Effect of Paraoxon and Sulfhydryl Reagents on ^{3}H-Quinuclidinol Benzoate and ^{3}H-Methylscopolamine with Synaptic Membranes of Rat Brain

A. I. Golovko, G. A. Sofronov, T. A. Kurganova, and T. V. Klyuntina

UDC 615.015.217:615.215.321.34]-092.9

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 118, № 7, pp. 51-53, July, 1994
Original article submitted December 2, 1993

Paraoxon is shown to reduce the density of M-choline receptors in the cortex of rat brain. Inhibition of ^{3}H-QNB is noncompetitive and reversible in nature. Sulfhydryl reagents do not affect this process. The mechanism of action of PO is thought to include direct effects on brain muscarine receptors.

Key Words: *paraoxon; ligands; muscarine receptors; sulfhydryl reagents*

In vivo and *in vitro* experiments have demonstrated the ability of organophosphorus inhibitors of acetylcholine esterase to interact with brain muscarine receptors in animals and to inhibit specific binding of ^{3}H-quinuclidinol benzoate (QNB), a specific ligand of M-choline receptors [6,7]. In particular, such a property has been reported for paraoxon (PO; phosphacol) [1].

The molecular mechanisms of interaction of PO with brain muscarine receptors remain unclear. These aspects of the physiological activity of PO are the subject of the present study.

MATERIALS AND METHODS

Experiments were carried out on male white rats weighing 180-220 g. Crude synaptosomes were isolated from the cerebral cortex and frozen for 18 h in 100 volumes of distilled water. After thawing and centrifugation (15 min, 20,000 g) the membranes were homogenized in 40 volumes of 50 mM K-phosphate buffer solution, pH 7.4, and centrifuged. The above procedure was repeated and the resultant pellet was resuspended in K-phosphate buffer solution.

S. M. Kirov Military Medical Academy, St. Petersburg

For radioligand assay 80-100 μg membranes were incubated in the presence of PO (10^{-6}-10^{-3} M) or sulfhydryl reagents dithiothreitol (10^{-9}-10^{-3}, Sigma, USA) or 5.5'-dithio-bis(2-nitrobenzoic acid) (DTNB, 10^{-9}-10^{-3}, Sigma) in 50 mM K-phosphate buffer solution, pH 7.4, at 20°C. The M-choline receptor ligands ^{3}H-QNB (NEN, Germany; 1.2 TBq/mM, 0.5 nM) or ^{3}H-methylscopolamine (Amersham, UK; 3.5 TBq/mM, 0.5 nM) were then added to the incubation medium. The samples were incubated for 2 h at 20°C and washed free of unbound ligand on GF/B filters (Whatman, UK). The PO-induced inhibition of specific binding of ^{3}H-QNB was analyzed in Lainuiwer-Burk and Scatchard coordinates. In both cases the membranes were preincubated with PO (5.5×10^{-5} M; 1.7×10^{-4} M, and 5×10^{-4} M) for 30 min before the radioligand was added. The effect of sulfhydryl reagents on PO binding to M-choline receptors was assayed in a different manner. Dithiothreitol or DTNB (both in a concentration of 10 μM) was added to the membrane suspension and 30 min later the organophosphorus compound (OPC, 200 μM) and another 30 min later the radioligand (0.5 nM) were added. Nonspecific binding was determined in the presence of 1 μM atropine.

0007-4888/94/0007-0734$12.50

The samples were assessed for radioactivity with a 1215 Rackbeta II counter. The quantitative parameters of ligand binding were calculated by regression analysis using the method of least squares. Each value represents the average of 3-5 independent experiments performed in triplicates.

RESULTS

PO inhibited binding of ^{3}H-QNB and ^{3}H-methylscopolamine with synaptic membranes of the rat cortex (Table 1), the affinity of the OPC to ^{3}H-QNB being somewhat higher.

Radioligand binding assay with ^{3}H-QNB in the Lainuiwer-Burk coordinates showed that PO inhibited the receptors in a noncompetitive manner. No marked effect of sulfhydryl reagents on ligand binding was noted.

PO inhibition of ^{3}H-QNB binding may result either from reduced density of the receptors or from their lower affinity. To answer this question we studied the effect of the OPC in ligand binding (the concentration of ^{3}H-QNB ranged from 0.1 to 1.0 nM). The obtained results were analyzed in Scatchard coordinates (Fig. 1).

PO did not change the affinity of M-choline receptors to ^{3}H-QNB, as is seen from the unchanged dissociation constant (K_d). At the same time, receptor density (B_{max}) progressively dropped with an increase of the concentration of inhibitor in the incubation medium (r=0.989).

These data suggest that PO inhibition of ^{3}H-QNB binding results from a reduced density of binding sites, not from their lower affinity. The possibility that PO irreversibly inhibits M-choline receptors was examined by preincubating the membranes with the OPC (30 min) followed by 5 washings-centrifugations. After that the density of ^{3}H-QNB binding sites was determined. Control samples were incubated with the same volume of buffer saline and treated in parallel with experimental samples (Fig. 2).

The data demonstrated that repeated washing completely restores the density of M-choline receptors, i.e., inhibition of nerve terminals is reversible. The reduced binding of the ligand in the presence of 170 μM PO without washing is shown for comparison.

The data on the effect of sulfhydryl reagents on PO-induced inhibition of binding of M-choline receptor ligands are shown in Table 2. As is seen from the table, DTNB somewhat potentiated the inhibiting effect of phosphacol, but no reliable data were obtained. It may be assumed that the sulfhydryl groups of the active site of the receptor do not affect OPC binding.

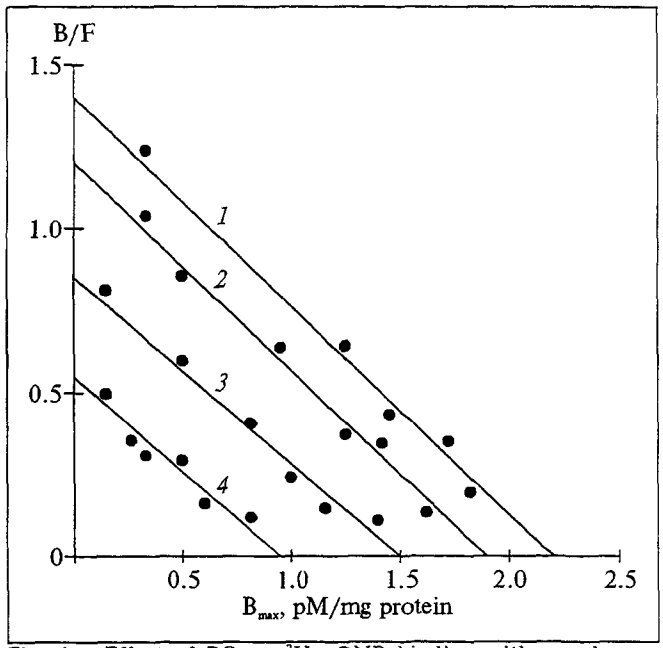

Fig. 1. Effect of PO on ^{3}H−QNB binding with membranes from rat cortex. Scatchard plots: *1*) control (K_d=0.166±0.020 nM; B_{max}=2.14±0.26 pM/mg protein); *2*) PO, 5.5×10^{-5} M (K_d=0.164±0.015 nM; B_{max}=1.90±0.17 pM/mg protein); *3*) PO, 1.7×10^{-4} M (K_d=0.173±0.026 nM; B_{max}=1.53±0.23 pM/mg protein); *4*) PO, 5.5×10^{-4} M (K_d=0.173±0.016 nM; B_{max}=0.95±0.09 pM/mg protein).

The obtained results on PO-dependent inhibition of ^{3}H-QNB and ^{3}H-methylscopolamine binding are in conformity to the data of other authori-

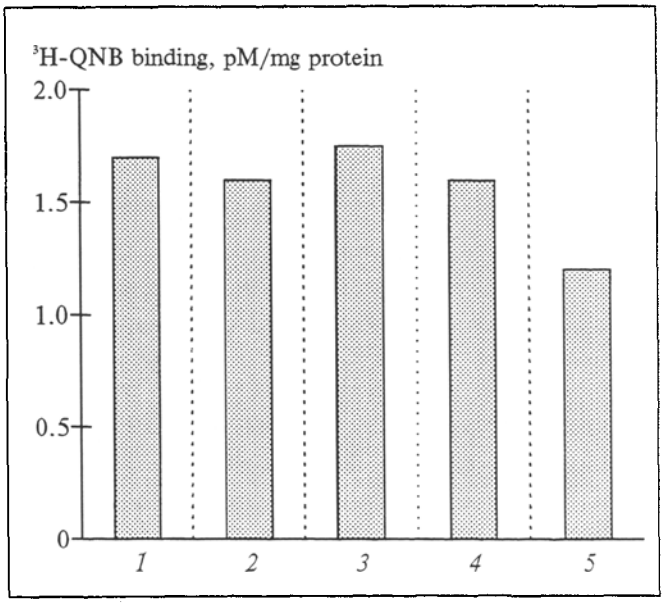

Fig. 2. Effect of preincubation of synaptic membranes from the cortex of intact rats with PO on ^{3}H−QNB binding (0.5 nM). *1*) control; *2*) PO, 5.5×10^{-4} M; *3*) PO, 1.7×10^{-4} M; *4*) PO, 5.5×10^{-5} M; *5*) PO, 1.7×10^{-4} M without washing. Binding in the control was 1.70±0.13 pM/mg protein; asterisk: p<0.01 in comparison with the control.

TABLE 1. Effect of PO and Sulfhydryl Reagents on Binding of ^{3}H-Quinuclidinol Benzoate and ^{3}H-Methylscopolamine (Both 0.5 nM) with Synaptic Membranes of Rat Cortex ($M\pm m$)

Compound	Ligand	
	^{3}H-QNB	^{3}H-methylscopolamine
PO	Inhibition, $IC_{50}=186\pm19$ μM; noncompetitive inhibition	Inhibition $IC_{50}=238\pm27$ μM;
Dithiothreitol	no effect	no effect
DTNB	no effect	no effect

TABLE 2. Effect of Sulfhydryl Reagents on PO-Induced Inhibion of Ligand Binding with Membranes from Rat Cortex ($M\pm m$)

Conditions	Ligand binding, fM/mg protein	
	^{3}H-QNB, 0.5 nM	^{3}H-methylscopolamine, 0.5 nM
Control	1536±117	641±59
PO, 200 μM	942±73**	359±29*
Dithiothreitol, 10 μM	1598±149	634±54
DTNB, 10 μM	1472±130	620±38
Dithiothreitol + PO	941±82**	384±17**
DTNB + PO	918±64**	359±32**

Note. *: $p<0.02$; **: $p<0.01$ in comparison with the control.

ties on the ability of organophosphorus inhibitors of acethylcholine esterase to block the central M-choline receptors [4,6,7]. On the other hand, PO has been reported not to affect the density of muscarine receptors in the caecum of guinea pigs [3]. This discrepancy may be attributed to structural and functional peculiarities of peripheral M-choline receptors [5].

No marked effect of sulfhydryl reagents on ligand binding was found. This is probably due to the fact that we used native membranes in our experiments, while the data on modulation of ^{3}H-QNB binding by dithiothreitol and DTNB were obtained on purified receptor preparations [2].

Thus, PO inhibited muscarine receptors of rat cortex in a reversible and noncompetitive manner. The reduced binding of ^{3}H-QNB is due to decreased receptor density without changes in receptor affinity. Sulfhydryl groups in the active site of the receptor are presumably not involved in PO reception, since sulfhydryl reagents did not affect the PO-induced inhibition of the binding of ^{3}H-QNB and ^{3}H-methylscopolamine, ligands of muscarine receptors. The mechanism of action of PO, apart from inhibition of acetylcholine esterase, probably involves direct effects on M-choline receptors.

REFERENCES

1. A. I. Golovko, G. A. Sofronov, T. V. Nikolaeva, and T. V. Klyuntina, *Byull. Eksp. Biol. Med.*, **114**, № 9, 290 (1992).
2. G. Berstein, K. Haga, T. Haga, and A. Ichiyama, *J. Neurochem.*, **50**, № 6, 1687 (1988).
3. A. L. Gardner, L. K. Choo, and F. Mitchelson, *Gen. Pharmacol.*, **19**, № 4, 583 (1988).
4. J. Kubinec, K. E. Vrana, and R. Roskoski, *Europ. J. Pharmacol.*, **136**, № 3, 295 (1987).
5. H. Kurtel, B. C. Yeden, A. Dedeoglu, *et al.*, *Arch. Int. Pharmacodyn.*, **308**, 39 (1990).
6. De-Lu Zhao, S. Pei, and C. Liu, *Acta Pharmacol.*, **6**, № 4, 227 (1985).
7. De-Lu Zhao, Z. Wang, S. Pei, and C. Liu, *Ibid.*, **4**, № 4, 225 (1983).