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The effect of organophosphorous insecticide Wofatox 50 EC on the adenylate cyclase activity of chicken embryo muscle

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The organophosphorous insecticides like parathion or W 50 (methylparathion)* are widely used for plant protection. However, above a certain level, these drugs also poison the warm blooded animals posing thereby potential danger to most wild animals and man [1, 2]. One of the main toxicological effects is the damage of muscles [3]. We also found the cervical muscles [4], including the musculus complexus major (unpublished), from chicken atrophized after treatment of eggs with W 50.

Although the organophosphates are well known inhibitors of various esterases, including most importantly the acetylcholinesterase, in many cases their toxicological effects (particularly if they are present at relatively low concentrations) do not appear to correlate with the inhibition of the cholinergic system [5–7].

The organophosphorous insecticides accumulate in membranes especially in muscle membranes [8]. Due to such asymetric distribution the membrane bound enzymes are the most likely targets for their effects. In the present work we tested the effect of W 50 on the activity of adenylate cyclase (AC), an important plasma membrane bound enzyme, in the chicken embryo musculus complexus major which is a main target of organophosphates.

(α-^{32}P)ATP (40–80 TBq/mmol) was obtained from Amersham International. ATP, cyclic AMP, GTP, GPP(NH)P, crystalline glucagon, creatine phosphate and creatine kinase were purchased from Sigma (St. Louis, MO). Neutral aluminium (90% active; 70–230 mesh) was obtained from Merck, Darmstadt, F.R.G. Wofatox 50 EC (active substance: 50% methylparathion, VEB Chemiechombinat, Bitterfeld, G.D.R.). Chicken eggs of the Shaver Starcross 288 strain were used.

Eggs were incubated in a Ragus automatic incubator and opened two days before hatching. The musculus complexus major was excised from the embryos and homogenized in 10 vol. of ice-cold 20 mM Tris/HCl buffer pH 7.6. For homogenization a motor-driven Waring blender at about 7000 rpm was used. The homogenates were filtered through two layers of cheesecloth and used immediately at 0.2 mg protein per assay for the measurement of AC activity. The latter was determined by measuring the conversion of (α-^{32}P)ATP (1 mM) to ^{32}P-cyclic AMP as described previously [9]. Formation of cAMP was linear for at least 15 min. Rat liver plasma membranes were prepared by the method of Neville [10]. Protein was determined according to the method of Lowry *et al.* [11].

AC activity in the muscle homogenate was only slightly stimulated by a relatively high concentration (10 μM) of GTP (Table 1). The most likely reason for this is that N_s, the regulatory protein through which GTP stimulates the enzyme, has GTP-ase activity [12, 13]; the resultant GDP, which binds strongly to N_s, blocks the stimulatory cycle [14, 15]. The small effect of GTP in this muscle is comparable to that observed when catalytic subunit preparations and pure N_s were reconstituted in phospholipid vesicles [16]. The non-hydrolysable analog of GTP, GPP(NH)P, has stronger stimulatory effect both in our case (Table 1) and in the reconstituted system [16]. The β receptor-specific agonist, isoproterenol, is known to increase the affinity of N_s for GTP thereby inducing and sustaining new cycles of stimulation [14–16]. Isoproterenol, according to this mechanism of action, is not supposed to have any effect in the absence of GTP which agrees with our observation (Table 1). In our system, however, isoproterenol also failed to stimulate the AC activity in the presence of GTP indicating that the β receptors were not coupled to N_s. Instead we observed the inhibition of GTP effect by isoproterenol (Table 1). No such inhibition of GTP effect occurred in the presence of 10 μM propranolol, a β receptor antagonist (not shown).

It is known that the β receptor interacts with N_i [16, 17], the inhibitory guanine nucleotide binding protein, through which GTP inhibits AC activity [18]. As a result isoproterenol occasionally inhibits AC activity (for example in fat cells [19]) presumably due to the dominance of N_i structure. A similar mechanism could also occur in the chicken embryo muscle.

Low concentrations (1–10 μM) of W 50 did not significantly affect the cyclase activity in the absence of effectors or in the presence of isoproterenol alone (Table 1).

* Abbreviations used: AC, adenylate cyclase; W 50, Wofatox 50 EC; N_s, the stimulatory guanine nucleotide binding protein; N_i, the inhibitory guanine nucleotide binding protein.

Enzymes: Adenylate cyclase or ATP:pyrophosphate lyase (cyclizing) (EC 4.6.1.1); creatinc kinase or ATP:creatine phosphotransferase (EC 2.7.3.2.).

Table 1. Effect of W 50 on the activity of adenylate cyclase of m. complexus major of chicken embryo

Cyclase effector	pmol cyclic AMP/mg protein/15 min Concentration of W 50 0	1 μM	10 μM
None	106 ± 8	82 ± 6	105 ± 5
GTP 10 μM	146 ± 12	121 ± 6	86 ± 4
GPP(NH)P 100 μM	192 ± 11	262 ± 8	351 ± 6
Isoproterenol 10 μM	117 ± 8	—	126 ± 7
Isoproterenol 10 μM GTP 10 μM	101 ± 7	124 ± 5	167 ± 10

Incubations were performed with 0.2 mg protein of muscle homogenate at 33° for 15 min. Results are the mean ± S.E.M. of three incubations in a representative experiment. Similar results were obtained in two other experiments.

Table 2. Effect of W 50 on the glucagon activated AC activity of liver plasma membrane

Cyclase effector	pmol cyclic AMP/mg protein/15 min Concentration of W 50 0	10 μM	100 μM
None	664 ± 31	687 ± 23	607 ± 22
GPP(NH)P 100 μM	1428 ± 14	1517 ± 17	1528 ± 33
Glucagon 0.1 μM GPP(NH)P 100 μM	2861 ± 26	3020 ± 29	3035 ± 17

Incubations with 50 μg membrane proteins were performed at 33° for 15 min. Results are the mean ± S.E.M. of three incubations. The experiment was repeated with another membrane preparation with similar results.

However, it abolished the stimulatory effect of GTP while stimulating the formation of cAMP in the presence of 100 μM GPP(NH)P. The reason for this differential effect of W 50 is not known: among other possibilities organophosphates may affect GTP-ase activity of N_s or may selectively change the affinity of N_s for GTP and GPP(NH)P. Since GTP alone does not seem to have a regulatory role *in vivo*, the inhibition of its effect described here is of limited significance.

While W 50 was not stimulatory when GTP or isoproterenol was alone, it became a moderate stimulator of AC in the concomitant presence of these two agents. To see whether the action of W 50 shows any tissue or hormone selectivity we tested its effect on the well characterized glucagon-stimulated AC system in rat liver plasma membranes. As shown in Table 2, the stimulatory effect of glucagon or that of GPP(NH)P was not significantly influenced even by 100 μM W 50.

In summary, the present results indicate that in the m. complexus major of chicken embryo, even in the final stage of embryonic life, the β receptor is functionally uncoupled from N_s. An apparent uncoupling may occur if N_i, as it occurs in some other tissues [20], would be more abundant than N_s: in this case interaction of β receptor with N_i would dominate (i.e. isoproterenol would be inhibitory). W 50 then could act either by inhibiting receptor-N_i interactions or facilitating receptor-N_s interactions. Since the glucagon-stimulated AC system (which lacks the inhibitory component; [21]) was not modulated by W 50, the former possibility is more likely but this point needs to be proven. In this context it is interesting to note that the function N_i is inhibited by phosphorylation [22]. A testable possibility is that organophosphates cause similar inhibition of N_i.

cAMP has important role in the regulation of cellular growth and differentiation [23]. It appears that unimpaired growth requires low cAMP concentration (probably regulated by hormones) at certain stages of cell cycle and development [23]. Since W 50 is membrane soluble and plasma membranes do not metabolize this drug it may have the potential to continuously block the hormone induced decrease of cAMP level. Further studies are required to prove this point.

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Butyrylcholinesterase inhibition by miracil D and other compounds

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Butyrylcholinesterase (BuChE*) is a serine hydrolase of unknown function. It generally occurs in the blood, tissues, and cytosol of organisms and cells, but it also appears to occur specifically in the superior cervical ganglion [1], the central visual pathway, and other areas of the mammalian brain and nervous system [2]. Its structure, ligand and inhibitor patterns resemble those of acetylcholinesterase (AChE), and considerable sequence homology has been detected between human serum BuChE and *Torpedo marmorata* AChE [3]. Both ChEs hydrolyze choline esters, although AChE preferentially hydrolyzes C2 substrates, whereas BuChE preferentially hydrolyzes C4 substrates. Both enzymes possess many inhibitors in common, but preferential inhibition of one enzyme or the other frequently occurs. Among the compounds which preferentially suppress BuChE are many heterocycles and esters which exert significant neuropharmacological effects, most probably through interaction with specific receptors. Included in this group are phenothiazines [4, 5], benzilates [6, 7], butyrophenones [8], imipramines [9], and phencyclidines [10]. We now wish to enlarge this group of preferential BuChE inhibitors through presentation of our findings on the thiaxanthone, miracil D (MD, [lucanthone], Fig. 1). This compound was found to be the most potent reversible BuChE inhibitor tested and was considerably more inhibitory than chlorpromazine. Both competitive and mixed inhibition were detected.

Materials and methods

Human serum BuChE was purified by procainamide affinity chromatography described by Ralston *et al.* [11]. The specific activity of the purified BuChE was approximately 570 units/mg protein. Horse serum BuChE was a product of the Worthington Chemical Co., Freefold, NJ. Catalytic activity was assayed by the method of Ellman *et al.* [12], using butyrylthiocholine (BTC) as substrate. An enzyme activity unit (U) is defined as the hydrolysis of 1 μmole of BTC per min in 0.1 M sodium phosphate buffer, pH 8.0, at 25°. Both BTC and 5,5′-dithiobis-(2-nitrobenzoic acid) were products of the Sigma Chemical Co., St. Louis, MO. Additional drugs and chemicals were obtained from component branches and members of the Walter Reed Army Institute of Research. Enzyme assays were conducted at 25° on a Gilford model 2600 spectrophotometer equipped with a Hewlett–Packard model 7225A plotter and a Gilford Thermal II printer. Enzyme (0.05 units/reaction system) was added last to start the reaction. Absorbance changes at 412 nm during the first minute were linear and were used to calculate reaction rates. Calculated average apparent inhibition constants are presented with their standard deviations, and linear regression analyses were conducted for all data in Figs. 2 and 3.

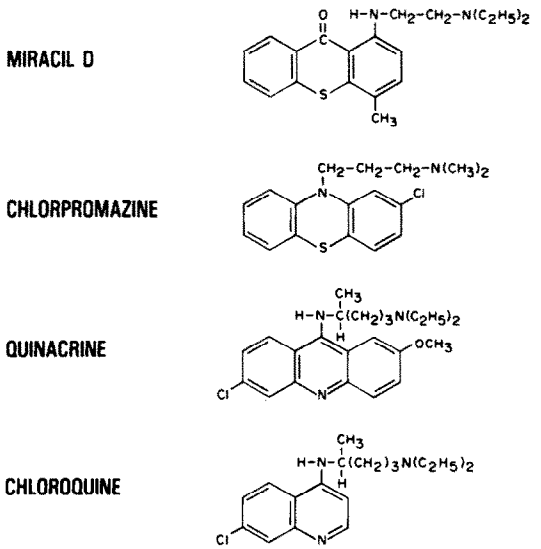

Fig. 1. Structures of miracil D (lucanthone) and additional compounds used in these studies.

* Abbreviations: BuChE, butyrylcholinesterase; AChE, acetylcholinesterase; MD, miracil D; BTC, butyrylthiocholine; and K_m, Michaelis-Menten constant.