CHOLINESTERASE INHIBITION IN TISSUES AND THE ACTION OF REVERSIBLE INHIBITORS

Ya. S. Smusin

Department of Forensic Medicine (Head – Candidate of Medical Sciences Ya. S. Smusin) Chelyabinskii Medical Institute and Laboratory of Pharmacology (Head – Professor M. Ya. Mikhelson), I. M. Sechenov Institute of Evolutionary Physiology, AN SSSR
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It is very difficult to determine the degree of inhibition of the action of cholinesterase if it is brought about by an inhibitor whose action is reversible. When cholinesterase activity in any tissue is determined by a biological or biochemical method, a homogenate of the tissue in water or saline has to be prepared and the inhibitor is thereby diluted. When it has been thus diluted, if the action is easily reversible, the inhibitor may fail to bring about the inhibition which it exerted in the "undiluted" tissue in vivo. For example, anesthetics inhibit cholinesterase in the concentrations in which it is present in the brain during anesthesia [2]. However, during the preparation of a brain homogenate of the anesthetized animal the tissue becomes diluted and the concentration of the anesthetic is reduced several times until it falls below threshold value, and the activity of the enzyme recovers to normal [2]. The complex formed between the anesthetic and the cholinesterase is very rapidly dissociated. The dissociation occurs to a certain extent during the preparation of the homogenate and the determination of the enzymatic activity of the tissue whenever the cholinesterase is inhibited by a reversible inhibitor, such as morphine, eserine, or proserine. It is only when eserine and (especially) proserine are used that the recovery of the enzymatic activity in the diluted homogenate proceeds slowly, so that there may be some recovery of inhibition. However, the inhibition is always weaker than it was in the living tissue.

When cholinesterase is demonstrated histochemically we have been able nevertheless to show a marked reduction of the characteristic cholinesterase stain in sections of tissues prepared from anesthetized animals [3]. In the histochemical determinations there is no dilution. The reaction takes place in the section which retains its normal structure. Possibly the anesthetic is not washed out of the test tissue, or is washed out very slowly, so that its inhibitory action on cholinesterase is shown.

In the present work we have attempted to find whether the reversible action of inhibitors (particularly anesthetics) on cholinesterase cannot be revealed by cutting out portions of tissue (muscle, nerve, brain) of an anesthetized animal; the tissue is not triturated, but placed in a solution of acetylcholine. The tissue of the anesthetized animal might destroy acetylcholine more slowly than the tissue of control animals.

EXPERIMENTAL METHODS

Both rectus abdominis muscles of a frog were cut, weighed, and placed in a beaker containing 10 ml of aerated $1 \cdot 10^{-5}$ acetylcholine in Ringer at 20°. The gradual reduction of concentration of acetylcholine in the fluid surrounding the muscle due to its hydrolysis was determined by testing small samples of this fluid at intervals on a frog rectus abdominis preparation. Determination of the time for half the acetylcholine to be hydrolyzed (T_{50}) enabled the index of cholinesterase activity (QChE) (mg of acetylcholine hydrolyzedper g of tissue per hour) of the muscle to be calculated. Under these conditions the QChE was always much smaller in triturated tissue [1], because in the intact muscle hydrolysis occurs only at the surface. However, under the same conditions we were able to compare the course of destruction of acetylcholine in tissue taken from a control and from an anesthetized animal. We also carried out experiments on the intact sciatic nerves of several frogs.

TABLE 1. Cholinesterase Activity in Normal Frogs and in Frogs Anesthetized with Alcohol; Evaluation Made with Pieces of Muscles and Nerves

QChE of control frogs			QChE under alcohol anesthesia	
muscles		nerves	muscles	nerves
0,072	0,1	0,26	0,07	0,27
0,071	0,043	0,225	0,16	0,067
0,164	0,44	0,22	0,161	0,22
0,1	0,2	0,122	0,117	0,12
0,133	0,155	0,34	0,039	0,13
0,14	0,17	0,168	0,07	0,135
0,1	0,105	0,13	0,106	0,2
0,12	0,061	0,17	0,065	0,072
0,06	0,044	0,226	0,07	
0,04	0,087	0,256	0,07	
0,065	0,1	0,364	0,133	
0,286	0,107	0,174	0,1	
0,226	0,11	0;225	0,049	
0,16	0,226	0,13	0,059	
			0,13	
Mean . .	0,132±0,033	0,215±0,02	0,093±0,024	0,152±0,059

TABLE 2. Spontaneous Hydrolysis of Acetylcholine in Ringer Solution

Time for half-breakdown (in hours)		
45,3	24,0	142,5
109,0	72,5	32,0
35,0	24,0	31,0
16,7	16,7	14,6
17,7	15,0	31,6
16,7	32,4	17,2
12,8	82,0	46,7
82,0		
Mean . .	40,0±7,4	

TABLE 3. Cholinesterase Activity of Ringer Solution Which Has Contained Muscle

QChE
0,007
0,020
0,061
0,024
0,023
0,013
Mean . . 0,025±0,008

In other experiments, under identical conditions we compared the cholinesterase activities of triturated muscles or nerves.

EXPERIMENTAL RESULTS

In the first set of experiments we compared the cholinesterase activities of muscles and nerves of frogs kept for 24 h under alcohol anesthesia with those of control frogs; the alcohol was diluted in water to 0.35 M/liter. As can be seen from Table 1, in muscles and nerves taken from anesthetized animals, hydrolysis of acetylcholine was 1.5-1.4 times slower than in the controls. About two hours were required for half of it to be destroyed.

If the tissues were not placed in acetylcholine solution, the time for the half-breakdown was 40 h (Table 2). It can be seen therefore that we were observing a true enzymatic and not a spontaneous hydrolysis of acetylcholine.

It was important to determine to what extent the hydrolysis occurred in the piece of tissue itself (or on its surface) and how much occurred through cholinesterase passing out of the tissue into the solution. The effective concentration of the inhibitor might be maintained only in the tissue; in the solution it was diluted several tens of times. The muscle was placed for two hours in a beaker containing 9 ml of Ringer's solution, and it was kept aerated. It was then removed, and to the solution 1 ml of $1 \cdot 10^{-4}$ acetylcholine was added [1]. The course of hydrolysis of acetylcholine under these conditions is shown in Table 3. On average, in the presence of muscle, the QChE had a value of

0.025 instead of 0.132. Therfore, not more than 22% of the hydrolysis of acetylcholine takes place through the passage of cholinesterase out of the tissue into the solution.

In another set of experiments we determined the course of hydrolysis of acetylcholine by the same muscle and nerves after they had been completely homogenized (Table 4).

TABLE 4. Normal Cholinesterase Activity, and Activity Under Alcohol Anesthesia; Determination in Homogenized Tissue

QChE control frogs		QChE under alcohol anesthesia	
muscles	nerves	muscles	nerves
3,9	0,6	3,5	3,06
4,1	2,14	5	4,5
6,9	1,94	7,2	1,5
9,7	1,55	10	3,54
5,3	2,59	9,7	1,927
1,4	2,1	8,7	2,548
4,7	1,7	1,88	
8	0,536	1,76	
8,7	2,532	2,82	
1	3,488	0,944	
1,84	2,2	0,644	
1,4	2,857	0,72	
2,22	4,7	1,022	
1,344	5	1,255	
3,3		0,093	
3,58		0,057	
2,127		0,17	
2,421			
2,285			
1,938			
Mean . . . 3,616±0,565	2,424±0,342	3,263±0,853	2,846±0,443

TABLE 5. Comparison of the Suppression of Frog Muscle Cholinesterase Activity by Proserine; Determination in a Piece of Tissue and in Homogenized Tissue

QChE			
"Undiluted" tissue		Method of preparing homogenate	
control	after treatment with proserine	control	after treatment with proserine
0,132±0,033 *	0,043	3,616±0,565 **	2,2
	0,033		3,8
	0,04		3,2
	0,05		2
	0,02		0,5
	0,019		
Mean . . 0,132±0,033	0,034±0,005	3,616±0,565	2,34±0,57
QChE control / QChE proserine	3,8	QChE control / QChE proserine	1,5

* Figures taken from Table 1.

** Figures taken from Table 2.

When we worked with triturated tissues of the control frogs we found a far more rapid hydrolysis than with separate pieces. The absolute value of the QChE obtained with triturated tissue was close to that given in [6]. Thus in these experiments we confirmed that it was impossible to demonstrate in triturated tissue inhibition of cholinesterase in vivo by reversible inhibitors such as anesthetics [6].

However, when working with complete pieces of tissue considerable suppression of cholinesterase in the tissues of anesthetized animals was found.

Suppression of cholinesterases by anesthetics is readily reversible. The activity of the enzymes is restored immediately after the concentration of the anesthetic falls below threshold value. It was important to prove the reliability of the method we have described for revealing the inhibition of cholinesterases using other inhibitors. We tested proserine which is a reversible inhibitor. However, the restoration of the activity of cholinesterase suppressed by proserine takes place extremely slowly. An injection of 170 mg/kg was given into the dorsal lymph sac, and caused paralysis of most of the frogs [2]. As can be seen from Table 5, when intact pieces of muscle were used the activity of the cholinesterases was reduced 3.8 times, and when the same muscle was triturated the reduction was only 1.5 times. The very small suppression occurring with trituration shows that the slowly reversible inhibition of cholinesterases by proserine did not disappear completely even after homogenization involving considerable dilution of the tissue. However, inhibition was much more strongly shown when pieces of muscle were used. Thus, in the case of proserine also, use of pieces of tissue gives a much more correct indication of the degree of suppression of cholinesterase in vivo than does the usual method of triturating tissue.

We have also carried out pilot experiments with 64 mg/kg of the irreversible cholinesterase inhibitor armin, anticipating that for it, the same degree of suppression would be shown with trituration as with intact pieces. However, here too the suppression was better shown with pieces of muscle. We had not anticipated this result. Perhaps in the case of armin too, under the conditions of our experiment there was a partial reactivation of cholinesterases which is better shown when the tissues are triturated than when intact muscles are used. The results with the irreversible inhibitor require further study.

Operation with "pieces of undiluted tissue" may also be used for the reversible cholinesterase inhibitors. We have used this method for determining the activity of cholinesterase in tissues of animals treated with morphine [4], and also in the tissues of cadavers of human subjects who had received lethal amounts of alcohol, barbiturates, or strychnine [5]. In all cases it was found that the suppression of cholinesterase activity in the tissue was more than half as great as in normal tissues. When the normal method of trituration and dilution of the tissue was used, no inhibition was found.

SUMMARY

The method is based on determination of the cholinesterase activity in a piece of tissue without preliminary homogenization thereof. This helps to avoid the inhibitor dilution with a marked reduction of its concentration, which invariably occurs in preparing tissue homogenates. With this method cholinesterase inhibition was detected in the muscular and nervous tissue of frogs anesthetized with ethyl alcohol. The usual method of preparation of tissue homogenates failed to demonstrate any inhibition under the same conditions.

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