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EFFECT OF PYRIDINE ALDOXIMES ON RESPONSE OF FROG RECTUS MUSCLE TO ACETYLCHOLINE

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The effects of pyridine-2, 3- and 4-aldoxime methiodide (PAM) on the isotonic contracture of the isolated frog rectus abdominis muscle elicited by acetylcholine have been studied. The potentiation to acetylcholine resulting from incubation with PAM isomers is reversible and may be related to the weak anticholinesterase property of these compounds. Concentrations of the isomers higher than those needed for maximal potentiation produce a reversible block of the muscle to acetylcholine. *p*H studies suggest that cations are responsible for the potentiating and blocking properties of the PAM isomers.

Pyridine-2-aldoxime methiodide (P2AM) is an effective therapeutic compound when used as an adjunct to atropine in the treatment of anticholinesterase poisoning (Askew, 1957; Wills, Kunkel, Brown, and Groblewski, 1957). However, Kewitz and Wilson (1956) reported that lethal doses of P2AM produced death from respiratory paralysis. Holmes and Robins (1955) found that this compound produced neuromuscular block in the isolated phrenic nerve-diaphragm preparation of the rat.

Because of the possible therapeutic importance of P2AM, the effects of this compound and its 3- and 4-isomers on a neuromuscular junction have been further investigated on the isolated frog rectus abdominis muscle.

METHODS

Solutions.—Frog Ringer: NaCl, 0.56; KCl, 0.0075; $CaCl_2$, 0.01; dextrose, 0.2% (w/v). Buffered with 0.02M-tris (hydroxymethyl) amino methane (TRIS) at *p*H 7.2. Oxygen was bubbled through the solution continuously during the experiments. The response of the rectus in Ringer buffered as above has been found comparable with that obtained in the conventional bicarbonate Ringer with the *p*H set by bubbling 95% O_2 and 5% CO_2. Frog Ringer buffered with TRIS possessed the advantage of permitting *p*H studies, with only negligible change in ionic strength.

Eserine salicylate (Merck) was used in a concentration of 2.5×10^{-5}M in frog Ringer. Acetylcholine (ACh) [Merck] was weighed and then dissolved in 5 ml. of cold acetate buffer *p*H 4.5 to yield a concentrated stock solution which was stored at 3° for not longer than one week. Aliquots of the stock were diluted with frog Ringer to the desired concentration immediately before use. The quantities of ACh used are expressed in μg./ml. of the free base.

Pyridine Aldoxime Methiodide Derivatives.—Samples of pyridine 2-, 3-, and 4-aldoxime methiodides were synthesized in the Chemotherapy Branch of Chemical Warfare Laboratories by Dr. Brennie E. Hackley, Jr., and were characterized by melting point and elemental analysis.

Measurement of Isotonic Contraction.—Frogs of either sex weighing 20 to 30 g. were used. The rectus abdominis muscle was dissected out and immersed in a temperature-regulated chamber (25° ± 0.2) of Ringer solution. ACh was added to yield a final volume of 4 ml. in the bath. A 1 min. contracture was recorded. The preparation was then washed four times with frog Ringer for periods of 30 sec. each, and the muscle was allowed to rest 9 min. before the next test solution of ACh was added.

The character of some of these experiments was such that the muscles were not tested for 1 hr. or more—as, for example, during incubation with an anticholinesterase. As the first response after a prolonged inactive period was often different from that produced when the contractures were elicited at the regular time intervals, the response after a longer rest period was always checked by successive measurements performed at 9 min. intervals. Two to three successive measurements generally sufficed to yield an identical rate of contraction (±1 mm./min.) to a constant dose of ACh.

RESULTS

Effect of P2AM upon the Response of the Rectus Abdominis to ACh. Relation to Cholinesterase Activity.—The rate of contracture of the isolated muscle to a given dose of ACh was measured both in the absence of P2AM as a control and then in the presence of the concentrations of P2AM listed in Table I. It may be seen that significant potentiation of the rate of contracture appeared at 1.0×10^{-4}M-P2AM and increased to a maximum at 7.5×10^{-4}M. Further increase in the concentration of P2AM resulted in a decrease in the rate of contracture to well below that of the control at 5.0×10^{-3}M-P2AM. This inhibitory effect on the response could be antagonized by doubling the concentration of ACh; both the potentiating and inhibitory effects were readily reversed on washing.

TABLE I

EFFECT OF P2AM ON THE CONTRACTURE PRODUCED BY ADDITION OF ACETYLCHOLINE TO ISOLATED FROG RECTUS ABDOMINIS MUSCLE

Conc. of P2AM (molar)	ACh Added (μg./ml.)	Rate of Contracture (mm./min.)
0	0·3	16·0
0	0·3	15·5
1.0×10^{-6}	0·3	16·0
1.0×10^{-5}	0·3	16·0
5.0×10^{-5}	0·3	18·0
1.0×10^{-4}	0·3	19·0
2.5×10^{-4}	0·3	24·0
5.0×10^{-4}	0·3	27·5
7.5×10^{-4}	0·3	29·0
1.0×10^{-3}	0·3	28·0
5.0×10^{-3}	0·3	11·5
5.0×10^{-3}	0·6	27·5
0	0·3	16·0
0	0·3	15·5

Measurements of the cholinesterase (ChE) activity (Fleisher, Corrigan, and Howard, unpublished observations) of the contralateral muscle from the same frog in 2.5 and 5.0×10^{-4}M-P2AM show a reduction of 23% and 40% respectively of the ChE activity obtained in the absence of P2AM, thus accounting, at least in part, for the observed potentiation of the rate of contracture at these concentrations of the oxime.

The site of the competitive antagonism between ACh and P2AM was studied further by measuring the responses of the isolated muscle to a constant dose of ACh (0.25 μg./ml.) in the presence of the successively greater concentrations of P2AM noted in Table II. After washing out the P2AM, and again obtaining the control response, the muscle was incubated with 2.5×10^{-5}M eserine for 1 hr. at 25° which completely inactivated the ChE activity. (Fresh muscles incubated with 2.5×10^{-5}M-eserine for periods exceeding 1 hr. showed no ChE activity.) The responses to the same dose of ACh were again obtained with the concentrations of P2AM used before, this time in the presence of 2.5×10^{-5}M-eserine. Table II shows maximum potentiation at 7.5×10^{-4}M-P2AM before incubation with eserine, followed by a decrease in the rate of contracture. After the ChE is wholly inactivated by eserine, the only effect observed in the presence of P2AM is antagonism to ACh, which increases markedly as the concentration of P2AM exceeds 1.0×10^{-3}M.

TABLE II

EFFECTS OF VARYING CONCENTRATIONS OF P2AM IN THE ABSENCE AND PRESENCE OF ESERINE UPON THE RESPONSE OF THE ISOLATED FROG RECTUS ABDOMINIS MUSCLE TO ACETYLCHOLINE

Conc. of P2AM (molar)	ACh Added (μg./ml.)	Rate of Contracture (mm./min.)	
		No Eserine	In 2.5×10^{-5} M Eserine
0	0·25	12	47
1.0×10^{-4}	0·25	14	46
2.5×10^{-4}	0·25	19	43
5.0×10^{-4}	0·25	22	41
7.5×10^{-4}	0·25	23	39
1.0×10^{-3}	0·25	22	34
2.5×10^{-3}	0·25	19	23
5.0×10^{-3}	0·25	13·5	12·5
5.0×10^{-3}	0·50	31	25
0	0·25	13	43
0	0·25	12	45

Effects of pH upon the Response of the Isolated Rectus Muscle to ACh in the Presence of P2AM or its 3- and 4-isomers.—The structure of P2AM and its 3- and 4-isomers is such that the ratio of the acid form of the molecule to the salt form would vary considerably with *p*H. It was of interest to study whether this variation in the ratio of anion to cation, produced by changes in the *p*H of the medium, could affect the response of the muscle to ACh. Control responses to ACh were first measured at *p*H 7.2, 8.0, and 8.6 and the rates of contracture varied no more than 2 to 3 mm./min. The effect of *p*H on further contractures produced by the addition of ACh in a final concentration of 0.5 μg./ml. to the incubation medium which contained progressively higher concentrations of P2AM was then studied on the same muscle at *p*H 7.2, 8.0, and 8.6. The results for all three *p*H values are shown in Fig. 1.

Similar studies were performed at *p*H 7.2, 8.0, and 8.6 with P3AM and P4AM respectively. From the known pK_a values for pyridine 2-, 3-, and 4-aldoxime methiodide (Wilson and Ginsberg, 1957) the proportion of the total concentration yielding the maximal rate of contracture which is in the cationic form may be calculated (Table III). Note that the concentration of cation associated with maximal potentiation of response remains roughly the same for P2AM and P3AM

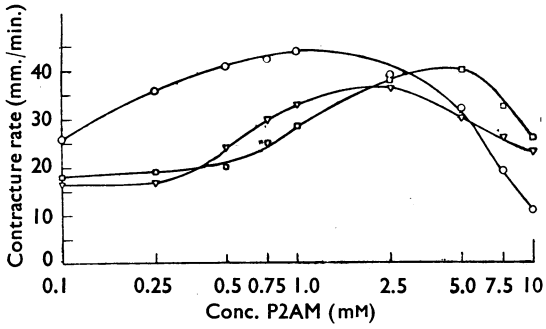

FIG. 1.—Effect of change of *p*H upon the response of the isolated frog rectus abdominis muscle to acetylcholine in the presence of increasing concentrations of P2AM. O *p*H 7.2; ▽ *p*H 8.0; □ *p*H 8.6.

while the total concentration of the compound varies over a fivefold range. The concentration of cation producing maximum response is more variable with P4AM, but still shows considerably smaller variation than the total concentration of the compound. The results suggest that potentiation is strongly influenced by, although not totally dependent on, the cation.

TABLE III

PROPORTION OF CATIONS OF PYRIDINE 2-, 3-, AND 4-ALDOXIME METHIODIDES AT *p*H 7.2, 8.0, AND 8.6

Compound	*p*K	*p*H	% of Cation	Conc. Yielding Maximal Rate of Contracture	
				Total Conc. (mM)	Molar Conc. of Cation (mM)
P2AM ..	8·0	7·2	86	1·0	0·86
		8·0	50	2·5	1·25
		8·6	20	5·0	1·00
P3AM ..	9·2	7·2	99	0·75	0·74
		8·0	94	0·75	0·71
		8·6	80	1·00	0·80
P4AM ..	8·6	7·2	96	0·75	0·72
		8·0	80	0·75	0·60
		8·6	50	2·50	1·25

Fig. 1 also shows a greater depression in the response to ACh for values of P2AM exceeding those required for the maximum contracture when the *p*H is 7.2 than at higher *p*H values. Since the cation is predominant at the lower *p*H of 7.2, an influence of this ion on the blocking of the response to ACh by P2AM is suggested.

DISCUSSION

Wilson and Ginsberg (1955) and Childs, Davies, Green and Rutland (1955) were the first to show the potent ChE-reactivating property of P2AM. The present paper reports other effects produced by this compound at the neuromuscular junction in the frog rectus abdominis muscle, namely an ACh-potentiating effect and an ACh-blocking property. The contracture produced by ACh in the presence of P2AM is therefore the result of these properties. Furthermore, in confirmation of the ineffectiveness of P2AM against eserine *in vivo* (Kewitz, Wilson and Nachmansohn, 1956), control experiments demonstrated no reactivating effects of P2AM against eserine-inactivated ChE of the isolated rectus muscle. Consequently, if the potentiating effect of P2AM is minimized by superimposition of the greater one produced by eserine, the blocking action of P2AM becomes predominant and can be shown to occur at concentrations where Holmes and Robins (1955) observed neuromuscular blocking action by this compound.

Both the potentiating and ACh-blocking potency of P2AM decrease with increase of *p*H from 7.2 to 8.6 concurrent with an increase in the proportion of anionic component from 14 to 80%. However, it is significant that at *p*H 7.2 there is a significant proportion of the anionic component of P2AM along with the cationic component which is presumed to contribute to reactivation by being bound or attracted to the anionic site on the ChE enzyme of the muscle. Among the three isomeric compounds, the proportion of available anionic component at physiological *p*H values varies as P2AM>P4AM>P3AM. The relative reactivating ability falls off even more rapidly at *p*H 7.2 (Fleisher, Howard and Corrigan, unpublished observation) indicating that either a certain minimal proportion of anionic component must be available for effective reactivation, or that other factors are also involved in the reaction of these compounds with inhibited ChE of the rectus muscle.

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