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The effects of P-2-AM on the release of acetylcholine from the isolated diaphragm of the rat

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Release of acetylcholine from rat isolated diaphragm preparations stimulated through the phrenic nerve was investigated in the presence of P–2–AM 0·01–100 mM. At 1 mM, the release of acetylcholine was increased by 157%, while higher concentrations resulted in a decrease. The findings indicate that the biphasic action of P–2–AM can be explained, at least in part, by variations in the quantal release of acetylcholine—an effect involving a presynaptic action.

Wilson & Ginsburg (1955) and Child, Davies & others (1955) reported that pyridine-2 aldoxime methochloride or methiodide (P–2–AM or PAM) can reactivate phosphorylated cholinesterases. Fleisher, Corrigan & Howard (1958), Holmes & Robins (1955) and Koelle (1957) demonstrated that such reactivation is observed at the motor end plates of skeletal muscles. Many authors have shown that, in addition to reactivation, PAM has a facilitating, and also a depressing or blocking action at the neuromuscular junction. The facilitation has been reported to result from a depolarizing effect (Wills, O'Leary & Oikemus, 1959), from an anticholinesterase activity (Holmes & Robins, 1955; Wagley, 1957; Fleisher & others, 1958; Fleisher, Moen & Ellingson, 1965; Goyer, 1968; Fleisher & Ellingson, unpublished results) or from an increase in the quantal release of acetylcholine (Edwards & Ikeda, 1962). The depressing effect on muscular contraction has been assumed to result from a direct toxic action on the muscle fibres (Holmes & Robins, 1955) or from a (+)-tubocurarine-like activity (Grob & Johns, 1958; Fleisher & others, 1958, 1965; Goyer, 1968; Fleisher & Ellingson, unpublished results). Recently I have found (unpublished results) that the antagonism of PAM to acetylcholine is non-competitive.

I have now examined whether the two effects of PAM at the neuromuscular junction could also be related to a presynaptic site of action, if so, the release of the chemical transmitter (acetylcholine) would then be modified. During these experiments, the rat phrenic nerve-diaphragm muscle preparation was used, as many workers (Burgen, Dickens & Zatman, 1949; Brooks, 1954; Straughan, 1960; Krnjević & Mitchell, 1961; Mitchell & Silver, 1963) have demonstrated that acetylcholine is released by this preparation when the motor nerve is stimulated.

EXPERIMENTAL

Innervated diaphragm and collection of acetylcholine

Phrenic nerve-diaphragm muscle preparations, from male Wistar rats, 200–250 g, were set up as described by Bulbring (1946), in a 7 ml bath containing Krebs solution of the following composition (g/litre): NaCl 6·92, KCl 0·35, $CaCl_2$ 0·28, $NaHCO_3$ 2·1, KH_2PO_4 0·16, $MgSO_4.7H_2O$ 0·30, glucose 2·0. The preparation was gassed

with 5% carbon dioxide in oxygen and the temperature was kept constant at 37 ± 0·1°. The phrenic nerve was stimulated by means of a Grass stimulator (S–8) delivering supramaximal rectangular pulses of 0·2 ms duration at a frequency of 0·1 or 25 Hz; the isotonic contractions were recorded by means of an E & M Physiograph. To prevent hydrolysis of acetylcholine released during nerve stimulation, the cholinesterases were inhibited by adding 5 μM neostigmine methylsulphate to the Krebs solution. Under the experimental conditions, it has been verified that such a concentration of neostigmine totally inhibits cholinesterases. Before each experiment, the preparation was allowed to rest for 30 min. The procedure was that of Straughan (1960), as modified by Cheymol, Bourillet & Ogura (1962).

(1) *Adaptation period*: the phrenic nerve was stimulated at a low frequency (0·1 Hz) for 10 min; the preparation was then allowed to rest for 20 min, after which time it was washed twice.

(2) *Stimulation period I*: the phrenic nerve was stimulated at 0·1 Hz for 3 min and at 25 Hz for an additional 20 min. The bath solution was then collected for the biological assay of acetylcholine.

(3) *Rest period*: the preparation was washed twice and allowed to rest for 10 min, after which the bath solution was discarded and the preparation was washed again.

(4) *Stimulation period II*: same as (2). (5) *Rest period*: same as (3).

(6) *Action of PAM*: without prior stimulation of the phrenic nerve, PAM was added to the bath in an amount to yield the required final concentration, and left in contact with the preparation for 20 min. The bath solution was then discarded and the preparation was washed once.

(7) *Stimulation period III*: same as (2), immediately after (6). (8) *Rest period*: same as (3). (9) *Stimulation period IV (recovery period)*: same as (2).

Estimation of acetylcholine

After each sample collection, the bath solution was refrigerated at 0°. Acetylcholine was estimated by the rat blood pressure preparation (male Wistar rats, 200–250 g), as described by Straughan (1958, 1960). The activity of 0·1 ml of the bath solution was evaluated against 0·1 ml of known concentrations of acetylcholine in Krebs solution containing neostigmine. The biological assay for acetylcholine was made on the same day as, and immediately after, collection of the test substance. The values for acetylcholine were expressed as ng of ions.

PAM was used at 0·01, 0·1, 1·0, 10 and 100 mM. A total of 31 experiments were made, with a minimum of three assays for each concentration of PAM.

RESULTS

Under my experimental conditions, the amount of acetylcholine (mean ± s.e.) released during stimulation period II and expressed as ng of ions is 52·5 ± 2·7. This compares with those of Straughan (1960) and Cheymol & others (1962). At concentrations of 0·01 and 0·1 mM (Fig. 1 and Table 1), PAM decreases the release of acetylcholine by 10·0 and 13·9% respectively; but these figures are not significant (P, 0·05). At 1·0 mM, the amount of acetylcholine released during nerve stimulation is greatly enhanced, the mean values for stimulation periods II and III being 48·0 and 123·2 ng, respectively, an increase of 157%. With higher concentrations

of PAM, the amount of acetylcholine released during nerve stimulation is decreased. At 10 mM, a 54·5% decrease was observed; with 100 mM a complete block occurred

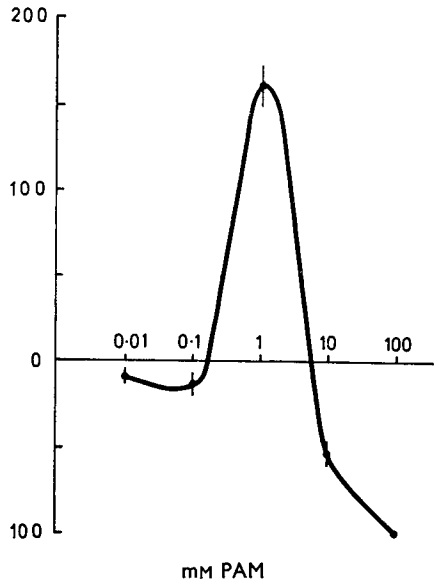

FIG. 1. Influence of various concentrations of PAM on the release of acetylcholine from rat isolated diaphragm preparations stimulated through the phrenic nerve. The amount of acetylcholine released is expressed as percentage of decrease or increase with respect to control experiments.

Table 1. *Release of acetylcholine (ng of ions) from rat phrenic nerve-diaphragm muscle preparations in presence of various concentrations of PAM*

Concentration of PAM (mM)	Stimulation period II $\bar{X}$	s.e.	Stimulation period III $\bar{X}$	s.e.	Stimulation period IV $\bar{X}$	s.e.	Value of *P* for II and III
0·01	50·400	3·960	45·920	5·429	45·280	4·613	>0·05
0·10	53·500	4·504	46·600	5·744	50·532	5·520	>0·05
1·00	48·000	8·092	123·200	21·200	50·000	7·776	<0·01
10·00	56·532	5·748	27·235	4·884	53·760	2·240	<0·01
100·00	48·532	3·732	0	0	50·400	5·600	<0·01

If the results obtained during stimulation period II and IV are compared (Table 1), there is no significant difference in the release of acetylcholine, regardless of the concentration of PAM used and the nature of the effect observed (increase or decrease in acetylcholine release).

DISCUSSION

As mentioned previously, many authors have demonstrated that PAM exerts a biphasic action at the neuromuscular junction. Most of these authors have explained the facilitating effect by an anticholinesterase activity, while they have interpreted the depressing effect as being caused by a (+)-tubocurarine-like activity. It appears that the anticholinesterase and tubocurarine-like activities do not serve as an adequate or the sole explanation for the facilitating or the depressing effect. Indeed, as shown by Goyer (1968), PAM (6 mM) did not influence muscular contractions, while it considerably inhibited the cholinesterases (60%). In the same series of experiments, PAM was at least 16 times less active as a cholinesterase inhibitor

in vitro than tubocurarine. Moreover, the tubocurarine-like activity appears when the cholinesterases are almost completely inhibited. It is probable that an additional mechanism or site of action is responsible for this biphasic activity.

According to Edwards & Ikeda (1962), PAM has a presynaptic site of action. In fact, these authors demonstrated on the frog nerve-muscle preparation that PAM (0·2–4·0 mM) increases the quantal release of acetylcholine. No decrease in its release was observed, as no concentration higher than 4 mM was used. At similar concentrations, our results confirm those of Edwards & Ikeda (1962), in that PAM (1·0 mM) increased the release of acetylcholine by 157% on the rat phrenic nerve-diaphragm muscle preparation and by 180% on the frog nerve-muscle preparation. These effects are only transient, however, as recovery is rapid and complete.

The concentrations of PAM that produce a neuromuscular block on the phrenic nerve-diaphragm muscle preparation of the rat (Goyer, 1968) and those that decrease the release of acetylcholine on the same preparation, are similar: with 10 mM, the amplitude of contraction is reduced by 20%, while the release of acetylcholine is decreased by 54·5%. At 25 mM, the amplitude of contraction is reduced by 100%, if the preparation is stimulated through its phrenic nerve, whereas the amplitude of contraction of the directly stimulated preparation is not modified.

The biphasic action of PAM can thus be explained, at least in part, by a presynaptic effect. The postsynaptic effects, namely, an anticholinesterase activity and a tubocurarine-like effect, are not the only nor perhaps even the major factors involved in the neuromuscular action of PAM. The prejunctional effects appear to play an important role.

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