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SOME ACTIONS OF TACRINE ON SLOW MUSCLES OF THE TOAD (BUFO MARINUS) AND THE CHICK

BY

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Tacrine is an inhibitor of cholinesterase comparable in potency with physostigmine. Its clinical applications include antagonism of curare (Gershon & Shaw, 1958), potentation of suxamethonium-induced relaxation of skeletal muscle (McCaul & Robinson, 1962), and antagonism of the sedative action of morphine (Stone, Moon & Shaw, 1961). The last property is relatively novel and is not displayed by all inhibitors of cholinesterase (Shaw & Bentley, 1953, 1955). For this reason, we have analysed the actions of tacrine on smooth and slow-contracting muscle in greater detail to assess whether these actions can be accounted for solely by inhibition of cholinesterase. The results with smooth muscle have been presented separately (de la Lande & Porter, 1963); the present report is confined to the comparison of the actions of tacrine and of potent anticholinesterases on the isolated rectus abdominus muscle of the toad and on the semispinalis muscle of the chicken (Child & Zaimis, 1960).

METHODS

The rectus abdominus muscle preparation of the toad (Bufo Marinus)

Paired muscles were set up separately in 5-ml. organ-baths containing (g in 1 l. of distilled water): NaCl 6.7, KCl 0.24, $CaCl_2$ 0.14, $MgCl_2$ 0.20, $NaHCO_3$ 1.68, NaH_2PO_4 0.49 and glucose 3.0. Drugs to be added to the preparation were made up in the same solution. The solutions were gassed with oxygen at room temperature (16 to 25° C).

The responses of the muscle to a drug were examined by replacing the above solution with the solution containing the drug and recording the contraction on a smoked drum by means of an isotonic lever (magnification $\times 7$, tension 1.5 g). The height of the contraction after a fixed interval of time (1.5 to 2 min) was recorded, after which the organ-bath was drained; the tension of the preparation was restored to the resting value by means of a weight or an electromagnet. After recording responses for different concentrations of the stimulant being investigated, the anticholinesterase was added to the reservoir of the solution bathing one of the preparations, and further measurements were made of the responses of both preparations to the stimulant. The only departure from this procedure was with tetraethyl pyrophosphate when the method of Hobbiger (1950) was used; the drug was added for an initial period of 45 min in a concentration of 1.5×10^{-6} M to the bathing solution and replenished at intervals of 15 min.

The change in sensitivity of the muscle to acetylcholine was assessed as follows: maximum responses (R) to acetylcholine were obtained, and the concentration (C_1) of acetylcholine which produced a response of one-half this magnitude (R/2) in 90 sec was measured or estimated from a dose/response curve. The muscle was then treated with an anticholinesterase and the concentration of acetylcholine (C_2) required to produce the former response (R/2) again estimated. When comparisons were made between acetylcholine and other stimulants, the concentrations of the latter were adjusted to provide contractions of approximately equal magnitude to that by acetylcholine. The term " sensitivity factor " is employed here to express the ratio C_1/C_2. Departures from the above procedure are referred to in the text.

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Isolated chicken semispinalis cervicis muscle

The semispinalis cervicis muscle of the chicken, aged between 3 and 4 days, was suspended in Tyrode solution at 40° C and gassed with a mixture of 95% oxygen and 5% CO_2 (Child & Zaimis, 1960). Acetylcholine or other stimulants were diluted in Tyrode solution and applied to the muscle for 100 sec using an automatic assay apparatus. The responses were recorded on a smoked drum by means of an isotonic lever (magnification $\times 5$, tension approximately 0.5 g). The preparation was washed twice after testing a stimulant, and 6 min were allowed for recovery to resting length before further application of a stimulant.

Toxicity in chickens

Tacrine in doses of 1.5 and 7.5 mg/kg was given intravenously to chickens, aged between 3 and 4 weeks. The presence or absence of neck retraction was used as an index of the depolarizing property of the drug (Child & Zaimis, 1960).

Drugs

These were tacrine, physostigmine salicylate or hydrochloride, neostigmine methyl sulphate, tetraethyl pyrophosphate, acetylcholine chloride and atropine sulphate. Solutions of the drugs were prepared immediately before each experiment with the exception of tetraethyl pyrophosphate, which was diluted with redistilled propylene glycol to provide a stock solution containing a concentration of 7 mM. This solution was kept at 4° C for 3 weeks and diluted as required.

RESULTS

Rectus abdominus muscle preparation of the toad

The response of the muscle to acetylcholine in low concentrations comprised a slow sustained contraction which reached a maximum within 3 to 15 min according to the concentration used. The sensitivity of the muscle to acetylcholine, as measured by the extent of the contraction obtained over a 90-sec period, remained constant for many hours. In the presence of tacrine, the sensitivity to acetylcholine was greatly increased, the effect of the drug becoming maximal in concentrations of 3.7×10^{-5} M (Fig. 1) above which no

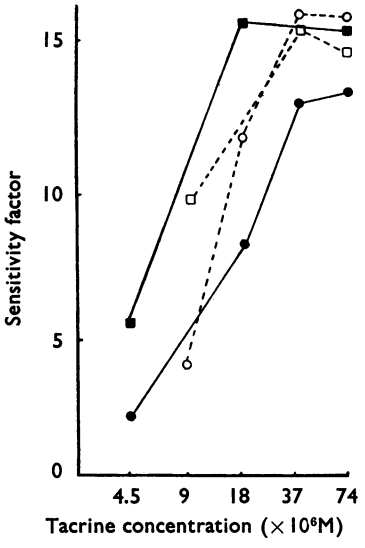

Fig. 1. The increase in sensitivity to acetylcholine of the rectus abdominus muscle of the toad with increasing concentrations of tacrine in four typical experiments.

further increase in sensitivity occurred. Relatively high concentrations of tacrine (above 3×10^{-3} M) caused a contraction resembling that with acetylcholine.

In the presence of tacrine (3.7×10^{-5} M), sensitivity to acetylcholine was greatly increased, the average increase in thirty-three experiments being 15.4-fold. Comparisons between the sensitivity factor obtained with tacrine and those obtained with other anticholinesterases are summarized in Table 1. In each instance one muscle of each pair was treated with

TABLE 1

COMPARATIVE SENSITIVITY FACTORS

The sensitivity factor (S.F.) for tacrine (4×10^{-5} M) was taken as unity. Values are means and standard deviations. * See Methods

Drug	Concentration ($\times 10^{-6}$ M)	No. of experiments	S.F. drug / S.F. tacrine
Tetraethyl pyrophosphate	1·5*	5	1·24±0·3
Neostigmine	3	4	0·95±0·14
Physostigmine	60	6	0·27±0·06

tacrine, and the other with the anticholinesterase being compared (neostigmine, physostigmine or tetraethyl pyrophosphate). The concentrations of drug were those which caused a maximal potentiation of responses to acetylcholine as judged by either the lack of further effect on increasing the concentration of the drug (in the case of tacrine and physostigmine) or the appearance of a contracture (as with neostigmine) which prevented further estimates of changes in sensitivity to acetylcholine.

The results show that tacrine has an effect similar to that of neostigmine or tetraethyl pyrophosphate, but is much more effective than physostigmine in increasing the sensitivity of the muscle to acetylcholine.

A series of comparisons was made between muscles treated with anticholinesterase alone, and anticholinesterase plus tacrine. The concentrations of each of the drugs were those above which no further increase in sensitivity to acetylcholine occurred. The results (Table 2) show that tacrine does not increase the sensitivity to acetylcholine after potentia-

TABLE 2

RATIOS OF MAXIMUM SENSITIVITY FACTORS

Values are means and standard deviations. TEPP=Tetraethyl pyrophosphate. * See Methods

Drug comparison	Concentrations ($\times 10^{-6}$ M)	No. of experiments	Ratio
TEPP + tacrine / TEPP	*1·5 + 40 / 1·5	4	0·96±0·07
Neostigmine + tacrine / Neostigmine	3+40 / 3	3	1·0 ±0
Physostigmine + tacrine / Physostigmine	25+40 / 25	6	2·65±0·4
Physostigmine + tacrine / Physostigmine	100+40 / 100	2	1·0 ±0

tion by tetraethyl pyrophosphate or neostigmine, but does so after potentiation by physostigmine.

However, four- to eightfold increases in concentration of physostigmine, while not producing further increases in sensitivity to acetylcholine, abolished the potentiating effect of tacrine (Table 2). This finding suggests that the inability of physostigmine to produce an increase in sensitivity of the order achieved by the remaining anticholinesterases is due to an anticholinergic action on the part of the drug. This is supported by the fact that the potentiation produced by tacrine alone (sensitivity factor=15.4) was greater than that produced by combinations of physostigmine and tacrine (sensitivity factor=10.9) (compare Tables 1 and 2).

Responses to other stimulants. The sensitivity of the muscle to carbachol was virtually unaffected by tacrine, tetraethyl pyrophosphate or neostigmine, but was depressed by physostigmine. Tacrine also had little effect on contractions caused by decamethonium (Figs. 2 and 3).

Rates of action. The time required to produce half the maximum increase in sensitivity to acetylcholine was used as an indication of the relative rates of action of the anticholinesterases. The relative times (means and standard deviations) were: tacrine, 18.5±13.6 min; physostigmine, 53.5±19.9 min; and neostigmine, 53.4±13.4 min.

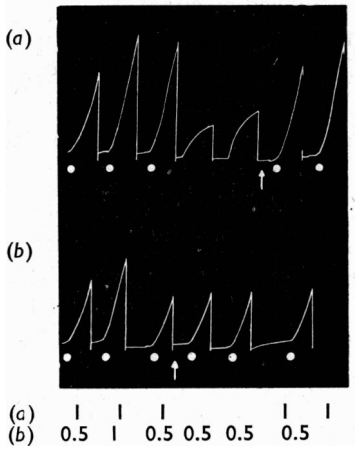

Fig. 2. The effect of tacrine on the responses of the rectus abdominus muscle to carbachol (*a*) and decamethonium (*b*) (at white dots, concentrations in μg/ml.). The response to stimulant was tested and recorded over a period of 2 min, once every 30 min. Tacrine (3.7×10^{-5} M) was added at the arrows and was present throughout the remainder of the experiment. Note: the two unmarked responses were produced by acetylcholine and are irrelevant to this study. (*a*) and (*b*) are from separate experiments.

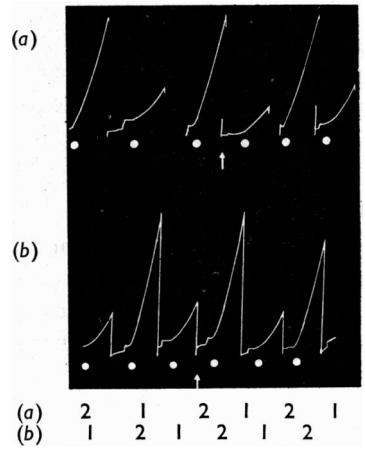

Fig. 3. The effect of tetraethyl pyrophosphate and neostigmine on the responses of the rectus abdominus muscle to carbachol (at the white dots, concentrations in μg/ml.). The response to carbachol was tested and recorded over a 2-min period once every 30 min. In (*a*) the preparation was treated with tetraethyl pyrophosphate as described in Methods. Approximately 90 min after the start of this treatment (at the arrow) the responses to stimulant were examined. In (*b*), neostigmine (6×10^{-6} M) was added at the arrow and was present throughout the remainder of the experiment. (*a*) and (*b*) are from separate experiments.

Isolated semispinalis muscle preparation of the chicken

Tacrine greatly increased the sensitivity of the isolated muscle to acetylcholine but did not produce a contracture even in relatively high concentrations (7×10^{-4} M). There was no increase but, instead, a slight decrease (approximately 15%) in the response to the depolarizing agent, decamethonium (Fig. 4). Neostigmine also increased the sensitivity of the muscle to acetylcholine in agreement with the findings of Tyler (1960) but, as with the rectus abdominus, there was relatively little margin between concentrations causing potentiation to acetylcholine and contraction of the muscle. Unlike tacrine, neostigmine (6×10^{-6} M) transiently potentiated the response of the muscle to decamethonium.

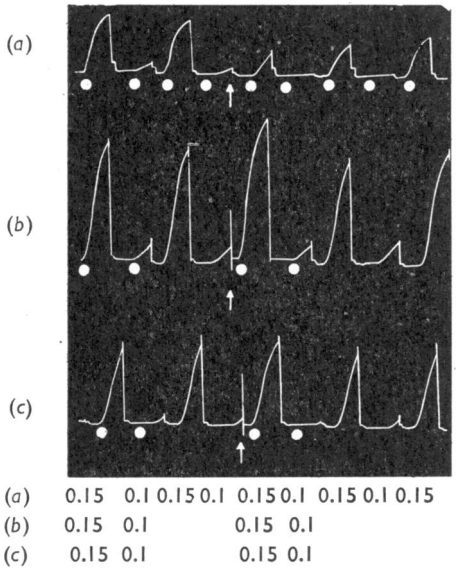

Fig. 4. The effect of tacrine and neostigmine on the response of the chick semispinalis cervicis muscle to decamethonium (at the white dots, concentrations in μg/ml.). The response to decamethonium was tested and recorded over a 100-sec period once every 8 min. Anticholinesterases were added at the arrows in the appropriate concentrations and were present throughout the remainder of the experiment. (*a*), Tacrine (3.7×10^{-5} M); (*b*), neostigmine (6×10^{-6} M); (*c*), neostigmine (3×10^{-6} M). At 30 min after the addition of anticholinesterases the sensitivity of the preparation to decamethonium was re-examined. (*a*), (*b*) and (*c*) are from separate experiments.

Intact chicken

Tacrine (1.5 mg/kg) potentiated the action of suxamethonium ($P<0.001$) in the chicken and, in higher doses (7.5 mg/kg), produced generalized muscle fasciculations, profuse salivation and lachrymation; however, neck retraction similar to that produced by depolarizing drugs was not observed.

DISCUSSION

When assessed by sensitivity factors alone, tacrine has an activity comparable to that of neostigmine or tetraethyl pyrophosphate while it is about four times more active than physostigmine. There is considerable evidence (Quilliam & Strong, 1949; Kirschner & Stone, 1950; Hobbiger, 1950) that physostigmine possesses anticholinergic properties

which may modify the effects of cholinesterase inhibition; by this criterion it appears that tacrine possesses fewer anticholinergic properties than physostigmine, and more closely resembles neostigmine and tetraethyl pyrophosphate. The only evidence of anticholinergic properties of tacrine in this study is slight depression of responses of the chick semispinalis cervicis muscle to decamethonium; this effect was not observed on the rectus abdominus muscle. The ability of tacrine to act in the presence of physostigmine may be interpreted also in the light of the anticholinergic action that physostigmine possesses. If the latter were manifest in concentrations of physostigmine below those causing complete inhibition of the enzyme, further inhibition by tacrine would increase sensitivity to acetylcholine. The latter action is consistent with the observation that an eight-fold increase in the concentration of physostigmine does not increase, but instead reduces the sensitivity to acetylcholine, and simultaneously abolishes the potentiating effect of tacrine. However, the problem arises that if the potentiating effect of tacrine is attributed to its action on cholinesterases then it must be assumed that physostigmine does not produce complete inhibition of the enzymes in the rectus muscle in relatively high concentrations (2×10^{-5} M). This concentration of physostigmine is routinely employed in pharmacological experiments when complete inhibition of cholinesterase is required and when the degree of inhibition may be readily tested. This apparently anomalous action of physostigmine may be a reflection of a peculiarity of toad cholinesterase, as others have observed a lack of potentiation of acetylcholine responses by physostigmine in a number of toad tissues (Boyd, Chang & Rand, 1960; Burnstock, O'Shea & Wood, 1963); also in this connection it may be mentioned that Hawkins & Mendel (1946) have demonstrated a physostigmine-resistant cholinesterase in frog brain.

The effect on responses to carbachol and decamethonium, and the ability to cause a contraction in the absence of another drug have been used as an indication of stimulant properties on muscle other than those arising from inhibition of cholinesterase. By these criteria, tacrine possesses little direct effects on muscle compared with neostigmine or tetraethyl pyrophosphate. The margin between concentrations of tacrine causing contraction and those producing maximum sensitization to acetylcholine is 100-fold. In the case of neostigmine it was impossible to measure the maximum sensitivity factor before contraction of the muscle intervened.

It appears therefore that the action of tacrine on the slow-contracting types of skeletal muscle used in this study is relatively uncomplicated compared with other representative anticholinesterases, and is mediated solely by inhibition of cholinesterase. This conclusion does not itself offer an explanation of the antagonism of morphine by tacrine on the central nervous system, but it accords well with a recent analysis of the interaction of tacrine and morphine on the guinea-pig isolated ileum (de la Lande & Porter, 1963) in which it was concluded that an antagonism of the inhibitory effects of morphine on cholinergic transmission by tacrine simply reflected the potent anticholinesterase action of the latter drug. In this study (de la Lande & Porter, 1963) attention was drawn to the rapid onset of action of tacrine on the isolated tissue and on purified acetylcholinesterase; the same property of tacrine is evident with respect to the rate at which maximum sensitization to acetylcholine is achieved on the rectus abdominus muscle.

An interesting implication of these findings relates to the clinical use of tacrine for prolonging the action of suxamethonium. In view of the similar mechanism of action of

decamethonium and suxamethonium, lack of potentiation of decamethonium on the isolated muscle represents evidence that potentiation of suxamethonium is indirect and arises from inhibition of suxamethonium hydrolysis by plasma cholinesterase. This explanation has been further verified in another experiment in which it was observed that the neck retraction induced in the chicken by decamethonium was unaffected by tacrine whilst that by suxamethonium was potentiated (Porter, unpublished).

Another interesting application of this study is the use of tacrine in the assay of acetylcholine. The speed of onset of action of the drug coupled with the associated lack of contraction of muscle such as occurs with neostigmine renders tacrine an extremely useful anticholinesterase for sensitizing the rectus abdominus muscle to acetylcholine for assay purposes. It has the advantage of producing much greater sensitization than physostigmine, and is free from hazards possessed by the organophosphonates. For these reasons, the isolated rectus abdominus muscle preparation of *Bufo marinus*, treated with tacrine (3.7×10^{-5} M), has been used routinely for the assay of acetylcholine in this laboratory. The method permits the assay of acetylcholine in concentrations at or above 5×10^{-9} M.

SUMMARY

1. The effects of tacrine, neostigmine, tetraethyl pyrophosphate and physostigmine on the response of the toad rectus abdominus muscle to acetylcholine, carbachol and decamethonium were investigated. Tacrine potentiated the response of the muscle to acetylcholine to the same extent as neostigmine, slightly less than tetraethyl pyrophosphate and approximately five-times more than physostigmine. The response to carbachol and decamethonium were unaffected by tacrine.

2. Tacrine potentiated the response of the rectus to acetylcholine in the presence of physostigmine (2.5×10^{-5} M) but had no effect in the presence of higher concentrations (10^{-4} M), or after treatment with tetraethyl pyrophosphate.

3. The responses of the semispinalis cervicis muscle of the chick resembled those of the rectus except that tacrine slightly depressed the response to decamethonium.

4. The results indicate that the action of tacrine in sensitizing slow contracting muscle to acetylcholine is solely by inhibition of cholinesterase.

5. Attention is drawn to the use of the tacrine-treated muscle for the assay of acetylcholine.

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