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Response of the rat ileum, uterus and vas deferens to carbachol and acetylcholine following repeated daily administration of a cholinesterase inhibitor

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Summary

1. Daily i.p. administration, for eight days, of the cholinesterase inhibitor disulfoton to rats produced mild to moderate signs of intoxication (tremors, incontinence and diarrhoea) but no deaths.

2. Segments of ileum taken from the treated rats were subsensitive to carbachol but the vas deferens and the uterus did not exhibit any change in sensitivity to carbachol.

3. The sensitivity to acetylcholine was increased in the ileum and vas deferens but not in the uterus.

4. Acetylcholinesterase activity was 60–70% inhibited in all three tissues.

Introduction

When animals are repeatedly exposed to sublethal amounts of cholinesterase inhibitors, there develops a state of reduced sensitivity (subsensitivity) to directly acting cholinergic agents which are not hydrolysed by cholinesterases. The first observation of subsensitivity was made by Brodeur & DuBois (1964) who found that the LD_{50} of carbachol was raised from 2·0 mg/kg to 3·9 mg/kg in rats treated for 30 days with the organophosphorus cholinesterase inhibitor, disulfoton (*O,O*-diethyl *S*-2-(ethylthio) ethyl phosphorodithioate).

Subsequent studies, both *in vivo* and *in vitro*, have shown that subsensitivity develops to both the stimulatory and inhibitory action of cholinergic agents following repeated exposure of animals to cholinesterase inhibitors. For example, reduced sensitivity to the negative chronotropic action of carbachol was observed in the anaesthetized rat (McPhillips & Dar, 1967) and in atria isolated from rats given repeated injections of disulfoton (Perrine & McPhillips, 1970). Segments of ileum from disulfoton-treated rats were subsensitive to the spasmogenic action of carbachol, oxotremorine and furtrethonium (McPhillips, 1969). Another example of subsensitivity was reported by Bito, Hyslop & Hyndman (1967) who found subsensitivity to the miotic action of carbachol and pilocarpine after chronic application of echothiophate and di*iso*propylfluorophosphate to the eyes of rabbits and cats.

In at least one instance repeated administration of a cholinesterase inhibitor has been shown to produce subsensitivity to a sympathomimetic agent. After repeated injections of physostigmine to cats, Emmelin (1964) found subsensitivity of the

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sensitivity by suggesting that acetylcholine accumulates as a result of acetylcholinesterase inhibition and the increased concentration of acetylcholine, to which effector cells are exposed, causes the responsiveness of the cells to decrease.

In most species cholinergic nerves exert a significant influence on the functional activity of the heart, intestine, iris and salivary gland (Henderson & Roepke, 1937; Ambache, 1955; Emmelin, 1961). The uterus (Miller & Marshall, 1965; Schofield, 1952) and vas deferens (Richardson, 1962), however, do not seem to be strongly influenced by cholinergic nerves. The purpose of the present study, therefore, was to see if subsensitivity of the uterus and vas deferens developed following repeated administration of disulfoton to rats. For purposes of comparison the ileum, which is known to exhibit subsensitivity, was studied at the same time.

Methods

Male and female albino rats (250–300 g) were used. They were housed in a temperature controlled room at 25° C and were permitted free access to food and water.

Disulfoton, 99% pure, was obtained from the City Chemical Company, N.Y. (USA). Inhibition of cholinesterases by this compound is not readily reversible since little recovery of activity takes place within the 24 h period following injection (Bombinski & DuBois, 1958; Brodeur & DuBois, 1964). Disulfoton was dissolved in a 4:1 mixture of propyleneglycol and ethanol, and administered intraperitoneally. Female rats received a dose of 1·0 mg/kg and males, a dose of 2·5 mg/kg because female rats are more sensitive to disulfoton than males (Bombinski & DuBois, 1958). The concentration of disulfoton was adjusted so that each animal received a volume of 0·5 ml/kg. Control rats received an equal volume of the propyleneglycol-ethanol mixture. All injections were given daily for 8 consecutive days. In addition, all female rats received a subcutaneous injection of diethylstilboestrol, 0·1 mg/kg, to induce oestrus. This injection was given approximately 2 h after the last dose of disulfoton.

The rats were killed by cervical dislocation 18–24 h after the last dose of disulfoton, and the tissues quickly removed and placed in a 50 ml organ-bath containing the appropriate physiological salt solution.

Uterus

The right horn of the uterus was removed, cut open longitudinally and placed in the organ-bath containing de Jalon's solution. The temperature of the bath was maintained at 32° C. The experimental conditions were essentially the same as those described by de Jalon, Bayo & de Jalon (1945).

Vas deferens

After removal of the vas deferens it was desheathed by carefully removing the serous coat together with the blood vessels which surrounded the smooth muscle. The tissue was immersed in Krebs–Henseleit solution and maintained at 37° C. The experimental conditions used were essentially the same as those described by Westfall, McClure & Fleming (1972).

Ileum

Approximately 2 cm of terminal ileum were removed and the contents gently flushed out. The tissue was then placed in the organ-bath containing Tyrode solution. Experiments on the ileum were carried out at room temperature, 22° C.

Each of the tissues was connected by nylon thread to an FT03 Grass force-displacement transducer and contractions of the tissues were recorded on a polygraph. Each tissue was left to equilibrate for 1 h before any drugs were added to the organ-bath. During this time the bathing solution was changed every 15 min and adjustments were made to maintain the resting tension. A resting tension of approximately 1 g was applied to the ileum and 0·5 g to the uterus and vas deferens. The bath solution was aerated with oxygen: carbon dioxide, 95%:5%.

Concentration-effect curves were obtained by increasing the concentration of carbachol and acetylcholine geometrically until a maximal response was reached. Cumulative concentration-effect curves were obtained with the ileum by addition of the next concentration when the response had reached a plateau. The time interval between changes of concentration was usually 30 s and never more than a minute. With the uterus and vas deferens, however, each concentration of carbachol was followed by a washout because contractions were not sustained in these tissues. The washout was accomplished by rinsing the bath twice at two successive 2-min intervals. The next concentration of carbachol was tested 2 min after the last wash.

Comparisons of sensitivity were based on EC_{50} values. The EC_{50} is the concentration of agonist necessary to produce 50% of the maximal contraction of the tissue. Each EC_{50} value was derived from an individual concentration-effect curve. Geometric mean EC_{50} values and 95% confidence limits were then computed for each group of data. Geometric means are reported because EC_{50} values have been shown to be log normally distributed (Fleming, Westfall & de la Lande, 1972).

Cholinesterase measurements

Cholinesterase activity was estimated by the radioisotopic method described by Siakotos, Filbert & Hester (1969). The tissues were rinsed and then homogenized with a Polytron homogenizer in 0·4 M sucrose to yield 5% (w/v) suspensions of ileum and vas deferens and 8% (w/v) suspensions of uterus. The substrates, acetylcholine and acetyl-β-methacholine, were incubated with the homogenates for 10 minutes. The final concentration of each substrate was 1 mM. Radioactivity was counted in a liquid scintillation spectrometer. Instead of Bray's solution, the scintillation fluid was the one described by Carter & Van Dyke (1971). The counting efficiency of ^{14}C was 75% as determined by use of ^{14}C-toluene as an internal standard. Cholinesterase activity is reported as μmol of substrate hydrolysed per g protein and per hour. Protein analyses were performed by the method of Lowry, Rosebrough, Farr & Randall (1951) which was adapted for the Technicon autoanalyser.

Hydrolysis of acetylcholine was interpreted as an estimate of total cholinesterase activity and hydrolysis of methacholine as an estimate of acetylcholinesterase activity.

For statistical analysis the one-way analysis of variance was applied to the data.

Results

After the second or third dose of disulfoton most of the rats exhibited some signs of intoxication consisting of slight tremors, incontinence and diarrhoea. There were no deaths. Signs were reduced by the eighth day.

In female rats treated for 8 days with disulfoton the sensitivity of the ileum to carbachol was reduced (Table 1). The EC_{50} increased approximately 2·5 fold. Uterine horns, taken from the same rats and studied at the same time, did not show any change in sensitivity to carbachol (Table 1).

TABLE 1. *Effect of repeated administration of disulfoton on the sensitivity of the rat isolated ileum, uterus and vas deferens to carbachol*

Sex	Tissue	Group	*n*	EC_{50}* μM	95% confidence limits
Female	Ileum	Control	11	0·27	0·23– 0·31
		Treated	9	0·67**	0·50– 0·87
	Uterus	Control	11	1·54	0·92– 2·55
		Treated	11	1·78	0·98– 3·19
Male	Ileum	Control	6	0·27	0·14– 0·50
		Treated	6	0·60**	0·39– 0·88
	Vas deferens	Control	9	4·20	3·20– 5·50
		Treated	10	6·20	3·00–12·90

Females received i.p. 1·0 mg/kg of disulfoton and males 2·5 mg/kg per day, for 8 consecutive days. *n* indicates number of rats per group. * Geometric mean. ** $P<0·05$.

The results obtained in male rats were comparable to those observed in females. The ileum developed subsensitivity to carbachol to a degree equal to that observed in the ileum from female rats. Vasa deferentia, which were taken from the same rats, however, did not show any change in sensitivity (Table 1).

Experiments were also carried out to see if repeated administration of disulfoton had any effect on the response of the uterus and vas deferens to acetylcholine. As shown in Table 2 there was a slight decrease in the EC_{50} of acetylcholine in uterine horns taken from treated rats relative to controls but the difference was not

TABLE 2. *Sensitivity of rat isolated uterus and ileum to acetylcholine after repeated administration of disulfoton*

Tissue	Group	*n*	ED_{50}* μM	95% confidence limits
Uterus	Control	6	2·7	0·3 –25·6
	Treated	6	1·6	0·3 –10·1
Ileum	Control	10	0·51	0·18– 0·99
	Treated	12	0·23**	0·13– 0·42

Disulfoton, 1·0 mg/kg, given i.p., for 8 consecutive days. *n* indicates the number of rats per group. * Geometric mean. ** $P<0·05$.

statistically significant. In the ileum there was a decrease in the EC_{50} of acetylcholine to approximately one-half of the control value. Figure 1 shows that there was also a marked change in the sensitivity of the vas deferens to acetylcholine.

A comparison of hydrolysis rates by tissue homogenates from untreated rats shows that the ileum hydrolyses acetylcholine and methacholine, 1 mM, faster than do the other two tissues. The activity of the vas deferens is somewhat less than that of the ileum and the uterus hydrolyses both acetylcholine and methacholine at the lowest rate.

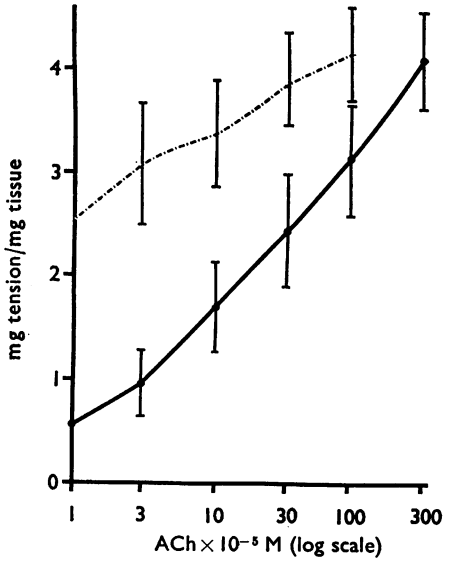

FIG. 1. Response of the isolated vas deferens to acetylcholine following repeated i.p. administration of disulfoton, 2·5 mg/kg per day for 8 consecutive days. There were 6 rats in both the control (——) and disulfoton-treated (- - - -) groups. Vertical bars represent standard errors.

The effect of disulfoton administration on cholinesterase levels is summarized in Table 3. There was marked inhibition of total cholinesterase and acetylcholinesterase activity in all three tissues; the rate of hydrolysis of both substrates was inhibited by more than 50% in each tissue.

TABLE 3. *Comparison of the cholinesterase activities of the rat ileum, uterus and vas deferens following repeated administration of disulfoton*

Tissue	Substrate	Group	*n*	(μmol hydrolysed/g of protein)/h ± S.E.	Per cent inhibition
Ileum	ACh	Control	6	1995·0 ± 82·5	75
		Treated	5	495·0 ± 220·5*	
	MCh	Control	6	124·0 ± 14·0	71
		Treated	5	36·0 ± 8·0*	
Vas deferens	ACh	Control	5	1485·0 ± 225·0	85
		Treated	5	225·0 ± 45·0*	
	MCh	Control	5	84·0 ± 2·0	64
		Treated	5	30·0 ± 4·0*	
Uterus	ACh	Control	6	384·0 ± 30·0	58
		Treated	6	163·5 ± 33·0*	
	MCh	Control	6	36·0 ± 6·0	60
		Treated	6	14·0 ± 2·0*	

Females received i.p. 1·0 mg/kg of disulfoton and males 2·5 mg/kg for 8 consecutive days. *n* represents number of rats per group. * $P<0·01$.

Discussion

It is generally accepted by those who have studied subsensitivity that the phenomenon is related to the increased exposure of effector cells to acetylcholine (Brodeur & DuBois, 1964; Emmelin, 1964; McPhillips & Coon, 1966; Reas & Trendelenburg, 1967; Bito, Dawson & Pétrinović, 1971). In most instances repeated administra-

tion of cholinesterase inhibitors is the method which has been employed to increase acetylcholine levels. In the present study repeated administration of the cholinesterase inhibitor disulfoton caused subsensitivity of the ileum to carbachol but failed to produce subsensitivity of either the uterus or vas deferens. Subsensitivity, therefore, does not occur in all tissues. Failure to develop subsensitivity does not appear to be the result of insufficient inhibition of acetylcholinesterase in any of the tissues because the degree of inhibition appeared to be similar in all three tissues. The activity of acetylcholinesterase, the critical enzyme, was reduced by 71% in the ileum and 60% in the uterus. While subsensitivity does not develop in the absence of cholinesterase inhibition it appears that inhibition of cholinesterase itself does not result in subsensitivity.

The fact that sustained cholinesterase inhibition in the uterus and vas deferens was not followed by subsensitivity to carbachol suggests that a sufficient amount of acetylcholine did not accumulate at cholinoceptors to cause the changes necessary for the development of subsensitivity. The amount of acetylcholine which accumulates would depend upon the amount released from nerve terminals and the amount disposed of by diffusion and by destruction by acetylcholinesterase. Since acetylcholinesterase in all three tissues was markedly depressed the amount of acetylcholine which would accumulate would depend primarily upon the amount released which in turn would be directly related to the number of impulses coming from the central nervous system. A critical factor in the development of subsensitivity, therefore, would seem to be the intensity of nervous activity in a particular tissue and the density of innervation.

There is no doubt that the ileum has a dense cholinergic innervation and receives a steady continuous flow of impulses from the parasympathetic nerves. If there is interference with the innervation of the gut then the motility of the gut is reduced. Decreased gastrointestinal motility and constipation are well known consequences of treatment with atropine (Henderson & Roepke, 1937 ; Ambache, 1955) and ganglion blocking agents (Paton & Zaimis, 1951). Cholinesterase inhibition, on the other hand, increases gut motility due to the accumulation of acetylcholine (Holmstedt, 1959) and subsequently leads to the development of subsensitivity.

The innervation of the vas deferens and uterus would suggest that these tissues do not receive a steady intense flow of impulses by way of parasympathetic nerves. Both of these tissues apparently have a rather sparse parasympathetic innervation which does not have a great deal of influence on the motility of these organs. The ratio of cholinergic and adrenergic nerve fibres to muscle cells in the rat uterus is apparently quite low (Adham & Schenk, 1969 ; Silva, 1966). This would go along with the low level of acetylcholinesterase activity which was found in the uterus in the present study. Moreover, Schofield (1952) and Pallie, Corner & Weddell (1954) believe that the autonomic innervation which is present plays only a minor role in uterine movement. They suggest that the primary role of uterine nerves is to control the blood supply and glandular secretions. Factors which modify the response of an organ to cholinergic nerve stimulation apparently do not affect the response of the uterus.

A similar analysis may also apply in explaining the failure of the vas deferens to develop subsensitivity. The acetylcholinesterase activity of this organ is higher than the activity of the uterus but still considerably less than the acetylcholinesterase

activity of the ileum. Comparisons based on total cholinesterase activity may be misleading. Risely & Skrepetos (1964) determined by histochemical means the distribution of cholinesterase in the rat vas deferens and found that cholinesterase was present in the epithelial cells and in the smooth muscle. Furthermore, Dawson & Rowlands (1959) observed that glycerylphosphorylcholine was an important component of the fluid in the vas deferens. The epithelial cholinesterase of the vas deferens therefore, may merely be involved in the metabolism of glycerylphosphorylcholine or related compounds and not in the metabolism of endogenous acetylcholine (Gerebtzoff, 1959). The amount of cholinesterase associated with nerves innervating smooth muscle cells of the vas may be quite small. This seems to be the case because Richardson (1962) demonstrated nerve endings in the rat vas deferens which contained a uniform population of nongranular vesicles and which may represent cholinergic vesicles. The number of such nerve endings, however, appeared to be small. If the smooth muscle of the vas deferens is innervated predominately by adrenergic neurones, as it seems to be, with only sparse cholinergic innervation, inhibition of acetylcholinesterase may produce only minimal elevation of the acetylcholine concentration, insufficient to produce subsensitivity.

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