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Paraoxon inhibits GABA uptake in brain synaptosomes ☆

Asghar Ghasemi [a,b,*], Ahmad Sadidi [c], Moslem Mohammadi [a], Ali Khoshbaten [a,d], Alireza Asgari [a,d]

[a] Department of Physiology and Biophysics, Baqiyatallah University of Medical Sciences, P.O. Box 19945-581, Tehran, Iran
[b] Prevention of Metabolic Disorders Research Center, Research Institute for Endocrine Sciences, Shaheed Beheshti University of Medical Sciences, Tehran, Iran
[c] Department of Neurosurgery, Baqiyatallah University of Medical Sciences, Tehran, Iran
[d] Research Center for Chemical Injuries (RCCI), Baqiyatallah University of Medical Sciences, Tehran, Iran

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Abstract

To investigate possible effect of paraoxon (10^{-9}–10^{-3} M) on GABA uptake, we used rat cerebral cortex synaptosomes. K_m and V_{max} of GABA uptake were determined in presence of paraoxon (10^{-3} M). Acetylcholine and its antagonists (atropine and mecamylamine) were used for evaluating cholinergic-dependency of uptake. Type of transporter involved was determined by using glial (beta-alanine) and neuronal (DABA) GABA uptake inhibitors. The results of the study showed that paraoxon at low doses (10^{-9}–10^{-6} M) increased and at high doses (10^{-5}–10^{-3} M) decreased GABA uptake. One millimolar paraoxon significantly decreased V_{max} (175.2 ± 4.23 vs. 80.4 ± 2.03, $P < 0.001$) of GABA uptake while had no effect on its K_m. DABA significantly decreased GABA uptake ($P < 0.001$) while beta-alanine had no effect. In conclusion, present data suggests that paraoxon probably acts as non-competitive antagonist of GABA uptake.

Keywords: Paraoxon; Synaptosome; GABA; Uptake; V_{max}; K_m

1. Introduction

Parathion is an organophosphate compound (OP) frequently used as an insecticide (Zhang and Malhotra, 2005) and due to its stability in aqueous solutions, is probably responsible for most accidental OP poisoning cases (Taylor, 1996). Paraoxon is the metabolic product of parathion (Zhang and Malhotra, 2005) which causes cholinesterase inhibition with low propensity to aging and good reactivatability (Thierman et al., 2005). Although the main mechanism for OP action is cholinesterase inhibition leading to accumulation of acetylcholine in the central and peripheral nervous systems (Cecchini et al., 2004), recent studies indicate that this is not the sole mechanism underlying the toxicity of OPs (Weinbroum, 2004; Rocha et al., 1996). It has been suggested that non-cholinergic mechanisms may play some roles in neurotoxicity of the paraoxon (Rocha et al., 1996). Since the major concern in OP poisoning is convulsions, which partially resist current treatments (Emerson et al., 1999), it has been suggested that neurotransmitter systems other than the cholinergic may be involved in OP-induced convulsions (McDonough and Shih, 1997); among these, the GABAergic system seems to be the foremost candidate. Shih et al. (1999) reported that almost all drugs that have beneficial effects

Abbreviations: AOAA, aminooxyacetic acid; BSA, bovine serum albumin; GABA, γ-aminobutyric acid; GAT, GABA transporter; L-DABA, L-diaminobutyric acid; LDH, lactate dehydrogenase; OP, organophosphate.

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* Corresponding author. Address: Department of Physiology and Biophysics, Baqiyatallah University of Medical Sciences, P.O. Box 19945-581, Tehran, Iran. Tel./fax: +98 21 22281561.
E-mail addresses: Ghasemi@erc.ac.ir, Ghasemi.asghar@yahoo.com (A. Ghasemi).

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against OP-induced convulsions increase inhibitory activity of the GABAergic system, whereas on the other hand it has been shown that the number of GABA transporters decrease in epileptic tissues (Bradford, 1995). Based on our knowledge, so far the effect of the paraoxon on GABA transporters have not been studied, hence this study was designed to determine the effect of paraoxon on GABA uptake in synaptosomes prepared from rat cerebral cortex. Synaptosomes are pinched-off nerve endings, with excellent biochemical and morphological preservation and consequently offer an invaluable system for studying neurochemical processes *in vitro* and provide highly functional preparations of nerve terminals (Duarte et al., 2004).

2. Materials and methods

2.1. Chemicals

[^{3}H]GABA (86 Ci/mmol) was purchased from Amersham Bioscience UK. Diethyl paraoxon, nipecotic acid, aminooxyacetic acid (AOAA), γ-amino-*n*-butyric acid (GABA), beta-alanine, L-diaminobutyric acid (L-DABA) and acetylthiocholine (ATC) were obtained from Sigma Chemical Co., Germany. 5,5′-Dithiobis 2-nitrobenzoic acid (DTNB) and bovine serum albumin (BSA) were prepared from Fluka (Swiss). Other materials prepared from Merck Company, Germany.

2.2. Animals

Male Wistar rats (200–250 g) were kept in 22 ± 2 °C and 12 h/12 h light dark cycle having access to food and water *ad libitium* during the course of study. All animal experiments were according to the established protocols by the Ethical Committee of the University.

2.3. Synaptosome preparation

Synaptosomes were prepared as previously described by Raiteri et al. (2003). In brief, cerebral cortex was dissected and homogenized in 0.32 M sucrose buffered with 100 mM phosphate, pH 7.4. In order to remove cell debris homogenate was centrifuged 5 min at 1000*g*. Supernatant recentrifuged at 12000*g* for 20 min. The pellet, containing synaptosomes, was resuspended in buffer solution containing (in mM): NaCl 125, KCl 3, $MgSO_4$ 1.2, $CaCl_2$ 1.2, $NaHCO_3$ 22, NaH_2PO_4 1, pH 7.4, saturated with 95% O_2 and 5% CO_2. Protein concentration was adjusted at 1 mg/ml.

2.4. Biochemical assays

Protein concentration was determined by Bradford method (Bradford, 1976) and BSA was used as standard. Lactate dehydrogenase (LDH) activity, as a biochemical proof for synaptosomal membrane integrity, was assessed with reduction of pyruvate to lactate (Moss and Henderson, 1994). Synaptosomal LDH activity was measured in presence (total activity) and absence (free activity) of 1% Triton X-100 and occluded activity expressed as percent of total. Acetylcholinesterase activity was determined by Ellman method as previously described by Dietz et al. (1973).

2.5. [^{3}H]GABA uptake assay

Synaptosomes (0.5 ml containing 0.5 mg protein), preincubated with 10^{-9}–10^{-3} M paraoxon (or buffer in case of control) for 20 min and then incubated with 400 nM GABA (1.5% of which was tritiated) at 37 °C for 10 min. AOAA (10 μM) was used in all experiment to prevent GABA catabolism. Reaction was stopped by adding 1 ml of cold saline after 10 min. Synaptosomes were centrifuged for 10 min at 10,000*g* twice and plate was solublized in 1% sodium dodecyl sulfate. Scintilator was added to each sample and their radioactivities were counted with liquid scintillation counter (Betamatic, Kontron, France). Specific GABA uptake was calculated as total uptake minus uptake in presence of 50 mM nipecotic acid (an inhibitor of GABA uptake). A few experiments were carried out in which sodium chloride was substituted by lithium chloride (equimolar) to clarify transporter-dependency of uptake. In order to clarify which type of GABA transporters were involved in GABA uptake, we used L-DABA as an inhibitor of neuronal GABA transporters (which preferentially inhibits GAT-1) and beta-alanine as a glial GABA uptake inhibitor (which dominantly inhibits GAT-2,3). Synaptosomes were preincubated with these two drugs 5 min prior to uptake assays. The results of triplicate experiments were done on synaptosomes prepared from three rats unless otherwise mentioned.

2.6. Kinetic studies

V_{max} and K_m of GABA uptake in synaptosomes were determined as previously reported (Sutch et al., 1999). In brief, GABA uptake was measured in a constant concentration of [^{3}H]GABA in presence of increasing concentration (0.1–100 μM) of GABA.

2.7. Statistical analysis

The results are given as mean ± SEM. Comparison between groups was done by paired *t*-test, Kruskal–Wallis with Mann–Whitney *U* test, and two-way analysis of variance if necessary. *P*-values less than 0.05 were considered significant. Data analyses were carried out by SPSS program except for calculating K_m and V_{max} for which Graph pad prism software was used.

3. Results

3.1. Biochemical analysis

When expressed as percent of total, occluded and free LDH activity were 87 ± 1% and 13 ± 1%, respectively

($P < 0.05$, $n = 8$). Paraoxon inhibited cholinesterase activity of synaptosomes, and this inhibition was concentration-dependent. Paraoxon IC_{50} for cholinesterase inhibition was 8.5 nM.

3.2. Sodium-dependency of uptake

When lithium chloride substituted sodium chloride, uptake was remarkably decreased to 15% of the initial value ($P < 0.001$, Fig. 1).

3.3. Effect of paraoxon on [^{3}H]GABA uptake

Paraoxon had a quasi-biphasic effect on [^{3}H]GABA uptake in rat cortical synaptosomes. Paraoxon increased the uptake in nanomolar concentrations (10^{-9}–10^{-6} M) and decreased it in micromolar concentrations (10^{-5}–10^{-3} M). Increase in the uptake was significant at 10^{-8}–10^{-6} M ($P < 0.01$), albeit an observable increment at greater concentrations. The situation reversed at 10^{-3} M paraoxon, where the decrease in uptake was statistically significant ($P < 0.05$, Fig. 2).

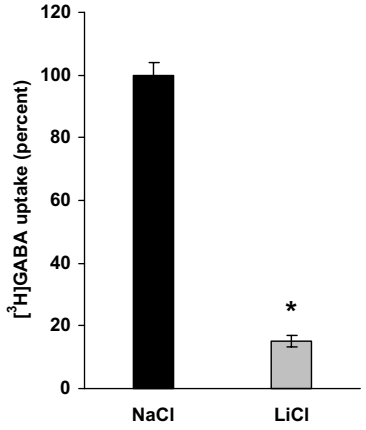

Fig. 1. Effect of lithium chloride substitution with sodium chloride on [^{3}H]GABA uptake by rat brain synaptosome. Data demonstrated that uptake was sodium dependent. $^*P < 0.001$.

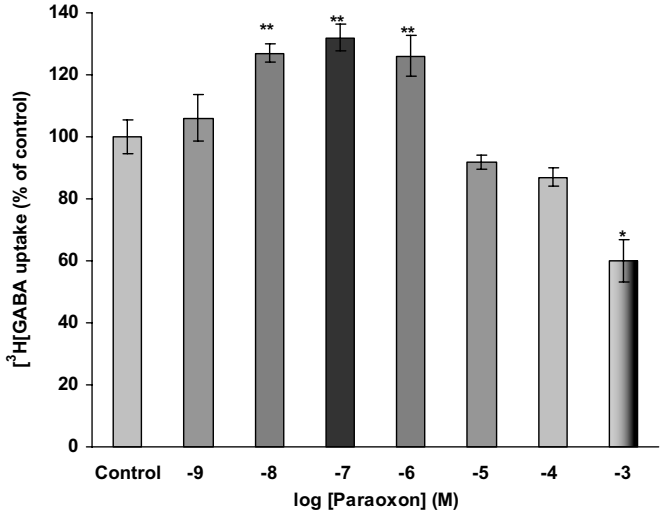

Fig. 2. Effect of paraoxon on [^{3}H]GABA uptake by rat brain synaptosome. $^*P < 0.05$, $^{**}P < 0.01$, compare to control. [^{3}H]GABA uptake in control group was 55 ± 3 pmol/mg protein.

3.4. Effect of paraoxn on K_m and V_{max} of GABA uptake

In control group K_m of GABA uptake (9.09 ± 0.92 μM) was not significantly different from paraoxon group (9.80 ± 1.02 μM); however, V_{max} dropped from 175.2 ± 4.23 pmol/mg protein/min in control group compared to 80.35 ± 2.03 in paraoxon group ($P < 0.001$) (Fig. 3).

3.5. Cholinergic-independency of GABA uptake

Acetylcholine had no effect on GABA uptake. On the other hand, neither atropine (10 μm) nor mecamylamine (100 μm) could reverse the inhibitory effect of paraoxon on GABA uptake (Fig. 4).

3.6. Effects of beta-alanine and L-DABA on GABA uptake

Beta-alanine at 100 μM had no apparent effect on GABA uptake; however, rising the concentration by 10-folds (1000 μM) decreased it significantly ($P < 0.001$). In addition, L-DABA (50, 500 μM) decreased GABA uptake significantly ($P < 0.001$) (Fig. 5).

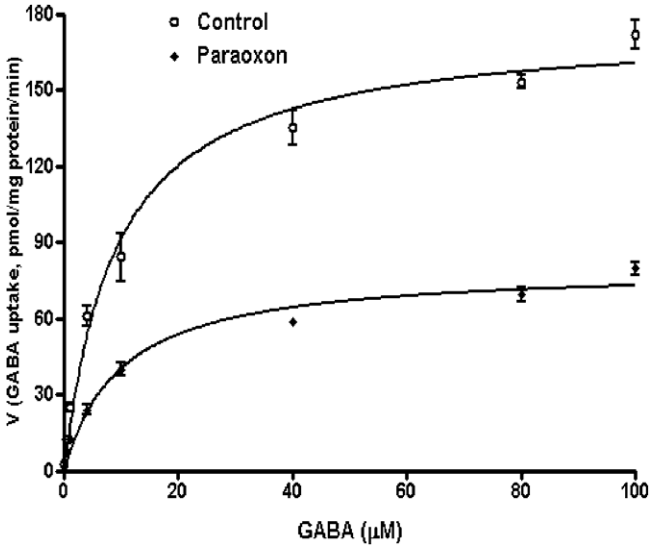

Fig. 3. Effect of paraoxon on K_m and V_{max} of GABA uptake in synaptosomes. GABA uptake was measured in a constant concentration of [^{3}H]GABA in presence of increasing concentration (0.1–100 μM) of GABA. Paraoxon had no effect on K_m (9.80 ± 1.02 vs. 9.09 ± 0.92 μM) while significantly decreased V_{max} (175.2 ± 4.23 vs. 80.35 ± 2.03 pmol/mg protein/min, $P < 0.001$).

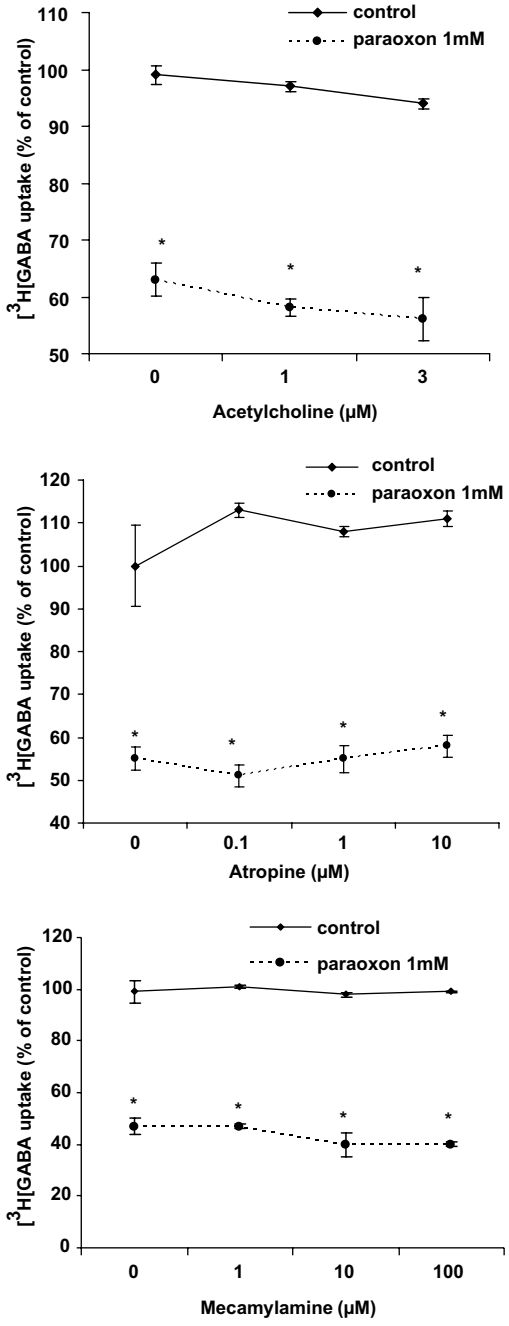

Fig. 4. Cholinergic-independency of GABA uptake. Acetylcholine, atropine and mecamylamine had no effect on GABA uptake by rat cerebral cortex synaptosomes ($^{*}P < 0.001$ compare to control group).

4. Discussion

In this study paraoxon inhibited cholinesterase activity in a dose dependent manner as predicted. We also observed that paraoxon at low doses increase and at high dose decrease GABA uptake in rat brain synaptosomes. Paraoxon (1 mM) inhibited GABA uptake in rat cerebral cortex synaptosomes, most probably mediated by decreasing maximum velocity of uptake through GAT-1. The membrane integrity of synaptosomes was confirmed by LDH experiments. The uptake was sodium dependent as the stoichiometry of GABA transporters has been reported to be 1GABA:2Na^+:1Cl^- (Lu and Hilgemann, 1999). Our experiments showed that equimolar substitution of Na^+ with Li^+ significantly attenuated uptake of [^{3}H]GABA which indicates Na^+-dependency of uptake as reported by others (Lu and Hilgemann, 1999). Increase in GABA uptake in submicromolar concentration may be due to an increase in the affinity of transporters, although it cannot be directly concluded from the present results.

Regarding inhibition of GABA uptake by 1 mM paraoxon, Szilagyi et al. (1993) showed that 1–2 mM tabun decreased [^{3}H]GABA uptake in guinea pig cerebral cortex, which is consistent with our results. Kinetic studies revealed that decrease in V_{max} of the uptake may be one possible mechanism underlying the inhibitory effect of paraoxon on GABA uptake. The pattern of inhibition represented seems non-competitive. The probability that paraoxon could directly affect GABA transporters or interrupt the uptake at another site or even internally, cannot be speculated from our experiments. Redistribution of GABA transporters between cell surface and cytoplasm is considered as a regulatory mechanism for transporters function (Whitworth and Quick, 2001). Therefore, it may be suggested that paraoxon, via transporters internalization, decreases GABA uptake. Bahena-Trujillo and Arias-Montano (1999) reported that phorbol esters decreased GABA uptake in rat substantia nigra synaptosomes and concluded that decrease in V_{max} is the underlying mechanism.

In this study neither acetylcholine nor its antagonists could alter the effect of paraoxon on GABA uptake giving a picture which shows that the effect of paraoxon is cholinergic-independent, although the same dose of paraoxon completely inhibited cholinesterase ($IC_{50} = 8.5$ nM). Rocha et al. (1996), working on electrophysiology of cultured hippocampal neurons have reported that effect of paraoxon on transmitter release is cholinergic-independent. Recently it has been reported that increase in the frequency of spontaneously generated action potentials by paraoxon occur independent of acetylcholinesterase inhibition (Vatanparast et al., 2006). It may be surprising that paraoxon at a dose higher than what is needed to inhibit cholinesterase activity, exerts some direct effects on other targets, however it is worth noticing that this case was reported for organophosphates previously and its importance remains to be elicited (Lotti, 1995).

The synaptic action of GABA is terminated mainly by reuptake into presynaptic terminals and glia mediated by GABA transporters. At least four GABA transporter proteins (GAT-1 to 4) have so far been cloned (Ng and Ong, 2002). Results of this study show that GAT-1 transporters are involved in this synaptosomal model. Beta-alanine (a glial GABA uptake inhibitor) could not prevent GABA uptake at 100 μM which has been reported to be sufficient for up to 90% inhibition of GAT-2 and GAT-3 (Sutch et al., 1999). On the other hand, DABA (a neuronal GABA uptake inhibitor) decreased GABA uptake significantly at a dose (50 μM) relatively near its IC_{50} (Wonnemann et al.,

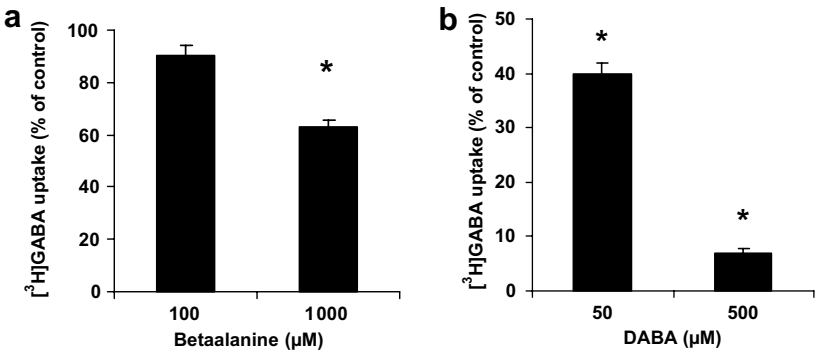

Fig. 5. Effect of glial (beta-alanine) and neuronal (DABA) GABA uptake inhibitors (a and b, respectively) on synaptosomal GABA uptake. Weak and strong inhibition of GABA uptake by beta-alanine and DABA respectively suggested that GAT-1 is involved (* $P < 0.001$ compare to uptake in control group).

2000). Strong and weak inhibition of uptake by DABA and beta-alanine, respectively, indicates that GABA was taken up by neuronal transporters (i.e., GAT-1).

Convulsion is one of the most concerning complications of organophosphate poisoning (Emerson et al., 1999). Induction of convulsion by paraoxon and on the same line inhibition of GABA uptake may seem paradoxic at first glance. However, a few notes are worth looking into. First, there is a report of a substance (Crotoxin) which inhibits GABA uptake but produces convulsion (Cecchini et al., 2004). Second, it is important to remember that GABA uptake inhibition could have proconvulsive effects as reported by Patrylo et al. (2001). Third and even more complex to interpret is the fact that synaptosomes have lower sensitivity to drugs and toxins, up to three orders of magnitude, as compared to intact systems (Dam et al., 1999). It is then speculated that if paraoxon inhibits GABA uptake in synaptosomes, it may well do so at lower doses *in vivo*. In conclusion, present finding shows that the GABAergic system may be the target of paraoxon in producing convulsions and it does so by at least affecting kinetics of GAT-1 transporters located in neuronal endings.

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