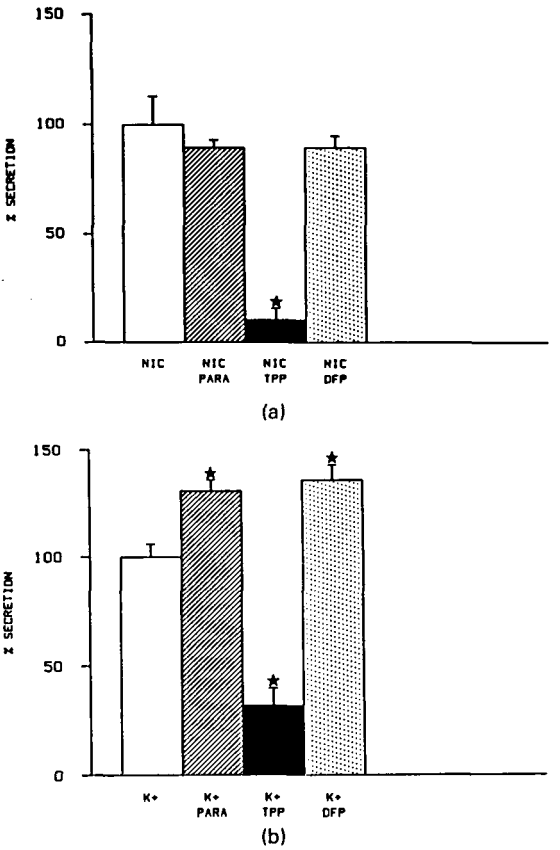

FIGURE 3. Effect of TPP and DFP on nicotine-, high potassium-, and veratridine-induced catecholamine secretion from adrenomedullary chromaffin cells. Cells were maintained in culture as previously described and were treated with either 100 μM TPP, 100 μM paraoxon, or 100 μM DFP for 24 h at 37°C in a controlled environment. Secretion experiments were carried out as described in the methods and values were normalized to 100% for comparison between secretagogues. Actual percent of endogenous catecholamine secreted are as follows: (a) 100% = 32.82 ± 4.28%; (b) 100% = 13.04 ± 1.09%.

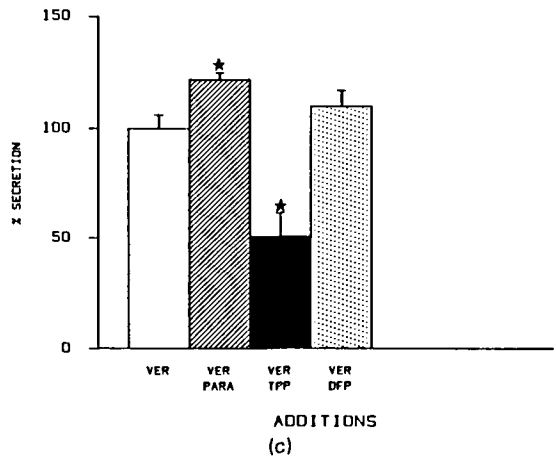

FIGURE 3. (*Continued*) Effect of TPP and DFP on nicotine-, high potassium-, and veratridine-induced catecholamine secretion from adrenomedullary chromaffin cells. Cells were maintained in culture as previously described and were treated with either 100 μM TPP, 100 μM paraoxon, or 100 μM DFP for 24 h at 37°C in a controlled environment. Secretion experiments were carried out as described in the methods and values were normalized to 100% for comparison between secretagogues. Actual percent of endogenous catecholamine secreted are as follows: (c) 100% = 48.69 ± 2.93%.

changes, that is, mitochondrial swelling and ATP synthesis inhibition, respectively. Both the Type I compound DFP and the nondelayed neurotoxicant paraoxon were ineffective. These results corroborate our current findings that TPP is a specific inhibitor of neural transmission. TPP inhibited catecholamine secretion from chromaffin cells in both a time- and dose-dependent fashion.

Chromaffin cells provide an excellent model to study neural transmission. Large quantities of adrenomedullary cells can be isolated as a rather homogeneous population and maintained in culture for up to 2 wk. The cells are derived from the neural crest and are sympathetic-like except for their lack of processes, that is, axons. They contain high concentrations of catecholamines and opiate peptides sequestered in intracellular vesicles, and exocytotically release their contents upon nicotinic stimulation of the cholinergic receptor.

TPP inhibited nicotine-induced catecholamine secretion in both a time- and dose-dependent fashion. An IC50 value with a micromolar concentration range of TPP is consistent with the high dose (1000 mg/kg) required to induce Type II OPIDN in rats (Veronesi et al., 1986; Carrington and Abou-Donia, 1988). To determine whether or not TPP selectively inhibited receptor-mediated exocytosis, various secretagogues were tested. Exocytosis can be elicited following stimulation of the acetylcholine receptor by nicotine. Secretion induced by nicotine is accom-

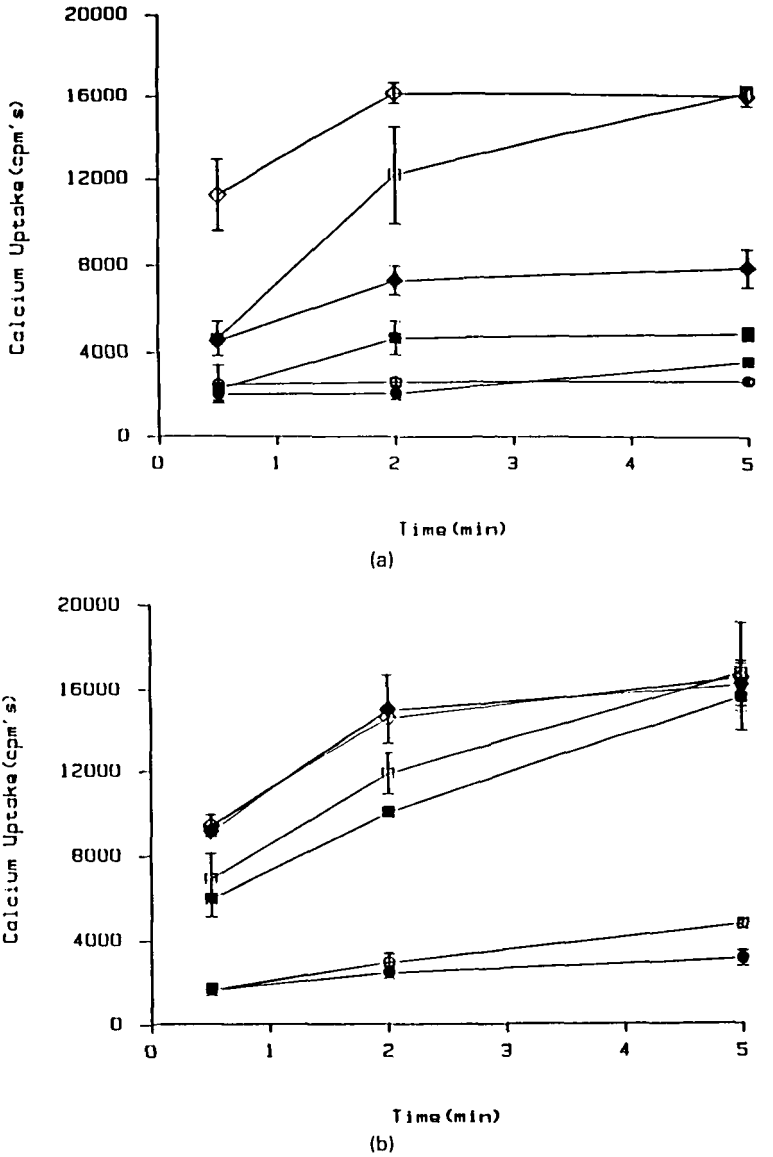

FIGURE 4. Effect of TPP and DFP on nicotine- and high potassium-evoked $^{45}Ca^{2+}$ uptake into primary cultures of chromaffin cells. Cells were cultured and treated as described in methods. $^{45}Ca^{2+}$ uptake was determined at the indicated time points and under the following conditions. (*a*) ○, 1% Ethanol; ●, 100 μM TPP; □, 10 μM nicotine; ■, 10 μM nicotine + 100 μM 5 TPP; ◇, 56 mM K^+; ♦, 56 mM K^+ + 100 μ TPP. (*b*) ○, No additions; ●, 100 μM DFP; □, 10 μM nicotine; ■, 10 μM nicotine + 100 μM DFP; ◇, 56 mM K^+; ♦, 56 mM K^+ + 100 μM DFP.

panied by both sodium and calcium influx via non-voltage-gated, receptor-linked ion channels into the cells. Secretion can also be induced by a high potassium concentration, such as 56 m*M*, which depolarizes the cell membranes and results in the uptake of calcium ions. Veratridine, an alkaloid neurotoxin, induces catecholamine secretion by increasing the inward flux of sodium through voltage-sensitive channels, which in turn leads to membrane depolarization and activation of calcium influx. TPP significantly inhibited secretion regardless of the secretagogue used; however, nicotine-induced secretion was inhibited most (about 90%), suggesting a partially receptor-mediated mechanism. Interestingly, both paraoxon and DFP stimulated potassium-induced secretion, while TPP inhibited secretion. TPP inhibition of secretion supports at least a partially receptor-mediated mechanism, because TPP is able to inhibit both receptor- and non-receptor-mediated secretions.

Consistent with the secretion data, TPP inhibited stimulated calcium uptake into the cells. A significant increase in potassium-evoked secretion occurred with calcium uptake into the cells in the presence of these neurotoxicants. Thus, despite a lack of correlation between increased secretion in the presence of DFP and changes in calcium uptake, there appears to be a correlation between TPP's inhibition of catecholamine secretion and calcium uptake. This correlation probably is not linked directly, since their time courses are very different.

Inhibition of catecholamine secretion and calcium uptake by TPP may be related to mitochondrial toxicity, which has been produced by TPP both in vitro (Knoth-Anderson et al., 1992) and in vivo (Konno et al., 1989). In chromaffin cells TPP induces mitochondrial swelling concurrently with an inhibition in ATP synthesis. Inhibition of ATP synthesis most likely results in the breakdown of ion gradients and in subsequent changes in the osmolality of the cell and its organelles. Changes in both the calcium levels and the osmolality of the cells alter their ability to secrete catecholamines via exocytosis (Livett, 1984; Hampton and Holz, 1983; Knoth et al., 1987). Ion deregulation and/or disruption in elemental homeostasis is known to alter numerous biochemical events (LoPachin and Saubermann, 1990). Inhibition of catecholamine secretion by TPP may be secondary to its cellular toxicity of chromaffin cells. Nevertheless, the resultant inhibition in synaptic transmission may be involved in the neurodegeneration produced in vivo. This study demonstrates the differential effects produced by Type I and Type II OPIDN compounds on exocytosis in chromaffin cells.

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