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Effect of tri-*o*-cresyl phosphate and methamidophos on ^{45}Ca uptake by brain synaptosomes in hens

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Abstract

The effect of tri-*o*-cresyl phosphate (TOCP) and methamidophos (MET) on potassium-stimulated ^{45}Ca uptake by brain synaptosomes in hens was studied. An in vivo test showed that TOCP increased potassium-stimulated calcium uptake 2 h after its administration, but that verapamil suppressed the enhancement of this calcium uptake. An in vitro test showed that lower concentrations of TOCP stimulated calcium uptake by synaptosomes, but that higher concentrations inhibited the uptake. In contrast, all tested concentrations of MET obviously inhibited calcium uptake; however, since calcium uptake was decreased by the administration of verapamil plus either TOCP or MET, the mechanism by which TOCP affects the voltage-operated calcium channel may be different from that of MET. The disruption of calcium homeostasis may be involved in organophosphate-induced delayed neurotoxicity (OPIDN). Calcium channel blocker may ameliorate OPIDN by maintaining calcium homeostasis in nerve cells.

Keywords: Organophosphate-induced delayed neurotoxicity (OPIDN); Tri-*o*-cresyl phosphate (TOCP); Methamidophos (MET); Verapamil; Calcium uptake

1. Introduction

Organophosphorus compounds are a diverse group of chemicals used primarily as pesticides, plasticizers, plastic softeners, flame-retardants, antioxidants, and hydraulic fluids. Exposure to almost all organophosphorus pesticides (OPs) can induce acute toxicity in human and animals due to inhibition of acetylcholinesterase (AChE). Single or multiple doses of some OPs, such as methamidophos (MET), dichlorvos and chlorpyrifos can additionally induce delayed effects; so-called organophosphate-induced delayed neurotoxicity (OPIDN), which is characterized by distal axonal degeneration and secondary demyelination of central and peripheral axons [1]. Tri-*o*-cresyl phosphate (TOCP) has been the prototype neuropathy-inducing agent, and adult hens are usually the animal model for experimental studies of OPIDN.

Although the inhibition and subsequent aging of neurotoxic esterase (NTE) has been proposed to

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be the initiating event in OPIDN [2], the events that occur between NTE inhibition and the appearance of clinical effects are not completely understood. Previous studies showed that neurodegeneration and the breakdown of cytoskeletal elements were accompanied by increased levels of axonal free calcium [3]. George et al. [4] confirmed that an influx of calcium into axons is associated with axonal degeneration and demyelination. Previous studies speculated that the free calcium level might rise during the development of OPIDN [5,6]. The administration of dichlorvos caused an increase in intrasynaptosomal Ca^{2+} in rat brain [7]. It has been proposed that NTE inhibition by OPs is associated with an increase in $[Ca^{2+}]_i$, which, in turn, may activate Ca^{2+}/calmodulin-dependent kinase leading to aberrant phosphorylation of cytoskeletal proteins and their accumulation, a common feature of many neurodegenerative diseases [5,7]. On the other hand, high concentrations of calcium can also activate calcium-dependent neutral proteases, especially calpain, which has been associated with the breakdown of cytoskeleton elements, thereby leading to Wallerian degeneration [8]. Furthermore, it has been shown that some calcium channel blockers, such as verapamil and nimodipine, can ameliorate the clinical symptoms and histological lesions of nerves during the development of OPIDN [9,10]. However, the mechanisms of both OPIDN development and the neutralizing effect of calcium channel blockers on OPIDN are not understood.

It is well known that there are several pathways regulating calcium concentration of which the calcium channels in membranes are very important. To understand the role of calcium in OPIDN, we investigated the effect of TOCP and MET, a pesticide widely used in China, on potassium-stimulated calcium uptake in the brain synaptosomes of adult hens.

2. Materials and methods

2.1. Chemicals

Tri-*o*-cresyl phosphate and physostigmine sulfate were purchased from BDH Chemicals (Poole, England). Bovine serum albumin (BSA), PPO, POPOP and verapamil were purchased from Sigma Chemicals (St. Louis, MO, USA). Methamidophos was obtained from the Shandong Pesticide Factory (Jinan, China). Coomassie brilliant blue G-250 was obtained from Fluka Biochemika (Buchs, Switzerland). $^{45}CaCl_2$ was purchased from Amersham–Phamacia Biotech (Little Chalfont, England).

2.2. In vivo test

2.2.1. Animal treatment

Adult Haisai laying hens (about 12 months old and weighting about 1.5 kg), purchased from the Dabei Poultry Farm (Beijing, China), were used in this study. Birds were housed one per cage and were acclimatized for at least 1 week prior to the start of this 21-day experiment. Hens were divided into three groups: a control (C) group, TOCP + verapamil (T + V) group, and TOCP (T) group. The hen house was maintained at about 22 °C with a light/dark cycle of 12 h each day. All hens of the T and T + V groups were given a single oral dose of TOCP (750 mg/kg) in a gelatin capsule while the control hens were given an empty gelatin capsule. Verapamil dissolved in saline was injected daily into the hens' breast muscle at a dose of 7.0 mg/kg for 4 days in a slight modification of the method described by el-Fawal et al. [9].

2.2.2. Preparation of crude synaptosomes

Synaptosomes were prepared from hen brain as previously described [11] with some modification. Briefly, hen brain tissue was homogenized in 0.35 M sucrose with a glass/Teflon homogenizer and then centrifuged at 1000*g* for 10 min at 4 °C. The resultant supernatant was then centrifuged at 10,000*g* for 30 min at 4 °C. The supernatant was discarded leaving the residual pellet of crude synaptosomes (P_2 fraction). The P_2 fraction was homogenized gently with 0.35 M sucrose to obtain a synaptosome suspension. The concentration of protein in the synaptosome solution samples was determined by the method of Bradford [12] using BSA as a standard and the solution was then diluted for immediate use in the ^{45}Ca uptake experiment.

2.2.3. $^{45}Ca^{2+}$ uptake in synaptosomes

$^{45}Ca^{2+}$ uptake in the synaptosomes was measured according to the method of Zhang et al. [13]. The sample solution (synaptosomes) was divided into two groups; one in high potassium (55 mM KCl) and the other in low potassium (5 mM KCl). Two hundred and fifty micrograms protein of the synaptosomes was added to standard medium, consisting of 120 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 30 mM Tris, 10 mM glucose, 0.1 mM $CaCl_2$, 1.5 mM Na_2HPO_4–NaH_2PO_4 (pH 7.4). The mixture was preincubated for 10 min at 30 °C. Fifty microliters of $^{45}CaCl_2$ (about 0.2 μCi) with 550 mM KCl was added to the high potassium group and the same amount of $^{45}CaCl_2$ with 550 mM NaCl to the low potassium group after which the mixtures were further incubated for 45 s at 30 °C. The final volume of the reaction system was 500 μl. The reaction was stopped by adding 2 ml of ice-cold standard medium, and the mixture was filtered immediately through glass microfiber filters (ϕ 0.8 μm). The filters were washed three times with 2 ml of ice-cold stop solution and then allowed to air dry. The radioactivity of ^{45}Ca on the filter was counted in 3 ml of scintillation solution (5 g PPO and 0.3 g POPOP in 1 liter toluene) by using Beckman LS 6000 liquid scintillation spectrometer. The net K^+-stimulated uptake was expressed as CPM values of the difference of $^{45}Ca^{2+}$ uptake across the synaptosomal membrane between the high potassium-stimulated and non-stimulated samples.

2.3. In vitro test

2.3.1. Animals

Healthy adult hens were used (see Section 2.2.1).

2.3.2. Preparation of brain synaptosomes

The synaptosome samples were prepared as described in Section 2.2.2.

2.3.3. $^{45}Ca^{2+}$ uptake in synaptosomes

$^{45}Ca^{2+}$ uptake was examined as described in Section 2.2.3, except that 50 μl of different concentrations of the tested organophosphate solution, with or without verapamil, was added to the reaction medium after 9 min preincubation at 30 °C, after which incubation was continued at 30 °C for 1 min before starting the reaction with $^{45}Ca^{2+}$.

2.4. Statistics

Data were generally expressed as mean ± standard error. Groups of data were compared by ANOVA and by post hoc analysis using Student–Newman–Keuls multiple range test. A difference between means was considered significant at $P < 0.05$.

3. Results

3.1. In vivo test

As shown in Table 1 and Fig. 1, by 2 h after TOCP treatment potassium-stimulated calcium uptake in synaptosomes had increased by up to 157% of the control ($P < 0.05$), while the calcium uptake value in the T + V group hens was 127% of the control ($P < 0.05$). This indicates that verapamil had begun to block calcium influx at this stage. By 4 h post administration, the amount of calcium uptake in the T group was 79% of the control, similar to that in the T + V group which was 80% of the control ($P < 0.05$). On day 1, calcium uptake in both the T and T + V groups was higher than that of the control group; on day 3, compared with the control, calcium uptake in the

Table 1
Effect of TOCP and verapamil on calcium uptake by synaptosomes in vivo[a]

Time after treatment	^{45}Ca uptake value ($\times 10^3$ CPM/mg protein)		
	Control	TOCP	TOCP + verapamil
2 h	28.2 ± 6.8	48.7 ± 2.2*	37.0 ± 4.3**
4 h	32.8 ± 2.5	26.3 ± 1.5*	26.2 ± 2.9*
1 day	31.1 ± 2.0	66.0 ± 2.5*	65.7 ± 4.9*
3 days	30.3 ± 0.6	23.3 ± 0.9*	42.0 ± 5.3*,**
5 days	34.4 ± 1.4	34.2 ± 2.1	36.7 ± 3.2
7 days	32.3 ± 1.4	37.5 ± 1.9*	38.7 ± 3.3*

[a] The data are means ± SE, $n = 3$.
* Significantly different from control group, $P < 0.05$.
** Significantly different from TOCP group, $P < 0.05$.

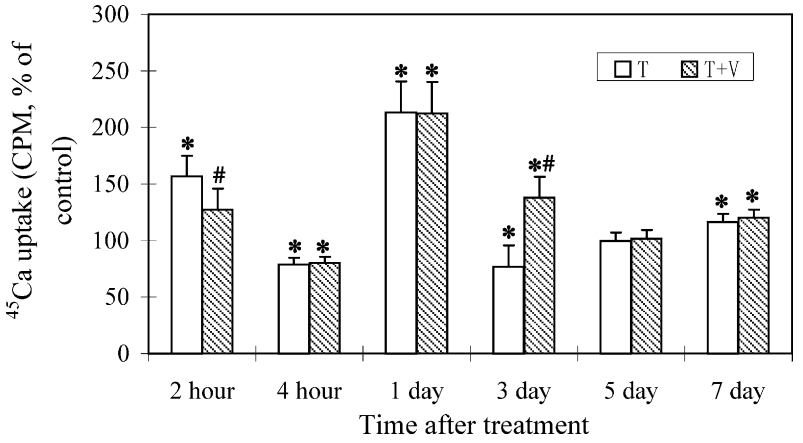

Fig. 1. Effect of TOCP and TOCP plus verapamil on calcium uptake by synaptosomes in vivo. The hens were treated as described in Section 2.2.1. The data are means ± SE ($n = 3$); *: $P < 0.05$, compared with control; #: $P < 0.05$, compared with TOCP alone.

T group declined, while that in the T + V group increased; calcium uptake levels in treated hens began to approach to those of the control group from the beginning of the fifth day.

The trend in calcium uptake ability in the T group was also similar to that in the T + V group. However, on day 3, it became distinctly different; the level of calcium uptake in the T group declined to its lowest level (77% of the control), while that in the T + V group increased by up to 137% of the control ($P < 0.05$).

3.2. In vitro test

As indicated in Table 2, calcium uptake in synaptosomes declined gradually with the increasing TOCP concentration. At higher concentrations, TOCP inhibited calcium influx; for example, the uptake was 63% of the control at a concentration of 1.0×10^{-4} M TOCP; but at lower concentrations, TOCP stimulated calcium influx; for example, at a concentration of 1.0×10^{-8} M, the uptake was 138.6% of the control ($P < 0.05$).

Table 2 also shows that calcium uptake decreased gradually as the concentration of MET increased. The calcium uptake value was 81% of the control ($P < 0.05$) at a concentration of 1.0×10^{-4} M MET; however, at lower concentrations, MET still inhibited calcium uptake.

As shown in Table 3, when TOCP (1.0×10^{-5} M) alone was added to the reaction system,

Table 2
Effect of different concentrations of TOCP and MET on calcium uptake by synaptosomes in vitro[a]

Concentration (M)	^{45}Ca uptake value ($\times 10^3$ CPM/mg protein)	
	TOCP	Methamidophos
1.0×10^{-8}	37.5 ± 1.9 (140%)*	24.7 ± 3.4 (93%)
1.0×10^{-7}	35.1 ± 7.2 (130%)	23.1 ± 3.3 (86%)
1.0×10^{-6}	28.4 ± 7.9 (106%)	24.6 ± 2.4 (92%)
1.0×10^{-5}	27.0 ± 3.6 (101%)	23.1 ± 3.3 (86%)
1.0×10^{-4}	20.5 ± 8.7 (76%)	21.6 ± 2.9 (81%)*

[a] Data in the table are means ± SE ($n = 3$). Numbers in parentheses represent the percentage of the control.

* Significantly different from control group ($26.8 \pm 0.6 \times 10^3$ CPM/mg protein), $P < 0.05$.

Table 3
Effect of organophosphates, with or without verapamil, on calcium uptake by synaptosomes in vitro[a]

Treatment	^{45}Ca uptake value ($\times 10^3$ CPM/mg protein)
TOCP (1.0 ± 10^{-5} M)	27.0 ± 3.6 (101%)
Methamidophos (1.0 ± 10^{-5} M)	23.1 ± 3.3 (86%)
Verapamil (2.0 ± 10^{-4} M)	16.2 ± 4.9 (60%)*
TOCP + verapamil	14.2 ± 0.5 (52%)*,**
Methamidophos + verapamil	7.5 ± 4.1 (28%)*,**,***

[a] Data in the table are means ± SE ($n \geqslant 3$). Numbers in parentheses represent the percentage of the control.

* Significantly different from control ($26.8 \pm 0.6 \times 10^3$ CPM/mg protein), $P < 0.05$.

** Significantly different from TOCP or methamidophos, $P < 0.05$.

*** Significantly different from verapamil alone, $P < 0.05$.

the synaptosomal calcium uptake was 101% of the control, but when verapamil (2.0×10^{-4} M) alone was present, the calcium uptake value was 60% of the control ($P < 0.05$). When both TOCP and verapamil were added to the reaction medium, the calcium uptake value was 52% of the control ($P < 0.05$); however, this value was not significantly different from that of verapamil group ($P > 0.05$), suggesting that TOCP and verapamil may not inhibit synergistically potassium-stimulated calcium uptake.

Table 3 also indicates that MET (1.0×10^{-5} M) alone inhibited calcium uptake, which occurred at a level of 86% of the control. When MET and verapamil were both added calcium uptake was 28% of the control ($P < 0.05\%$). These results also suggest that MET and verapamil have a synergistic effect on potassium-stimulated calcium uptake.

4. Discussion

It is well known that calcium is an intracellular messenger in signal transduction and consequently plays a significant role in a host of cellular functions. Previous research found that neuropathic organophosphates might disturb the calcium regulation system of cells, such as the Ca^{2+}–ATPase and voltage-operated calcium channel (VOCC) in the cell membrane [6].

Although no delayed neuropathy in the brains of hens treated with TOCP or MET was found, we believe that the physiological function of neurons in the brain was affected by the OPIDN-causing compounds. Our data showed that the organophosphorus compound TOCP can cause changes in the K^+-stimulated calcium uptake ability of synaptosomes that may be associated with the VOCC of brain synaptosomes. The in vivo test showed that the amount of calcium uptake in synaptosomes had increased 2 h after the administration of TOCP, which suggests that calcium influx through VOCC might increase. However, the calcium uptake value was relatively low, indicating that verapamil had begun to inhibit calcium influx through the VOCC. We speculate that calcium influx increased during the early onset period of OPIDN (i.e., 2 h after treatment), indicating that the free calcium concentration in synaptosomes may also have increased. On day 3, calcium uptake in the T + V group was higher (not lower) than in the T group, possibly because the target synaptosomes were surrounded by a higher concentration of verapamil due to the continuous administration of the latter. It is known that very high concentrations of verapamil, e.g., 200 μM, can inhibit the Na^+ channel and then inhibit the depolarization of neural cells [14]. In this case, the VOCC may close. High K^+ abruptly stimulated the depolarization of synaptosomes which may have kept the VOCC open too long thereby causing an increased influx of calcium.

The in vitro test showed that the effect of TOCP on the K^+-stimulated calcium uptake of synaptosomes was concentration dependent. Lower concentrations of TOCP stimulated calcium uptake by synaptosomes, but higher concentrations inhibited it. In contrast, all tested concentrations of MET, an insecticide widely used in China, obviously inhibited calcium uptake; however, the fact that calcium uptake was decreased by the administration of verapamil plus either TOCP or MET suggests that the mechanism by which TOCP affects VOCC may be different from that of MET. Verapamil and MET at certain concentrations can synergistically inhibit calcium uptake by synaptosomes, resulting in lower calcium uptake value than that produced by the MET or verapamil treatment alone. These results suggest that the two chemicals can co-inhibit calcium uptake by synaptosomes.

It is well known that TOCP is a typical toxicant that can induce delayed neurotoxicity in several species including man; however, MET has been found to induce delayed neurotoxicity in humans but not in the hen [15]. Our data suggested that the mechanism through which MET induces delayed neurotoxicity might be different from that of TOCP. At usual concentrations, TOCP stimulated the calcium uptake of brain synaptosomes that may lead to a high level of intracellular calcium, which is perhaps the cause of the progression of the delayed neurotoxicity; however, MET at usual concentrations inhibited the calcium uptake of brain synaptosomes in hens, which may lead to a lower concentration of intracellular calcium and is

perhaps one of the reasons that the signs of delayed neurotoxicity are usually not observed in hens exposed to MET. We speculate that TOCP might cause changes in K^+-stimulated calcium channel openings resulting in increased calcium influx followed by a rise in the free calcium concentration of neural cells. The calcium channel blocker verapamil can inhibit this calcium influx by blocking calcium channels. Therefore, calcium channel blockers may ameliorate OPIDN because they can maintain calcium homeostasis in nerve cells. However, further in vivo research is required to understand the effect of MET on the calcium channels of synaptosomes during the development of the delayed neurotoxicity induced by MET.

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