J. Pharm. Pharmacol., 1964, **16**, 557–560. Received March 9, 1964

The effect of some ganglionic stimulants and blocking drugs on acetylcholine release from the mammalian neuromuscular junction

L. BEANI, CLEMENTINA BIANCHI, G. BIEBER AND F. LEDDA

The effect of nicotine, dimethylphenylpiperazinium (DMPP), hexamethonium and pempidine on release of acetylcholine from the guinea-pig phrenic nerve diaphragm preparation has been investigated. Neither nicotine nor DMPP, 2×10^{-5}, modified acetylcholine release from the hemidiaphragms at rest or indirectly stimulated at 50/sec: therefore their neuromuscular blocking action has only a postjunctional origin. Hexamethonium, 4×10^{-4}, significantly reduced the output of transmitter from preparations stimulated at 50/sec at 38°. It did not affect the release of acetylcholine from hemidiaphragms at rest or the acetylcholine content of the muscle. The presynaptic effect of hexamethonium is probably related to its linkage with "receptors" present on the surface of the nerve endings. Pempidine, 1×10^{-4}, diminished the release of acetylcholine from the preparations at rest or stimulated either at 50 or at 6/sec. The effect was related to the frequency and to the temperature. Moreover, the drug reduced the acetylcholine content of the muscle. This effect may be the result of non-specific metabolic inhibition or of an impairment of choline transport system.

ACCORDING to Paton & Zaimis (1949, 1951) and Hesleff & Unna (1954), hexamethonium interrupts ganglionic and neuromuscular transmission solely by acting on the postjunctional membrane as a competitive blocking agent. However, this statement is not fully accepted by Riker & Szreniawsky (1959) who suggest that the nerve endings are an additional site of action of the drug. It has also been suggested that the tertiary amine, pempidine, blocks ganglionic transmission by acting at both pre- and post-synaptic sites (Corne & Edge, 1958). Dimethylphenylpiperazinium (DMPP), like nicotine, possesses both stimulant and blocking actions on autonomic ganglion cells and at the neuromuscular junction (Ling, 1959) and there is evidence that these two drugs also may exert an action on nerve endings, either facilitating (Lee & Shideman, 1959) or inhibiting (Wilson, 1962) transmitter release.

In the experiments now described we have studied the pre-junctional action of hexamethonium, pempidine, nicotine and DMPP directly, by measuring their effect on acetylcholine release from motor nerve endings and on tissue stores of acetylcholine. These experiments have been briefly reported elsewhere (Beani, Bianchi, Bieber & Ledda, 1962).

Experimental

The experiments were made on phrenic nerve-hemidiaphragm preparations from guinea-pigs weighing 250–350 g. Each preparation was suspended in 3 ml of oxygenated Tyrode solution. Both hemidiaphragms from each animal were prepared; one was treated with the chosen drug and the other served as a control.

The methods for (i) estimation of transmitter release, and (ii) detection of acetylcholine stores, have been previously described (Beani & Bianchi, 1961; Beani, Bianchi & Ledda, 1962) and may be summarised as follows:

From the Department of Pharmacology, University of Florence, Florence, Italy.

(i) The preparations, pre-incubated with 500 μg/ml dyflos (DFP) for 150 min, were indirectly stimulated by rectangular pulses (0·1 msec duration, at a voltage twice the threshold) for five 10 min periods of high frequency stimulation, interspersed with usually 20 min periods of rest. Immediately before every period of high frequency stimulation, the height of contraction at 15/min was briefly tested. The contractions of both preparations were recorded on smoked paper with an isotonic lever, amplification 1:7, load 2 g. The acetylcholine released during stimulation or rest was estimated on the guinea-pig ileum without any special sensitising procedure. The effect of drug on the transmitter output was examined at 33° and 38° and at stimulation rates of 6 and 50/sec.

The drug was added to one preparation of each pair after the end of the second 20 min period of rest, left in contact for 30 min before starting the third period, and maintained during the third and fourth period of stimulation and rest. After repeated washing, the fifth period of stimulation was carried out. Every experimental point was determined on six pairs of preparations.

(ii) The tissue stores of acetylcholine were estimated at the end of the fourth stimulation period (50/sec, 33°) by the method of Bentley & Shaw (1952) as modified by Beani, Bianchi & Ledda (1962). The estimations were made on the hemidiaphragms treated with drugs found to modify the release of acetylcholine and in the contralateral preparations of the same animals, which acted as controls. Every experimental point was determined on ten pairs of hemidiaphragms. Amounts of acetylcholine are given as chloride; the final concentration of the drugs is (w/v) as base.

TABLE 1. EFFECT OF GANGLIONIC STIMULANT AND BLOCKING DRUGS ON NEUROMUSCULAR TRANSMISSION AND ACETYLCHOLINE RELEASE AND TISSUE STORES

Drug	Indirect contraction at 15/min before the 3rd and 4th period, as % of the control		Stimulation rate/sec	Acetylcholine release in 3rd and 4th period of stimulation from treated preparations as % of expected values*		Tissue ACh/hemidiaphragms (ng±s.d.) after the 4th period of stimulation, 50/sec, 33°	
	33°	38°		33°	38°	Controls	Treated
Nicotine 2×10^{-5}	0	0	50	98·2±19·6	102·1±21·2	not estimated	not estimated
DMPP 2×10^{-5}	0	0	50	104·1±26·5	105·0±32·9	not estimated	not estimated
Hexamethonium 1×10^{-4}	42	37	6	108·4±17·6	83·1±16·9	117·4±15	122·8±33·7
			50	89·4±13·9	57·9±14·6		
Pempidine 1×10^{-4}	26	14	6	70·0±24·7	55·8± 6·3	113·5±22	83·1±29·9●
			50	50·7±18·9	44·7±17·1		

● = Statistically different ($0{\cdot}02 > P > 0{\cdot}01$) from the control group.
* = Results are means of 6 experiments.

Results

The evaluation of the drug effect on the transmitter release was made by comparing the absolute values of acetylcholine released in the third and fourth stimulation and rest periods, from treated and untreated

preparations. The drug effect was also assessed by expressing the mean of the acetylcholine released during the third and fourth periods from every treated preparation, as a percentage of the "expected" value. The "expected" value was calculated from the average releases from both test and control preparations in the first and second period, and from the average release from the controls in the third and fourth periods.

The reliability of these evaluations is based upon the observation that the absolute release from the untreated right and left hemidiaphragms of the same animal is nearly equal in any given period.

Nicotine and DMPP, 2×10^{-5}, did not modify the release of acetylcholine either at 33 or at 38°, from preparations stimulated at 50/sec (Table 1) or at rest (values not given).

Lower stimulation frequencies were not used because we have found that, in general, the effect of a drug able to inhibit the release of acetylcholine, is directly related to the frequency of stimulation (Beani & Bianchi, 1961; Beani, Bianchi & Ledda, 1963).

Hexamethonium, 4×10^{-4}, significantly ($P < 0.001$) reduced the output of acetylcholine only in the fourth period from the hemidiaphragms at 38° and stimulated at 50/sec (Table 1). Acetylcholine output was still depressed during the fifth period of stimulation, although the drug had been washed out and the contractions elicited by stimulation at 15 min had fully recovered. At 33° and 50/sec, inhibition of acetylcholine output was evident ($P < 0.01$) only after washing, although a slight reduction was present in the fourth period. When the lower stimulation rate is employed (6/sec) the effect of hexamethonium was not significant either at 33 or at 38°. The resting release was never reduced by the drug. The reduction of acetylcholine release was brought about by hexamethonium at a concentration insufficient to block neuromuscular transmission completely and the drug had no effect on acetylcholine stored in the tissue, at the end of the fourth stimulation period at 50/sec, 33° (Table 1).

Pempidine, 1×10^{-4}, strongly reduced the output of acetylcholine from the stimulated preparations. The effect was greater when higher stimulation rate and temperature were employed (Table 1). After washing out the drug, the difference between the acetylcholine released from the control and the treated groups remained significant in the fifth period at 50/sec, either at 33 or at 38°. Pempidine also decreased the acetylcholine released from the preparations at rest (values not given), the effect being most pronounced at 38°: in the group stimulated at 6/sec, the amount released in 10 min of rest was 5.8 ± 1.8 ng in the controls, and 3.3 ± 0.7 ng in the treated group ($P < 0.01$).

A noteworthy difference between hexamethonium and pempidine was that the latter, even in a concentration insufficient to block neuromuscular transmission completely, significantly reduced the tissue stores of acetylcholine (Table 1).

Discussion

The drugs were added to the preparations 30 min before starting the third period of stimulation, to allow their uniform distribution in the

tissue. Consequently, no information was obtained about their initial effect on acetylcholine release: the short acetylcholine-like activity of nicotine and DMPP on the guinea-pig diaphragm, for instance, may result not only from stimulation of the motor end-plate, but also from stimulation of the motor nerve endings. This last effect, if present, was not detected in our experimental procedure. However, it is clear that neither nicotine nor DMPP reduces acetylcholine output during the long-lasting neuromuscular block, the mechanism of which is "competitive" (Beani, Bianchi & Conti, 1960) and limited to the post-junctional membrane.

The inhibition of acetylcholine release by hexamethonium was only evident at 38° and at the higher stimulation rate. Hexamethonium was without effect on acetylcholine release at rest, or on tissue stores.

This effect may suggest that the drug slowly combines with (and dissociates from) "receptors" present on the surface of the axon. The consequence may be a reduced ability of the motor nerve endings to follow the high frequency impulses. Therefore the mechanism of the presynaptic effect of hexamethonium may be similar to that proposed for tubocurarine (Beani & Bianchi, 1961) although the inhibition brought about by the former increases at higher instead of at the lower temperatures. The effect of pempidine has a different pattern. It reduced the output of acetylcholine even at 33° and at the lower stimulation rate. Moreover, it diminished the release from the unstimulated preparations and the acetylcholine stores. Mitchell & Silver (1963) have shown that a great part of the transmitter released from the muscle at rest does not have a nervous origin. Pempidine does not inhibit choline-acetyltransferase (Parkinson, 1959) and its effect on the acetylcholine release and storage may therefore be a consequence of a non-specific metabolic effect or of an impairment of choline transport mechanism (Birks & McIntosh, 1961). Pempidine appears to reduce the safety factor of neuromuscular transmission, chiefly through its presynaptic effect; this may be the reason for the observed sensitisation towards curarising agents (Beani & others, 1960).

References

Beani, L., Bianchi, C. & Conti, G. (1960). *Boll. Soc. Ital. Biol. Sper.*, **36**, 1664–1668.
Beani, L. & Bianchi, C. (1961). *Ibid.*, **37**, 1150–1154.
Beani, L., Bianchi, C. & Ledda, F. (1962). *Ibid.*, **38**, 320–323.
Beani, L., Bianchi, C., Bieber, G. & Ledda, F. (1962). *Ibid.*, **38**, 1443–1446.
Beani, L., Bianchi, C. & Ledda, F. (1963). Second International Pharmacological Meeting, *Biochem. Pharmacol.* Suppl., vol. **12**, 166.
Bentley, G. A. & Shaw, F. H. (1952). *J. Pharmacol.*, **106**, 193–199.
Birks, R. & MCIntosh, F. C. (1961). *Canad. J. Biochem. Physiol.*, **39**, 787–827.
Corne, S. J. & Edge, N. D. (1958). *Brit. J. Pharmacol.*, **13**, 339–349.
Lee, W. C. & Shideman, F. E. (1959). *J. Pharmacol.*, **126**, 239–249.
Ling, H. W. (1959). *Brit. J. Pharmacol.*, **14**, 505–511.
Mitchell, J. F. & Silver, A. (1963). *J. Physiol.*, **165**, 117–129.
Paton, W. D. M. & Zaimis, E. J. (1949). *Brit. J. Pharmacol.*, **4**, 381–400.
Paton, W. D. M. & Zaimis, E. J. (1951). *Ibid.*, **6**, 155–168.
Parkinson, J. (1959). *Nature, Lond.*, **184**, suppl. 8, 554–555.
Riker, W. K. & Szreniawsky, Z. (1959). *J. Pharmacol.*, **126**, 233–238.
Thesleff, S. & Unna, K. R. (1954). *Ibid.*, **111**, 99–113.
Wilson, A. B. (1962). *J. Pharm. Pharmacol.*, **14**, 700.