Br. J. Pharmac. Chemother. (1967), **31**, 74–81.

THE EFFECT OF CALCIUM IN REMOVING THE BLOCKING ACTION OF BRETYLIUM AND GUANETHIDINE

BY

J. H. BURN AND F. WELSH

From the Department of Pharmacology, Washington University Medical School, St. Louis, Missouri, U.S.A.

(*Received March* 2, 1967)

Acetylcholine releases catecholamines from the chromaffin cell of the adrenal medulla by making the membrane of the cell permeable to calcium ; calcium then enters the cell and releases catecholamines (Douglas & Rubin, 1963). Acetylcholine releases noradrenaline from the sympathetic post-ganglionic fibre in the same way ; the fibre is made permeable to calcium ; calcium then enters and releases noradrenaline (Burn & Gibbons, 1965).

Bretylium and guanethidine block the release of noradrenaline from the sympathetic fibre when this is effected by acetylcholine, but not when it is effected by tyramine (Burn and Gibbons, 1965). Since bretylium and guanethidine are antagonists of acetylcholine at the neuromuscular junction (Dixit, Gulati & Gokhale, 1961 ; Burn & Seltzer, 1965), it may be that their blocking action is explained by preventing calcium from entering the fibre. In their presence acetylcholine may fail to make the fibre permeable to calcium. Earlier experiments (Burn & Seltzer, 1965) with other substances suggested this, and observations have now been made with bretylium and guanethidine.

METHODS

Isolated loops of ileum were taken from freshly killed rabbits together with the adjacent mesentery, and the main artery supplying the loop was drawn into a pair of circular platinum electrodes of the pattern described by Burn & Rand (1960a). The periarterial nerves were stimulated. When the loop was suspended in a bath at 32° C pendular movements were recorded and these were inhibited by stimulation. Maximal shocks were used of 1 msec duration. The loop was suspended in Locke solution which was gassed with oxygen containing 5% CO_2.

The hearts were removed from freshly killed rabbits and the atria were dissected. They were set up in Locke solution at 29° C which was gassed with oxygen and 5% CO_2. The rate of the spontaneous beats was counted with a stop watch. Acetylcholine bromide, nicotine acid tartrate, bretylium tosylate, guanethidine sulphate and procaine hydrochloride were used, and concentrations are expressed in terms of these salts, except for nicotine where the concentration is that of the base.

RESULTS

Rabbit ileum

The effect of bretylium on the rabbit ileum is shown in Fig. 1. Two control inhibitions were recorded in response to stimulation at 20/sec, the calcium concentration being 2.2

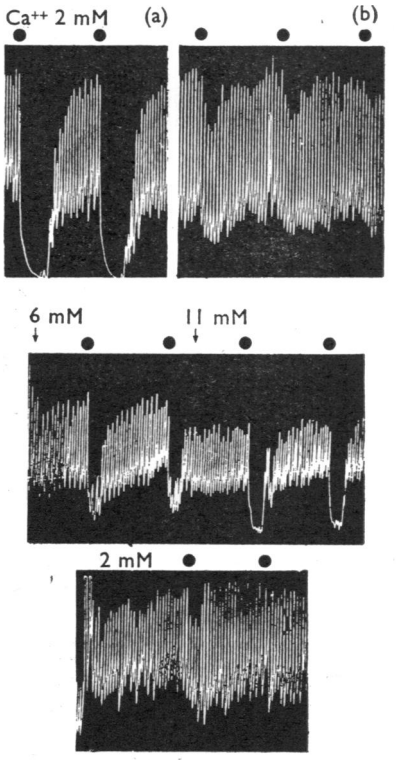

Fig. 1. Rabbit ileum, Finkleman preparation. Stimulation at black dots. In the upper panel (a) are two control inhibitions following stimulation at 20/sec for 30 sec. In (b) bretylium 10^{-5} was added, and the response to stimulation became very small. In the middle panel the calcium concentration was raised first to 6.6 mM and then to 11 mM. The response to stimulation returned. In the lower panel, the calcium concentration was reduced again to 2.2 mM, and stimulation was almost ineffective.

mM, and then bretylium (10^{-5} g/ml.) was added. After 15 min, three stimulations were applied and it was seen that the inhibition was almost abolished. The calcium concentration was raised to 6.6 mM. Stimulation then produced inhibition again. After raising the calcium concentration to 11 mM, the effect of stimulation was further restored. When the calcium was reduced to 2.2 mM once more and the bretylium concentration was maintained, stimulation had almost no inhibitory action.

The effect of guanethidine

A similar result with guanethidine is shown in Fig. 2. Stimulation was applied at frequencies of 3/sec and 6/sec. Both caused practically complete inhibition when the calcium concentration was 2.2 mM as shown in the top panel. Guanethidine (10^{-6} g/ml.) was then added to the bath, and 15 min later stimulation at 3/sec and at 6/sec had very little effect. When the calcium concentration was raised to 11 mM, guanethidine having then been present for 1 hr, stimulation at both frequencies was almost as effective as at

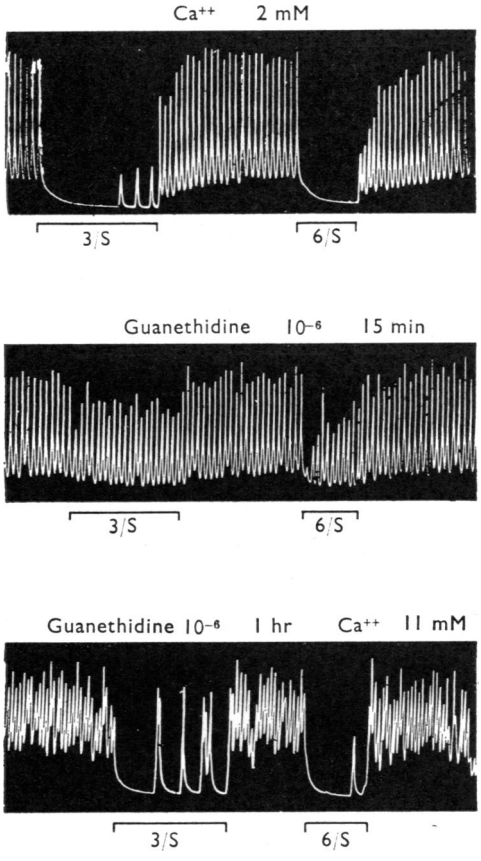

Fig. 2. Finkleman preparation. The upper panel shows the response to stimulation at 3/sec and 6/sec in the presence of calcium 2.2 mM. In the middle panel guanethidine 10^{-6} was added and the responses 15 min later are recorded. In the lower panel are the responses when guanethidine had been present for 1 hr, but calcium had been raised to 11 mM.

first. The same result was obtained in each of 6 experiments, though there was considerable variation in the height to which the calcium concentration had to be raised to remove the block.

The action of procaine

The effect of sympathetic stimulation was diminished by adding procaine hydrochloride to the bath when the calcium concentration was 2.2 mM. The addition was made in steps, and procaine reduced the amplitude of the pendular movements without reducing the effect of stimulation up to a concentration of 3×10^{-5} g/ml. Higher concentrations reduced the effect of stimulation also; at 1.2×10^{-4} g/ml. the effect of stimulation was still just visible. The calcium concentration was raised at this point to 6.6 mM, and then to 22 mM. The effect of stimulation was not increased, and indeed was less.

The isolated atria

Experiments were carried out on isolated rabbit atria with atropine present in the bath. As Burn & Gibbons (1965) observed, when the calcium concentration was 2.2 mM, acetylcholine (50 μg/ml.) caused no appreciable rise in rate. When the calcium concentration was 6.6 mM, however, a substantial rise in rate was seen. The increase having been recorded, the bath fluid was changed and guanethidine (5 μg/ml.) was added to the bath. After 20 min the atrial rate returned to its previous level, but when acetylcholine was added as before, the rate did not rise appreciably. The bath fluid was changed again, the guanethidine being replaced and the calcium concentration raised to 11 mM. When acetylcholine was added, the rate then rose as it did before guanethidine was added. In Table 1 are the mean results of 5 experiments in each of which the same observations were made. Thus guanethidine prevented the rise in rate which was caused by acetylcholine, but when the calcium concentration was raised, the block was removed.

TABLE 1

EFFECT OF THE CALCIUM CONCENTRATION ON THE ACTION OF GUANETHIDINE ON RABBIT ATRIA STIMULATED IN THE PRESENCE OF ATROPINE BY ACETYLCHOLINE (50 μg/ml.) AND ALSO BY NICOTINE (13 μg/ml.)

In experiments with acetylcholine, atropine was 5 μg/ml.; in experiments with nicotine, atropine was 1 μg/ml.

[Ca^{++}] (mM)	Guanethidine (μg/ml.)	Rate/min (mean±S.E.) Initial	With acetylcholine
6·6	0	58±2·7	100±4·1
6·6	5	61±3·1	62±6·0
11·0	5	58±1·4	104±3·0
		Initial	With nicotine
2·2	0	87±3·9	131±4·4
2·2	1	88±4·7	98±7·3
4·4	1	84±3·7	135±6·7

Table 1 also shows the mean results of 4 experiments in which guanethidine blocked the rise of rate caused by nicotine. Nicotine, 13 μg/ml., was observed to cause a rise when the calcium concentration was 2.2 mM. This rise was absent or diminished in the presence of guanethidine (1 μg/ml.), but when the calcium concentration was raised to 4.4 mM the block was removed, and the rise was seen again.

DISCUSSION

The action of acetylcholine in releasing noradrenaline from the spleen was explained by Ferry (1963) as due to stimulation of the sympathetic fibres. He showed that when acetylcholine was injected into the splenic artery of the cat, antidromic impulses passed along the splenic nerves; he therefore supposed that orthodromic impulses would pass into the spleen and liberate noradrenaline in the usual way. This explanation of the action of acetylcholine has been shown to be wrong by results in three papers. Hertting & Widhalm (1965), Wolner (1965) and Fischer, Weise & Kopin (1966) have all shown that when the cat spleen is perfused, or when the vessels of the cat's tail are perfused, a concentration of bretylium can be found which blocks the effect of stimulation of the sympathetic fibres but does not block the effect of acetylcholine.

Despite this evidence some have suggested that acetylcholine might act on nerve terminals when the rest of the nerve was blocked by the local anaesthetic action of bretylium. From the figures of Hertting & Widhalm this would mean that to block the nerve terminals a concentration of bretylium would be needed from five to 20 times as great as that required for other parts of the nerve. This is a difference of sensitivity for which there is no evidence. Moreover Exley (1957) compared xylocholine or TM10 with a similar compound in which the methyl groups attached to the quaternary nitrogen were replaced by ethyl groups. The two compounds had the same local anaesthetic action, but the ethyl compound had no adrenergic blocking action. Therefore the adrenergic blocking action was not related to the local anaesthetic action.

Thus it can be said that acetylcholine releases noradrenaline directly from the post-ganglionic fibre just as it releases catecholamines from the adrenal medulla.

Burn & Gibbons (1964a, 1965) have shown that the release of noradrenaline from the post-ganglionic fibre by acetylcholine depends on the concentration of calcium outside the fibre. Thus the increase in the rate of isolated rabbit atria produced by acetylcholine was nil when the calcium concentration was 2.2 mM, was 19% when it was 6.6 mM, and was 46% when it was 13.2 mM. This again resembles the release of catecholamines from the adrenal medulla by acetylcholine (Douglas & Rubin, 1963), and the conclusion can be drawn that the effect of acetylcholine is to make the compartment of the post-ganglionic fibre in which noradrenaline is held, permeable to calcium. The experiments described in the present paper show that the block by bretylium and guanethidine of the action of acetylcholine also depends on the external concentration of calcium. Thus an increase in calcium concentration, which increases the effect of acetylcholine in releasing noradrenaline, diminishes the effect of bretylium and guanethidine in blocking the release. In contrast to bretylium and guanethidine, block produced by procaine was not diminished by raising calcium even to the concentration of 22 mM. This suggests that bretylium and guanethidine act by opposing the action of acetylcholine ; that while acetylcholine acts by making the membrane of the post-ganglionic fibre permeable to calcium, bretylium and guanethidine may prevent this increase in permeability from taking place.

Substances with a blocking action

The first substance to be shown to block the response to the sympathetic impulse was acetylcholine. Brücke (1935) and Coon & Rothman (1940) demonstrated this in the pilomotor motor muscles of the cat's tail, and Burn & Rand (1960b) in the vessels of the rabbit's ear. Block is also caused by nicotine (Bentley, 1962) and by DMPP (dimethylphenylpiperazinium). Thus de la Lande & Rand (1965) have shown that DMPP blocks the response to sympathetic impulses to rabbit ear vessels in a concentration as low as 4×10^{-6} g/ml., and that this block, like that produced by guanethidine, is removed by dexamphetamine.

Other substances which block the response to the sympathetic impulse include d-tubocurarine and dihydro-β-erythroidine (Burn & Seltzer, 1965) and also mecamylamine and pempidine (Burn & Gibbons, 1964b). Thus it is evident that the response to the sympathetic impulse is blocked by agents which block the action of acetylcholine at the neuromuscular junction and at the sympathetic ganglion.

The action of bretylium and guanethidine

What then is the relation of bretylium and guanethidine to the substances just mentioned? Both these substances block neuromuscular transmission, but their actions differ, as Green & Hughes (1966) have shown. In the rat diaphragm the action of bretylium was found to be much greater at high rates of stimulation of the phrenic nerve than at low ones, and the block was antagonized by choline. The effect of frequency of stimulation on the block produced by guanethidine was different, being similar to that for tubocurarine, and the block was unaffected by choline. Since, in addition, concentrations of guanethidine (but not of bretylium) which cause neuromuscular block reduce the response of the frog rectus muscle to acetylcholine, the action of bretylium appeared to be chiefly presynaptic (at the neuromuscular ending), while that of guanethidine was postsynaptic.

Green & Hughes say " these observations . . . could easily be fitted to the hypothesis of Burn & Rand . . . that the adrenergic neurone blocking agents interfere with a supposed intermediary function of acetylcholine in adrenergic nerves; it could be postulated that bretylium acts largely by suppressing the release of acetylcholine, and that guanethidine competes with acetylcholine ".

It is also known that bretylium (Boura & Green, 1959) and guanethidine (Maxwell, Plummer, Schneider, Povalski & Daniel, 1960) not only cause neuromuscular block in skeletal muscle, but also block in the sympathetic ganglion.

Release of bretylium by sympathetic stimulation

Observations have been made by Fischer *et al.* (1966) in the cat spleen after injecting the cat with labelled bretylium. They found that when they perfused the spleen, stimulation of the splenic nerves caused a release of labelled bretylium, though the injection of acetylcholine did not. They made similar observations in the perfused heart, stimulating the stellate ganglion. Their observations indicated that the bretylium released by stimulation was not bound at the sites where noradrenaline was stored. They said that bretylium may act by replacing a cholinergic link in the sequence of events leading to transmitter release which occur when a sympathetic nerve impulse arrives at the nerve ending.

Noradrenaline release by bretylium and guanethidine

There is still a further connexion between bretylium and guanethidine on the one hand, and acetylcholine on the other—namely, that an initial large dose of either bretylium or guanethidine causes release of noradrenaline, leading, for example, to increase of rate and amplitude in isolated rabbit atria, though not in atria from a rabbit treated with reserpine. Thereafter, they cause the usual block of sympathetic impulses. Thus just as acetylcholine can both release noradrenaline, and in high concentration block sympathetic impulses, so bretylium and guanethidine have the same double action, though with them the stimulant action is exceptional, and the blocking action usual. Their double action on the adrenergic fibre is reminiscent of the double action of decamethonium at the neuromuscular junction. Decamethonium first stimulates and then blocks.

The effect of calcium on the block

If bretylium and guanethidine act as antagonists of acetylcholine, they must occupy the sites to which acetylcholine attaches itself in making the membrane permeable to calcium, and so make the membrane impermeable to calcium. When they have neutralized the action of acetylcholine at a given concentration of calcium, raising the concentration should make the membrane permeable again. This is what has been observed.

SUMMARY

1. When a loop of rabbit ileum is prepared together with the attached mesentery, and stimulation is applied to the periarterial nerves, inhibition of the pendular movements occurs. The fibres stimulated are post-ganglionic sympathetic fibres. Bretylium and guanethidine block the inhibitory action. If the calcium concentration is raised the block is removed. When the calcium concentration is lowered once more, the block reappears.

2. The inhibition can be blocked by procaine, but the block is not removed by raising the calcium concentration, even when it is raised to 22 mM.

3. Acetylcholine, acting in the presence of atropine, causes an increase in the rate and amplitude of isolated rabbit atria, and this increase depends on the calcium concentration, being greater as the calcium concentration rises. The increase at a given calcium concentration is blocked by guanethidine, but when the calcium concentration is raised, the block is removed.

4. The actions of bretylium and of guanethidine have been discussed in the light of these and other observations.

This work was communicated to the British Pharmacological Society on April 8, 1967. One of us (J. H. B.) wishes to thank Dr. O. H. Lowry for the opportunity to work in this department. The work was supported by a grant (5T1-NB-5221) from the National Institute of Neurological Diseases and Blindness, U.S.A.

REFERENCES

BENTLEY, G. A. (1962). Studies on sympathetic mechanisms in isolated intestinal and vas deferens preparations. *Br. J. Pharmac. Chemother.*, **19**, 85–98.

BOURA, A. L. A. & GREEN, A. F. (1959). The actions of bretylium: adrenergic neurone blocking and other effects. *Br. J. Pharmac. Chemother.*, **14**, 536–548.

BRÜCKE, F. T. (1935). Über die Wirkung von Acetylcholin auf die Pilomotoren. *Klin. Wschr.*, **14**, 7–9.

BURN, J. H. & GIBBONS, W. R. (1964a). The part played by calcium in determining the response to stimulation of sympathetic postganglionic fibres. *Br. J. Pharmac. Chemother.*, **22**, 540–548.

BURN, J. H. & GIBBONS, W. R. (1964b). The sympathetic postganglionic fibre and the block by bretylium; the block prevented by hexamethonium and imitated by mecamylamine. *Br. J. Pharmac. Chemother.*, **22**, 549–557.

BURN, J. H. & GIBBONS, W. R. (1965). The release of noradrenaline from sympathetic fibres in relation to calcium concentration. *J. Physiol., Lond.*, **181**, 214–223.

BURN, J. H. & RAND, M. J. (1960a). The relation of circulating noradrenaline to the effect of sympathetic stimulation. *J. Physiol., Lond.*, **150**, 295–305.

BURN, J. H. & RAND, M. J. (1960b). Sympathetic post-ganglionic cholinergic fibres. *Br. J. Pharmac. Chemother.*, **15**, 56–66.

BURN, J. H. & SELTZER, J. (1965). Substances blocking sympathetic post-ganglionic fibres and the neuromuscular junction. *J. Physiol., Lond.*, **179**, 569–576.

COON, J. M. & ROTHMAN, S. (1940). The nature of the pilomotor response to acetyl choline; some observations on the pharmacodynamics of the skin. *J. Pharmac. exp. Ther.*, **68**, 301–311.

DIXIT, B. N., GULATI, O. D. & GOKHALE, S. D. (1961). Action of bretylium and guanethidine at the neuromuscular junction. *Br. J. Pharmac. Chemother.*, **17**, 372–379.

DOUGLAS, W. W. & RUBIN, R. P. (1963). The mechanism of catecholamine release from the adrenal medulla and the role of calcium in stimulus-secretion coupling. *J. Physiol., Lond.*, **167**, 288–310.

EXLEY, K. A. (1957). The blocking action of choline 2 : 6-xylyl ether bromide on adrenergic nerves. *Br. J. Pharmac. Chemother.*, **12**, 297–305.

FERRY, C. B. (1963). The sympathomimetic effect of acetylcholine on the spleen of the cat. *J. Physiol., Lond.*, **167**, 487–504.

FISCHER, J. E., WEI E, V. K. & KOPIN, I. J. (1966). Interactions of bretylium and acetylcholine at sympathetic nerve endings. *J. Pharmac. exp. Ther.*, **153**, 523–529.

GREEN, A. F. & HUGHES, R. (1966). Effects of adrenergic neurone blocking agents on voluntary muscle stimulated at different frequencies. *Br. J. Pharmac. Chemother.*, **27**, 164–176.

HERTTING, G. & WIDHALM, S. (1965). Über den Mechanismus der Noradrenalin-Freisetzung aus sympathischen Nervenendigungen. *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.*, **250**, 257–258.

DE LA LANDE, I. S. & RAND, M. J. (1965). A simple isolated nerve-blood vessel preparation. *Aust. J. exp. Biol. med. Sci.*, **43**, 639–656.

MAXWELL, R. A., PLUMMER, A. J., SCHNEIDER, F., POVALSKI, H. & DANIEL, A. I. (1960). Pharmacology of [2-(octahydro-1-azocinyl)-ethyl] guanidine sulphate (SU-5864). *J. Pharmac. exp. Ther.*, **128**, 22–29.

WOLNER, E. (1965). Versuche über die Innervation der Pilomotoren. *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.*, **250**, 437–450.