RECIPROCAL POTENTIATING ACTION OF DEPOLARIZING DRUGS ON THE ISOLATED FROG RECTUS ABDOMINIS MUSCLE

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Subthreshold concentrations of the depolarizing agents, acetylcholine and decamethonium, potentiated isotonic contractures of the isolated frog rectus abdominis muscle elicited by effective doses of either of these drugs or by choline in the absence of the anticholinesterases, sarin, or dyflos. Comparable potentiation of the contracture produced by decamethonium or choline could be obtained by alternate stimulation with acetylcholine in the absence of dyflos or sarin. Furthermore, dyflos alone did not effect potentiation in the absence of alternate stimulation with acetylcholine. Finally, the inhibitory action of tubocurarine, as measured by depression of the contracture elicited by decamethonium and acetylcholine, was not antagonized by sarin. It was concluded that the enhancement of the response to acetylcholine produced by dyflos and by sarin was brought about exclusively by their action as inhibitors of cholinesterase. The ability of dyflos and of sarin to potentiate contractures elicited by decamethonium or by choline may be attributed to a facilitation of the action of the depolarizing compounds by subthreshold concentrations of acetylcholine available in the presence of these anticholinesterases.

Cohen and Posthumus (1955) observed that dyflos (di-isopropyl phosphorofluoridate) and sarin (isopropyl methylphosphonofluoridate) potentiate the effects of decamethonium and choline on the isolated frog rectus abdominis muscle. Because decamethonium and choline are not esters, and are therefore not hydrolysed by cholinesterase, they concluded that dyflos, and other anticholinesterases, promote a "sensitization" of acetylcholine receptors independent of any inhibition of cholinesterase activity. They further inferred that direct, non-enzymatic sensitization "is at least partly responsible for the enhanced response of the muscle toward acetylcholine after the administration of anticholinesterases." In view of the fundamental nature of their conclusions concerning the mechanism of action of anticholinesterases, a similar study on the frog rectus has been conducted in our laboratory.

METHODS

Frog Ringer Solution. — Composition: NaCl, 0.56% ; KCl, 0.0075% ; CaCl₂, 0.01% ; dextrose

0.2%. The solution was buffered with 0.02 M-tris-(hydroxymethyl-amino)methane (Tris buffer) at pH 7.2 and was bubbled with oxygen.

Depolarizing Drugs. — Acetylcholine chloride, choline chloride, and decamethonium bromide were made up in frog Ringer solution. The test solutions of acetylcholine were freshly prepared before each experiment.

Anticholinesterase Drugs. — Dyflos (di-isopropyl phosphorofluoridate, DFP) [Merck Ampoules]. Sarin (isopropyl methylphosphonofluoridate), obtained from Chemical Research Division of Chemical Warfare Laboratories; purity was 95% or better according to an analysis of acidity. All solutions of anticholinesterases were made up freshly before use in frog Ringer solution.

Measurement of Isotonic Contracture. — The method of preparation of the isolated muscle, addition of reagents and measurement of muscle contracture have been previously described (Fleisher, Howard, and Corrigan, 1958). Responses of the muscle to any dose of a stimulating drug were repeated until successive measurements yielded an identical rate of contracture (± 1 mm./min.) before the addition of a second depolarizing substance (for example, see Table I).

RESULTS

Effect of Subthreshold Concentrations of Depolarizing Agents upon the Contracture Produced by Addition of a Second Depolarizing Agent

A reproducible rate of contracture of the muscle was obtained to a particular dose of decamethonium to serve as a control for the response elicited by the same dose of decamethonium in the presence of a subthreshold concentration of acetylcholine. Control measurements showed no contraction following addition of subthreshold concentrations of acetylcholine, decamethonium and choline which were approximately half of the minimal effective dose.

TABLE I
POTENTIATING EFFECT OF SUBTHRESHOLD CONCENTRATIONS OF DEPOLARIZING SUBSTANCES ON THE CONTRACTURE PRODUCED BY THE ADDITION OF A SECOND DEPOLARIZING AGENT ON ISOLATED FROG RECTUS ABDOMINIS MUSCLE

Expt. No.	Dose ($\mu\text{g./ml.}$)	Contracture (mm./min.)
1	50 $\mu\text{g.}$ decamethonium	20
	50 " " " " " "	20
	(0.05 $\mu\text{g.}$ acetylcholine for 1.5 min.	No contracture
	(Followed by 50 $\mu\text{g.}$ decamethonium	27
	(0.05 $\mu\text{g.}$ acetylcholine for 1.5 min.	No contracture
2	(Followed by 20 $\mu\text{g.}$ decamethonium	20
	1 $\mu\text{g.}$ acetylcholine	18.0
	1 " " " " " "	18.5
	(6 $\mu\text{g.}$ decamethonium for 1.5 min.	No contracture
	(Followed by 1 $\mu\text{g.}$ acetylcholine ..	28.5
3	1,000 $\mu\text{g.}$ choline	21
	1,000 " " " " " "	21
	(0.05 $\mu\text{g.}$ acetylcholine for 1.5 min.	No contracture
	(Followed by 1,000 $\mu\text{g.}$ of choline	26.5
	(0.05 $\mu\text{g.}$ acetylcholine for 1.5 min.	No contracture
4	(Followed by 800 $\mu\text{g.}$ of choline ..	21
	1 $\mu\text{g.}$ acetylcholine	20.5
	1 " " " " " "	20
	(70 $\mu\text{g.}$ choline for 1.5 min. ..	No contracture
	(Followed by 1 $\mu\text{g.}$ acetylcholine ..	23

Results shown in Table I indicate that an increased response to decamethonium may be produced by as little as 0.05 $\mu\text{g./ml.}$ of acetylcholine (Expt. No. 1). Similarly the responses to acetylcholine and to choline were potentiated by the presence of subthreshold concentrations of decamethonium and of acetylcholine (Expt. Nos. 2 and 3).

There was little or no potentiation to acetylcholine in the presence of a subthreshold concentration of choline (Expt. No. 4).

Effect of Sarin on the Potentiation of Acetylcholine Produced by Decamethonium.—A constant initial response was obtained to acetylcholine and then in the presence of a subthreshold amount of decamethonium. After washing, the response to further addition of acetylcholine was elicited twice in the absence of decamethonium at the usual interval of 1 min. Table II shows that the enhancement of response disappears rapidly after the decamethonium is washed out.

Incubation with sarin 5×10^{-7} M for 1.5 hr., and testing in the presence of 10^{-7} M sarin, resulted in a ten-fold sensitization of the muscle to acetylcholine, without its response to decamethonium

TABLE II
EFFECT OF SARIN ON THE RESPONSE PRODUCED BY THE ADDITION OF ACETYLCHOLINE TO ISOLATED FROG RECTUS ABDOMINIS MUSCLE IN THE PRESENCE OF A SUBTHRESHOLD CONCENTRATION OF DECAMETHONIUM

In B the muscle was incubated with sarin, 5×10^{-7} M for 1.5 hr. followed by measurement of responses in 10^{-7} M-sarin.

Dose ($\mu\text{g./ml.}$)	Contracture (mm./min.)
A. Before sarin	
1 $\mu\text{g.}$ acetylcholine	22
1 " " " " " "	23
(2 $\mu\text{g.}$ of decamethonium for 1.5 min.	No contracture
(Followed by 1 $\mu\text{g.}$ acetylcholine	32
1 $\mu\text{g.}$ acetylcholine	26
1 " " " " " "	24
B. After sarin	
0.1 $\mu\text{g.}$ acetylcholine	24
0.1 " " " " " "	25
(2 $\mu\text{g.}$ of decamethonium for 1.5 min.	No contracture
(Followed by 0.1 $\mu\text{g.}$ acetylcholine	36
0.1 $\mu\text{g.}$ acetylcholine	20

being affected (Table II). Addition of acetylcholine to the sarin-treated muscle in the presence of previously added decamethonium showed further potentiation over and above that produced by sarin. After washing, the response elicited by acetylcholine, in the continued presence of 10^{-7} M sarin but in the absence of decamethonium, was less than the initial response after sarin.

Effect of Sarin on the Potentiation Produced by Acetylcholine Toward Choline.—The responses of the muscle to choline were observed before and after incubation of the muscle with sarin 10^{-5} M. The response was increased after incubation with sarin, but returned to the control value during the next two additions of choline to the muscle despite the continued presence of the anticholinesterase. When choline was added in the presence of a subthreshold concentration of acetylcholine, the response was comparable to that obtained immediately after incubation with sarin (Table III).

Effect of a Previous Response to Acetylcholine on the Response to Decamethonium or Choline in the Absence of and After Incubation with Anticholinesterase

In the experiments of Cohen and Posthumus (1955) the frog rectus muscle was made to contract to acetylcholine, decamethonium, and choline in

TABLE III
EFFECT OF SARIN ON THE RESPONSE PRODUCED BY THE ADDITION OF CHOLINE TO ISOLATED FROG RECTUS ABDOMINIS MUSCLE. EFFECT OF A SUBTHRESHOLD CONCENTRATION OF ACETYLCHOLINE

In B the muscle was incubated with 10^{-5} M-sarin for 30 min. followed by measurement of responses in 10^{-7} M-sarin.

Dose (μ g./ml.)	Contracture (mm./min.)
A. Before sarin	
1,000 μ g. choline	30.0
1,000 " " " " " " " " " " " "	29.5
B. After sarin	
1,000 μ g. choline	34.5
1,000 " " " " " " " " " " " "	32.5
1,000 " " " " " " " " " " " "	29.5
{ 0.005 μ g. acetylcholine for 1.5 min.	No contracture
{ Followed by 1,000 μ g. choline	34.0
{ 0.005 μ g. acetylcholine for 1.5 min.	No contracture
{ Followed by 800 μ g. choline	29.5

TABLE IV
EFFECT OF ACETYLCHOLINE ON THE CONTRACTURE PRODUCED BY DECAMETHONIUM IN THE ABSENCE OF AND AFTER INCUBATION WITH DYFLOS
In B the muscle was incubated in 10^{-6} M dyflos for one hour followed by measurement of responses in 10^{-6} M dyflos.

Expt. No.	Dose (μ g./ml.)	Contracture (mm./min.)
1	Not treated with dyflos	
	21 μ g. decamethonium ..	24
	21 " " " " " " " " " " " "	24
	1.25 μ g. acetylcholine ..	44
	21 μ g. decamethonium ..	36
	1.25 μ g. acetylcholine ..	50
	10.5 μ g. decamethonium ..	25
	21 " " " " " " " " " " " "	33.5
	21 " " " " " " " " " " " "	26
2	A. Before dyflos	
	21 μ g. decamethonium ..	19
	21 " " " " " " " " " " " "	17
	B. After dyflos	
	21 μ g. decamethonium ..	19
	21 " " " " " " " " " " " "	18
	0.15 μ g. acetylcholine ..	37
	21 μ g. decamethonium ..	24
	0.15 μ g. acetylcholine ..	34
	14 μ g. decamethonium ..	16
	21 " " " " " " " " " " " "	20.5
	21 " " " " " " " " " " " "	19

that order, using frog Ringer solution to wash the tissue between contractions. After incubation with dyflos or sarin, the same procedure was repeated. By this method they observed a 1.5 to 2-fold enhancement of response towards decamethonium and a 4-fold enhancement towards choline after incubation with the anticholinesterases mentioned.

These results have been confirmed in the present experiments. However, additional experiments were performed in which contractures were also obtained by decamethonium and choline before measuring those elicited by acetylcholine. The results of representative experiments are shown in Tables IV and V. Expt. No. 1 in each table shows that the control responses obtained to decamethonium and to choline are potentiated after a contracture elicited with acetylcholine in the absence of dyflos. This potentiation was temporary, since further responses, obtained by either decamethonium or choline not preceded

TABLE V

EFFECT OF A PREVIOUS RESPONSE TO ACETYLCHOLINE ON THE CONTRACTURE PRODUCED BY CHOLINE IN THE ABSENCE OF AND AFTER INCUBATION WITH DYFLOS

In 2B the muscle was incubated with 10^{-5} M dyflos for one hour followed by measurement of responses in 10^{-6} M dyflos.

Expt. No.	Dose (μ g./ml.)	Contracture (mm./min.)
1	Not treated with dyflos	
	800 μ g. choline	23
	1,000 ,, ,,	29
	2 μ g. acetylcholine	21.5
	1,000 μ g. choline	36.5
	2 μ g. acetylcholine	22.5
	800 μ g. choline	30
	1,000 ,, ,,	31.5
	1,000 ,, ,,	29
2	A. Before dyflos	
	1,000 μ g. choline	22
	1,000 ,, ,,	22.5
	B. After dyflos	
	1,000 μ g. choline	22.5
	0.4 μ g. acetylcholine	33.5
	1,000 μ g. choline	27.5
	0.4 μ g. acetylcholine	32
	800 μ g. choline	23
	1,000 ,, ,,	22

TABLE VI

EFFECT OF TUBOCURARINE UPON THE RESPONSE OF THE ISOLATED FROG RECTUS MUSCLE TO DECAMETHONIUM

In the experiments with sarin the rectus muscle was incubated for 30 min. with 10^{-5} M sarin followed by measurement of responses in 5×10^{-7} M sarin.

Expt. No.	Contracture Produced by 25 μ g./ml. of Decamethonium			
	Before Sarin		With Sarin	
	Without Tubocurarine	With 2×10^{-7} M Tubocurarine	Without Tubocurarine	With 2×10^{-7} M Tubocurarine
	(mm./min.)	(mm./min.)	(mm./min.)	(mm./min.)
1	18	11	18	11
2	23	14	21	13
3	19	11	20	11

TABLE VII

EFFECT OF TUBOCURARINE UPON THE RESPONSE OF THE ISOLATED FROG RECTUS MUSCLE TO ACETYLCHOLINE

In the experiments with sarin the rectus muscle was incubated for 30 min. with 10^{-5} M-sarin followed by measurement of responses in 10^{-6} M-sarin.

Expt. No.	Before Sarin			With Sarin		
	Acetylcholine Concentration ($\mu\text{g.}/\text{ml.}$)		Desen- sitiza- tion Ratio	Acetylcholine Concentration ($\mu\text{g.}/\text{ml.}$)		Desen- sitiza- tion Ratio
	No Tubo- curarine	With 5 $\times 10^{-7}\text{M}$ Tubo- curarine		No Tubo- curarine	With 5 $\times 10^{-7}\text{M}$ Tubo- curarine	
1	1.5	3.5	2.3	0.08	0.2	2.5
2	1.5	5.0	3.3	0.075	0.25	3.3
3	1.0	3.0	3.0	0.07	0.20	2.9
4	2.0	5.5	2.8	0.20	0.60	3.0

by a response to acetylcholine, decreased to the control value.

Expt. No. 2 in Tables IV and V shows that no potentiation over the control responses upon the addition of decamethonium and choline respectively after incubation with dyflos occurred. Subsequent measurement of a response to decamethonium or choline following a contracture elicited by acetylcholine yielded a degree of potentiation no greater than that obtained in the absence of anticholinesterase. This potentiation, like that obtained following an acetylcholine response in the absence of dyflos, faded rapidly if alternate stimulation with acetylcholine is not maintained. Similar results were obtained with other rectus muscles undergoing incubation with sarin instead of dyflos.

Effect of Sarin upon the Response to Decamethonium and Acetylcholine of the Rectus Muscle Treated with Tubocurarine, Decamethonium and Acetylcholine

A reproducible rate of contracture in response to the addition of 25 μ g./ml. of decamethonium was first obtained. Then the response of the rectus muscle to the same concentration of decamethonium in the presence of 2×10^{-7} M tubocurarine was measured, followed by washing with frog Ringer solution until the initial response was again obtained. The muscle was then

incubated with 10^{-5} M sarin for 30 min. and the response to 25 $\mu\text{g./ml.}$ of decamethonium again elicited in the presence of 5×10^{-7} M sarin. As in earlier experiments, the rectus muscle displayed no enhanced response after incubation with sarin. The rate of contracture to 25 $\mu\text{g./ml.}$ of decamethonium was then measured in the presence of 5×10^{-7} M sarin and 2×10^{-7} M tubocurarine. If the anticholinesterase had a direct potentiating action upon receptor sites in the muscle some antagonism of the previous depression of the decamethonium response by tubocurarine, in the presence of an excess of cholinesterase inhibitor, could be expected. Table VI shows that no such antagonism was demonstrable for the rectus muscles of three frogs treated in the manner described.

In a similar experiment, the effect of sarin upon the response of curarized rectus muscle to acetylcholine was determined. The concentration of acetylcholine needed to yield a constant response was determined for each of the conditions noted in Table VII. For example, in Expt. No. 1 the presence of 5×10^{-7} M tubocurarine necessitated 2.3 times the initial concentration of 1.5 $\mu\text{g./ml.}$ of acetylcholine to maintain the constant response (36 ± 1 mm./min.) obtained before treatment with sarin. The ratio of the doses required to give equal responses before and after tubocurarine is called the "desensitization ratio." After incubating with sarin, only 1/19 of the original concentration of acetylcholine (0.08 $\mu\text{g./ml.}$) was required to give the initial contracture. But after the addition of tubocurarine (5×10^{-7} M) to the medium containing the sarin, the concentration of acetylcholine required to maintain the initial contracture increased 2.5-fold over that needed in sarin alone. The other experiments shown in Table VII, carried out in the same way, agreed with Expt. No. 1—that is, they show no direct antagonism of sarin to tubocurarine over and above that due to its property of inactivating cholinesterase.

DISCUSSION

The frog rectus abdominis muscle is believed to contain slow muscle fibres which respond to acetylcholine (and other depolarizing substances) with slow contractile changes (Brecht and Epple, 1952; Kuffler and Vaughan Williams, 1953). Such slow muscle fibre systems in frog ilio-fibularis muscle show true mechanical facilitation—that is, during a series of stimuli successive nerve volleys add increasing amounts of tension (Kuffler and Vaughan Williams, 1953).

The application of an appropriate concentration of acetylcholine (or other suitable depolarizing agent) mimics the effect of stimulating the nerve supply of such muscles (Burgen and MacIntosh, 1955). Consequently it may be postulated that the potentiation between subthreshold concentrations of one depolarizing agent and an effective concentration of another develops by a facilitatory mechanism comparable to that demonstrated for the frog ilio-fibularis muscles. This explanation would include the potentiation of the muscle response to acetylcholine by decamethonium before treatment with dyflos. It serves also to explain the other examples of potentiation developed in the presence of subthreshold concentrations of the depolarizing agents studied. In addition, the presence of anticholinesterases was found to be unnecessary for the potentiation produced by the presence of subthreshold concentrations of acetylcholine and decamethonium, as well as butyryl and succinyl choline (Fleisher, Corrigan and Howard—unpublished observations), on the response elicited by addition of effective doses of the same drugs and of choline to frog rectus muscle.

Cohen and Posthumus (1955) observed no further sensitization by decamethonium toward acetylcholine after treatment with anticholinesterases. We found a significant potentiation in the response of sarin-treated muscle to acetylcholine in the presence of a subthreshold concentration of decamethonium (Table II) over and above the increased sensitization to acetylcholine induced indirectly by inactivation of cholinesterase (Miquel, 1946). Table II also shows that the relative increase in response to acetylcholine developed in the presence of identical subthreshold concentrations of decamethonium is the same before and after sarin. If the anticholinesterase possessed the property of sensitizing the receptor sites so as to yield an enhanced response to decamethonium, then a significantly greater effect of subthreshold doses of decamethonium could have been expected after incubation with sarin. The small and temporary potentiation obtained to choline following sarin incubation (Table III) may be attributed to accumulation of endogenous acetylcholine in the presence of an anticholinesterase (Fatt and Katz, 1952).

In addition sarin showed no antagonism to tubocurarine over and above the anticurare activity derived from its anticholinesterase action. That is, the inhibitory action of tubocurarine on receptor sites as measured by its depression of the response of the rectus muscle to decamethonium

or acetylcholine was not antagonized by the cholinesterase inhibitor (Tables VI and VII). It would be expected that if sarin acted on the rectus muscle exclusively as an anticholinesterase, and tubocurarine specifically reduced the depolarizing action of applied acetylcholine (del Castillo and Katz, 1956), then the degree of desensitization to acetylcholine produced in the rectus muscle by identical partially curarizing concentrations of tubocurarine would be approximately the same with and without sarin. The results in Table VII strongly support this conclusion for the rectus muscle under the conditions tested. The direct depressant action of some anticholinesterases on receptor sites observed by other investigators has usually been found with concentrations considerably greater than those required for complete inhibition of cholinesterase activity (Eccles and MacFarlane, 1949; Kirschner and Stone, 1951; and Axelsson, Gjone, and Naess, 1957).

The consistent potentiation of the contracture of the muscle elicited by decamethonium and choline following a previous response to acetylcholine and washing, before incubation with an anticholinesterase, may be explained on the basis of a residual sensitization of receptors, or subthreshold depolarization following the response to stimulation with acetylcholine. This hypersensitivity was transient, and usually disappeared after 20 to 30 min. soaking in frog Ringer. This explanation is

supported by the observation of a chemical component in slow muscle-fibre activity which can restore tension or shortening after temporary collapse (Kuffler and Vaughan Williams, 1953).

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