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Role of muscarinic signal transduction and CREB phosphorylation in dichlorvos-induced memory deficits in rats: An acetylcholine independent mechanism

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ABSTRACT

The present study was designed to explore the alternative mechanism (other than AChE inhibition) for chronic, low-level exposure to dichlorvos, an organophosphate, *in vivo*. Dichlorvos, at a dose of 1.0 and 6.0 mg/kg body weight (b.wt.) for 12 weeks, showed impairment in neurobehavioral indices viz. rota rod, passive avoidance and water maze tests. Though high dose of dichlorvos had a detrimental effect on acetylcholinesterase activity, no significant inhibition was seen with low dose of dichlorvos. Western blot analysis and immunofluorescence studies showed a significant reduction in the expression of M_1, M_2 and M_3 muscarinic receptor subtypes in high dose group animals, whereas in low dose group animals only the M_2 receptor subtype was reduced significantly. Further, the signal transduction cascade linked to these receptor subtypes was affected in high dose group animals whereas in low dose group only adenylyl cyclase-linked signaling pathway was impaired. Finally, the phosphorylation of CREB, a memory enhancing transcription factor, was significantly reduced in both low dose and high dose group animals. Thus, the present study reveals the significance of M_2 muscarinic receptor linked adenylyl cyclase signaling pathway and phosphorylation of CREB in the development of neurobehavioral impairments after chronic low-level exposure to dichlorvos.

1. Introduction

Organophosphate (OP) compounds, well-known cholinesterase inhibitors, continue to be the major cause of morbidity and mortality in third world countries. In developing countries, over 3 million poisonings occur annually due to these pesticides, out of which 220,000 are fatal (Eddleston et al., 2002). In addition to their use in the control of insects and pests, some cholinesterase inhibitors are used as therapeutic agents (Cummings et al., 2008) as well as chemical warfare agents (Balali-Mood and Balali-Mood, 2008; Jett, 2007). Dichlorvos (DVPP), an organophosphorus pesticide, has been used as a crop protectant and as a general public health insecticide since 1961. Because of its anticholinesterase properties DVPP shows both nicotinic and muscarinic signs and symptoms in the peripheral and central nervous systems which include nausea, vomiting, lacrimation, salivation, bradycardia, miosis and finally death may occur due to respiratory failure (Deer et al., 1993).

Humans exposed to low levels of OP agents in industrial or agricultural settings have reported difficulty in concentration as well as memory impairment, long after the exposure has ceased (Jett et al., 2001; Rohlman et al., 2007). Fiedler et al. (1997) have shown that long-term use of OPs without any evidence of acute poisoning appears to produce subtle changes in neuropsychological test performance, such as, slower reaction time. Several consequences of chronic, low-level exposure to pesticides are not directly attributable to the accumulation of acetylcholine since tissue acetylcholinesterase activity returns to normal level in about 3–4 months, whereas the neurotoxic effects such as neurobehavioral impairments remain long after the cessation of OP exposure (Milby, 1971; Eskenazi et al., 1999). Farahat et al. (2003) have demonstrated that low-level exposure to OP pesticides in rats impaired their cognitive functions without any significant effect on acetylcholinesterase activity, suggesting an alternative mode of action for OP pesticides after chronic low-level exposure.

It has been shown that nanomolar concentrations of various organophosphate compounds cause a significant reduction of muscarinic receptors both in neonate and adult rats (Costa et al., 1982; Yamada et al., 1983; Guo-Ross et al., 2007). Another potential cellular target of organophosphates that has been identified by several laboratories, is adenylyl cyclase and hence the synthesis of cAMP. Studies have demonstrated that Chlorpyrifos (CPF) and other organophosphates inhibit the synthesis of cAMP (Ward and Mundy, 1996). Stimulation of adenylyl cyclase catalyzes the synthesis of cAMP that increases cAMP-dependent protein kinase

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(PKA)-mediated phosphorylation of several proteins, including cAMP responsive element binding protein (CREB) (Shaywitz and Greenberg, 1999). CREB is phosphorylated on its Serine-133 residue by a variety of kinases including PKA, and then is translocated to the nucleus where it serves as a transcription factor for genes that carry the CRE consensus sequence, 5′-TGACGTCA-3′, in their promoter region. Numerous studies have indicated that CREB is also critical to several forms of use-dependent synaptic plasticity and transcription-dependent forms of memory (Bourtchuladze et al., 1994). As dichlorvos is known to impair motor and memory functions, we wanted to investigate the mechanism underlying these effects, may be via an alteration in the signal transduction pathways coupled to muscarinic receptors that may further lead to phosphorylation of CREB and hence affect the memory functions independent of AChE inhibition.

2. Materials and methods

Dichlorvos was purchased from Hindustan Ciba Geigy Ltd., Mumbai, India. Polyclonal antibodies for muscarinic receptor subtypes were obtained from Santa Cruz Biotech., USA. Phosphoplus CREB (Ser-133) antibody was obtained from Cell Signaling Technology Inc., Beverly, MA, USA. Protein kinase C assay kit was purchased from Upstate Cell Signaling Solutions, USA. cAMP enzyme immunoassay kit was purchased from R&D System, USA. [^{32}P] ATP (specific activity 5000 mCi/mmole) and [^{3}H] ATP were obtained from Board of Radiation & Isotope Technology (BRIT), Mumbai, India. All other chemicals used in the study were of the highest quality available.

2.1. Animals and their use

Male albino rats (Wistar strain) in the weight range of 100–120 g were housed in polypropylene cages and kept in a well-ventilated room under hygienic condition. Animals were provided standard rat pellet diet (Hindustan Lever Ltd., Bombay, India) and water *ad libitum*. Ethical clearance for killing of animals was duly obtained from the institute's ethical committee.

2.2. Experimental design

The animals were divided into following three groups (6 animals/group). Dichlorvos dissolved in corn oil was injected subcutaneously for 12 weeks. Doses were based on the previous studies carried out in the lab.

Control group: Animals received an equal volume of corn oil (vehicle) as administered to the animals of dichlorvos-treated group.
Low dose group: Animals received 1 mg/kg b.wt./day dichlorvos for 12 weeks.
High dose group: Animals received 6 mg/kg b.wt./day dichlorvos for 12 weeks.

Animals were examined for any deficit in motor and memory functions at 6th and 12th weeks. Additionally, serial estimations of serum cholinesterase activity were performed in these animals. Finally, animals were anaesthetized with sodium pentathol and sacrificed by decapitation. The whole brain was isolated, rinsed in ice-cold physiological saline (0.9% NaCl) and used for different experiments.

2.3. Neurobehavioral studies

2.3.1. Motor function test

Rota rod test was carried out by the method of Dunham and Miya (1957). Animals were initially trained to maintain themselves on the rotating rod for a period of more than 3 min. Subsequently after a period of 24 h, the animals were again screened for their ability to remain on the rotating rod for three successive trials of 3 min each.

2.3.2. Memory function tests

2.3.2.1. Passive avoidance test. Experiments were performed by the method of Piala et al. (1959) using a shuttle box apparatus, which consisted of a unit with a dark unlit chamber and an illuminated chamber separated by a controllable door. The floor consisted of a metal grid wired to deliver shocks of controlled intensities and durations. First day each rat was placed into the illuminated compartment and allowed to explore both chambers of the apparatus for 5 min. On second day, the rats were placed into the illuminated chamber of the apparatus, one at a time. As soon as rat entered into the dark chamber, the door was closed, and a foot shock was applied (0.1 mA, 40 V). After the shock, the rat was removed and returned to its home cage. On day 3, each rat was placed into the illuminated chamber individually, and the latency to enter into the dark chamber was measured, which served as a measure of retention of avoidance response.

2.3.2.2. Morris water maze test. This test was carried out by the method of Morris (1984).

2.3.3. Acquisition test

A water tank of 2.1 m diameter was filled with water up to 8 cm from the top of the tank; a nontoxic white paint was dispersed in the water to make it opaque. A platform with an 8 cm × 8 cm top surface was placed in the middle of one quadrant about 24 cm from the side. The top surface of the platform was submerged about 1 cm below the surface of the water. All rats were given four training trials (acquisition) on days 1–4. During each training trial the rat was placed into the water with its nose facing the side of the tank at one of the four randomly selected locations corresponding to each quadrant of the maze, and then it was released. The time spent in searching the hidden platform was recorded.

2.3.4. Retrieval test

On day 5, the platform was removed, and each rat was placed in the center of the tub facing the same direction and allowed to swim for 90 s. The time spent in the target area (where the platform had been positioned on days 1–4) was recorded. The 4-day acquisition test is considered a measure of spatial reference memory, and the retrieval test is considered a measure of the strength of spatial memory.

2.4. Biochemical studies

2.4.1. Preparation of synaptic plasma membrane

Synaptic plasma membranes (SPMs) were prepared from the rat brains by discontinuous sucrose density gradient centrifugation by the method of Jones and Matus (1974). The proteins were quantitated by the method of Lowry et al. (1951).

2.4.2. Acetylcholinesterase assay

The acetylcholinesterase activity was assayed in the serum/SPMs according to the method of Ellman et al. (1961). For brain AChE, butyryl cholinesterase was inhibited by the addition of 10 μM ethopropazine to the assay mixture and the change in absorbance was measured at 412 nm for 2 min at 30 s intervals.

2.4.3. Western blot analysis for muscarinic receptor subtypes

The membrane protein was prepared as described by Wang et al. (2001) and resolved on 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) and transferred onto nitrocellulose (NC) membrane. The protein blots were incubated with respective primary antibodies (M_1 and M_3, 1:100 and M_2 1:150) at 4 °C overnight, followed by incubation with horseradish peroxidase-conjugated anti-goat IgG antibody (1:1000, Bangalore Genei, India) for 1 h. Immunoreactive protein was visualized by diaminobenzidine (DAB) and relative concentrations of proteins were detected by densitometry analysis. NC membranes were stripped with stripping solution (0.5 M Tris–HCl, pH 6.8, 10% SDS and β-merceptoethanol) and reprobed with GAPDH (Santa Cruz 1:5000) at 37 °C for 1 h as loading control.

2.4.4. Immunofluorescence staining of muscarinic receptors in different regions of rat brain

About 30 μm thin sections of rat brain cerebral cortex and hippocampus were prepared and rinsed in phosphate buffer saline (PBS) three times for 5 min each. Respective primary antibodies were added in a dilution of 1:50 and the slides were incubated for 1 h at 37 °C. Further the slides were washed three times with PBS and incubated with secondary antibody (1:20, FITC labeled) for 30 min at 37 °C. Again, the slides were washed three times for 5 min each in PBS and visualized under a fluorescence microscope.

2.4.5. Phospholipase C activity

Phospholipase C activity was measured by the method of Bergers et al. (1989). Brain tissue was homogenized for 20 s in 50 mM Tris–HCl buffer (pH 7.4) followed by centrifugation at 3000 × *g* for 10 min. The supernatant was re-centrifuged at 40,000 × *g* for 10 min. To obtain the cytosolic fraction, the above supernatant was again centrifuged at 100,000 × *g* for 60 min and the resulting supernatant was stored at −70 °C until used further.

The phospholipase C activity was assayed in a reaction volume of 200 μl containing 50 μl [^{3}H] labeled PIP_2, 50 μl enzyme sample and 100 μl HEPES buffer (100 mM HEPES (pH 7.0) and 10 mM LiCl). The contents were incubated at 37 °C for 30 min. The reaction was stopped by cooling on ice followed by the addition of 1 ml chloroform–methanol–HCl (100:100:6) and 0.3 ml of 1 M HCl. The phases were separated by centrifugation and an aliquot (0.5 ml) of upper phase was transferred into a scintillation vial containing aqueous scintillation fluid and radioactivity measured.

2.4.6. Ca^{2+}/CaM-dependent protein kinase assay

Ca^{2+}/Calmodulin-dependent phosphorylation was quantitated according to the method of Suwita et al. (1986). The standard assay system for Ca^{2+}/CaM kinase contained 50 mM MOPS buffer (pH 7.0), 10 mM $MgCl_2$, 0.25 mg/ml BSA, 1.0 mg/ml histone IIS, 100 mM [γ-^{32}P] ATP, 5 μg calmodulin and 0.1 mM $CaCl_2$ (where required) in a final volume of 50 μl. Reaction was initiated by addition of 20 μl enzyme preparation. Following an incubation of 5 min, 25 μl aliquots were drawn and spotted on phosphocellulose strips (P81, 1 cm × 2 cm). The control samples contained 20 μM EGTA in the reaction mixture in addition to other constituents. Filters were immersed in 75 mM phosphoric acid to terminate the reaction. The strips were washed thrice