Br. J. Pharmac. (1971), **42**, 352–363.

Factors affecting the action of guanethidine on adrenergic neurones

O. D. GULATI AND SAROJA JAYKAR*

Department of Pharmacology, Medical College, Baroda, India

Summary

1. The uptake of guanethidine by adrenergic neurones has been studied indirectly by testing the ability of various procedures to prevent or reverse adrenergic neurone blockade in the periarterially stimulated isolated ileum preparation.

2. Adrenergic neurone blockade was prevented but not reversed by equilibration with guanethidine ($3{\cdot}3 \times 10^{-6}$M) at low temperatures (10° C), in the absence of sodium or in the presence of tetrodotoxin ($0{\cdot}3 \times 10^{-6}$M) or noradrenaline ($1{\cdot}2 \times 10^{-3}$M).

3. Calcium (5×10^{-2}M) both prevented and, to some extent, reversed the adrenergic neurone blocking action of guanethidine.

4. Equilibration with guanethidine in the presence of mersalyl ($0{\cdot}6 \times 10^{-7}$M) or in the absence of potassium or calcium could neither prevent nor reverse adrenergic neurone blockade.

Introduction

Evidence supporting the existence of an amine-concentrating mechanism at the level of the neuronal cell membrane has been presented by Malmfors (1965) and Carlsson & Waldeck (1965). Dexamphetamine and cocaine block the uptake of noradrenaline and guanethidine into sympathetic nerves (Iversen, 1964; Chang, Costa & Brodie, 1965; Muscholl, 1961; Callingham & Cass, 1962; Day, 1962). Chang *et al.* (1965) and Obianwu, Stitzel & Lundborg (1968) have suggested that guanethidine is taken up into sympathetic nerve endings by the uptake process for noradrenaline.

Using the Finkleman (1930) preparation of the rabbit ileum the uptake of guanethidine was investigated in more detail in this study. The rationale of the experiments depended upon the ability of different procedures or agents to prevent the adrenergic neurone blocking action of guanethidine. Prevention of adrenergic neurone blockade would in all probability be due to block of uptake of guanethidine. The approach though indirect was justified by the results obtained.

Methods

Segments of ileum, from adult rabbits of either sex weighing 1·5–2 kg, were prepared with their sympathetic nerves intact by the method of Finkleman (1930). A segment of ileum 2–3 cm long was set up in a 33 ml organ bath containing McEwen's (1956) solution of the following composition (expressed in mM/litre):

* Present address: 3245 East Jefferson Avenue, Detroit, Michigan, USA.

NaCl, 129·9 ; KCl, 5·6 ; $CaCl_2$, 1·6 ; $NaHCO_3$, 25 ; NaH_2PO_4, 9·5 ; glucose, 11·1 ; sucrose, 13·1.

The solution was maintained at 35·5° ±1° C and movements of the ileum were recorded by an isotonic frontal writing lever on a smoked drum. The load on the lever was 750 mg and the relaxations were magnified 10 times. The periarterial nerves were stimulated by bipolar platinum electrodes at supramaximal voltages and frequencies of 2, 5, 10, 20 and 60 Hz with square wave pulses of 5 ms duration for 45 s every 4 minutes.

As a rule two preparations were set up simultaneously from the same ileum. After eliciting control responses to different frequencies one preparation was treated with the test drugs for a specified period of time. Both the preparations were then exposed to guanethidine ($3·3 \times 10^{-6}$M) for 10 minutes. The preparations were washed several times with McEwen's solution and the responses to nerve stimulation were recorded. The effects of the test drug on responses to nerve stimulation were studied by setting up a third preparation from the same ileum and exposing it to the test drug for a specified time.

In some experiments, calcium, potassium or sodium was omitted from the bathing medium. Whenever sodium was omitted, the osmotic pressure of the solution was maintained with sucrose and pH with potassium bicarbonate. In some experiments excess calcium (5×10^{-2}M) was added without compensation and hence the solution was hypertonic.

The temperature of the bath fluid was lowered (where necessary) by putting ice cubes in the outer jacket.

The drugs used were guanethidine sulphate (Ciba, Basle), mersalyl, tetrodotoxin (Sankyo Company Ltd., Tokyo), sodium edetate, calcium chloride and noradrenaline bitartrate (Unichem Labs., Bombay).

Results are expressed in terms of percentages of the inhibition of contractions achieved during the first measurement of the frequency-response relations (control response). In most experiments maximal (but incomplete) inhibition was obtained at a frequency of 20 Hz but in some experiments maximal inhibition was only obtained at 60 Hz.

Statistical procedures used are those outlined by Burn, Finney & Goodwin (1952).

Results

Adrenergic neurone blocking action of guanethidine

The inhibition of the contractions of the rabbit ileum produced by mesenteric nerve stimulation showed variations from preparation to preparation. Therefore two preparations were set up simultaneously as described in **Methods**. Control responses to nerve stimulations were elicited for each set of experiments separately. Following this, one preparation was exposed to guanethidine for 10 min, and washed five to six times before eliciting the responses to nerve stimulation at different frequencies. Guanethidine ($3·3 \times 10^{-6}$M) produced substantial, to complete, block of responses of the ileum to periarterial sympathetic nerve stimulation at frequencies ranging from 2 Hz to 60 Hz. The blocking action lasted for more than 3 hours. This is in confirmation of the results of Burn & Welsh (1967).

Effect of various drugs and procedures on the adrenergic neurone blocking action of guanethidine

Low temperature

In five control preparations at 35·5° C guanethidine produced complete adrenergic neurone blockade.

In five experiments the tissue was bathed for 10 min in McEwen's solution maintained at 10° C. The tissue relaxed and all pendular movements ceased. While the tissue was still maintained at 10° C, it was exposed to guanethidine ($3{\cdot}3 \times 10^{-6}$M) for 10 minutes. The tissue was then washed several times with the McEwen's solution at 35·5° C until it regained its tone and pendular movements. Responses to stimulation were then elicited (in McEwen's solution at 35·5° C) at various frequencies. The nerve stimulation was as effective as in control preparations (Fig. 1).

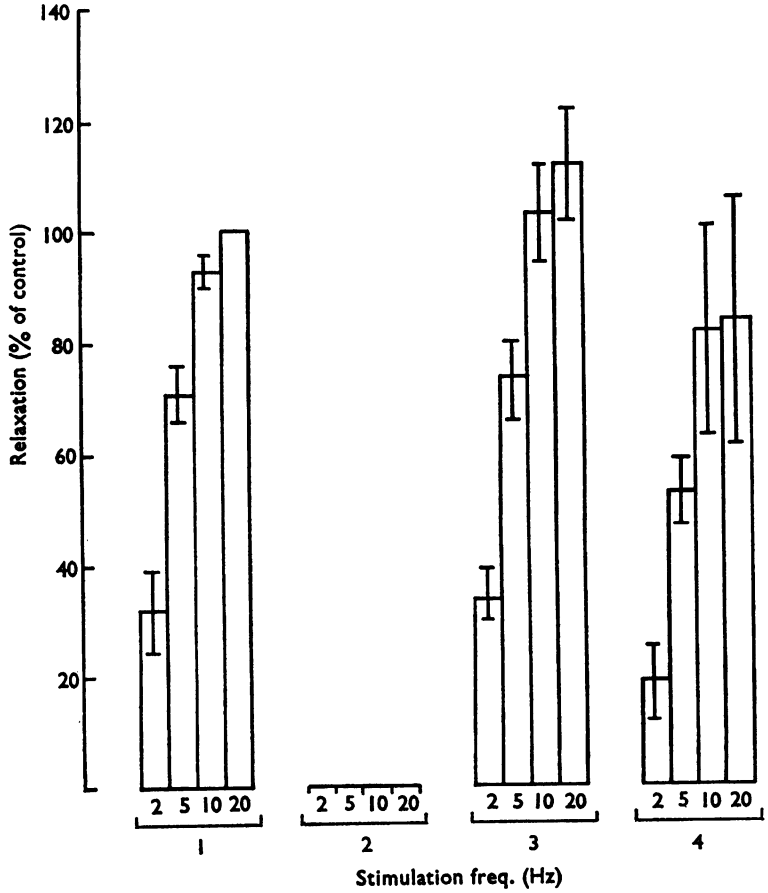

FIG. 1. Responses of Finkleman preparations to periarterial nerve stimulation at various frequencies, which are indicated below each column in Hz. In this, and subsequent figures, the responses are expressed as a percentage of the inhibition achieved in the first control frequency-response curve. Panel 1 shows control responses and panel 2 shows responses after exposure of the preparations to guanethidine ($3{\cdot}3 \times 10^{-6}$M) for 10 minutes. Responses shown in panels 3 and 4 were elicited after exposing the preparations to McEwen's solution for 10 min at 10° C and 15° C respectively, followed by exposure to guanethidine ($3{\cdot}3 \times 10^{-6}$M) for 10 min at the same temperatures. Responses shown were recorded at a bath temperature of 35·5° C. Vertical lines indicate standard errors.

In three experiments the temperature was maintained at 15° C for 10 min before exposing the tissue to guanethidine. The responses (elicited as described for 10° C) to nerve stimulation were not significantly different from the control responses ($P>0{\cdot}1$) (Fig. 1). Thus, lowering the temperature to 10° C or 15° C completely prevented the adrenergic neurone blocking action of guanethidine.

In the five preparations in which guanethidine had completely blocked the responses to nerve stimulation the preparation was bathed in McEwen's solution at 10° C for 20 minutes. Responses to nerve stimulation continued to remain totally blocked.

Deprivation of sodium

In five control preparations guanethidine totally abolished responses to nerve stimulation at frequencies of 2, 5 and 10 Hz. The mean response at 20 Hz was $7{\cdot}4 \pm 4{\cdot}9\%$ of the maximal response.

The test preparations were exposed to sodium-free McEwen's solution for 30 minutes. This produced slight relaxation and the inhibitory responses to nerve stimulation could not be elicited. After five to six washes with normal McEwen's solution, the inhibitory responses to nerve stimulation were restored. The prepara-

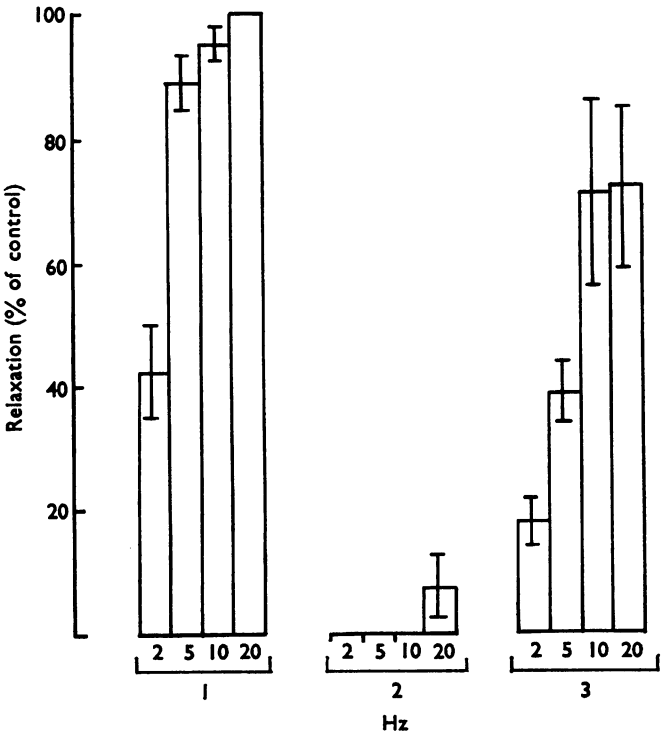

FIG. 2. Responses of Finkleman preparations to periarterial nerve stimulation at various frequencies. The responses are expressed as % of control response. Panel 1 shows control responses and panel 2 shows responses after exposure of the preparations to guanethidine ($3{\cdot}3 \times 10^{-6}$M) for 10 minutes. Responses shown in panel 3 were elicited after exposing the preparations to sodium-free McEwen's solution for 30 min followed by exposure to guanethidine ($3{\cdot}3 \times 10^{-6}$M) for 10 min in the same medium. Osmolarity of the tissue was maintained with equimolar amount of sucrose. Responses were recorded in normal McEwen's solution. Vertical lines indicate standard errors.

tions were treated with guanethidine while still bathed in sodium-free medium. This was followed by five to six wash-outs with normal solution. The adrenergic neurone blocking action of guanethidine was prevented to a large extent (five experiments ; Fig. 2). Exposure of the preparation to sodium-free solution for 40 min (after eliciting a panel of control responses and a panel following guanethidine) did not reverse the adrenergic neurone blocking action of guanethidine (two experiments).

Tetrodotoxin

In five control preparations guanethidine blocked the responses to periarterial nerve stimulation at all the frequencies tested (Fig. 3).

In five other preparations made from the ilea of the same rabbits tetrodotoxin ($0{\cdot}3 \times 10^{-6}$M for 20 min) completely abolished the responses obtained at various frequencies but the tone and the peristaltic movements were not inhibited. After five to six washes the responses at frequencies 2, 5, 10 and 20 Hz recovered to $47{\cdot}5 \pm 9\%$, $65{\cdot}7 \pm 6{\cdot}3\%$, $76{\cdot}9 \pm 9{\cdot}7\%$ and $72{\cdot}8 \pm 4{\cdot}9\%$ of the maximal responses, respectively.

In five preparations tetrodotoxin ($0{\cdot}3 \times 10^{-6}$M) was placed in the bath for 20 min before exposure to guanethidine. The tissue was washed five to six times. The

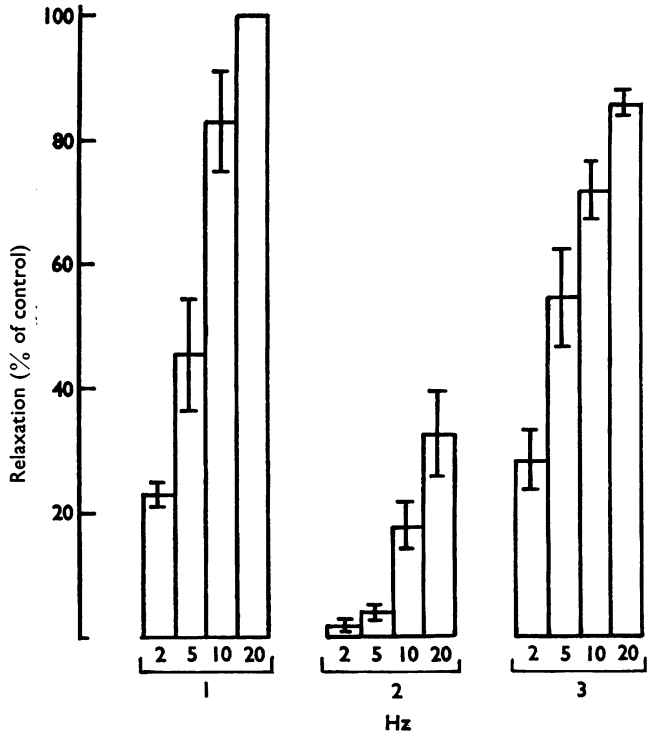

FIG. 3. Responses of Finkleman preparations to periarterial nerve stimulation at various frequencies. The responses are expressed as % of control response. Panel 1 shows control responses and panel 2 shows responses after exposure of the preparations to guanethidine ($3{\cdot}3 \times 10^{-6}$M) for 10 minutes. Responses shown in panel 3 were elicited after exposing the preparations to tetrodotoxin ($0{\cdot}3 \times 10^{-6}$M) for 20 min followed by exposure to guanethidine ($3{\cdot}3 \times 10^{-6}$M) for 10 min in the presence of tetrodotoxin and five to six wash-outs. Vertical lines indicate standard errors.

adrenergic neurone blocking action of guanethidine was almost completely prevented (Fig. 3). However, tetrodotoxin ($0{\cdot}3 \times 10^{-6}$M) placed in the bath for 30 min after guanethidine pretreatment failed to reverse the action of guanethidine (two experiments).

Noradrenaline

In four control preparations guanethidine produced complete block of responses to nerve stimulation. In four other preparations made from the ilea of the same rabbits, noradrenaline ($1{\cdot}2 \times 10^{-3}$M) was added to the bath followed immediately by the addition of guanethidine. After 10 min the preparations were washed several times until the tone and pendular movements returned, since noradrenaline relaxed the gut and totally abolished the pendular movements. There was a marked reduction of the neurone blocking action of guanethidine. The data are shown in Fig. 4.

Noradrenaline ($1{\cdot}2 \times 10^{-3}$M for 15 min) failed to reverse the adrenergic neurone blocking action of guanethidine (one experiment).

In three experiments the responses of control preparations to nerve stimuli were completely blocked by guanethidine. Exposure of test preparations to noradrenaline ($1{\cdot}2 \times 10^{-3}$M) for 5 min followed by exposure to guanethidine as above failed to prevent the neurone blockade.

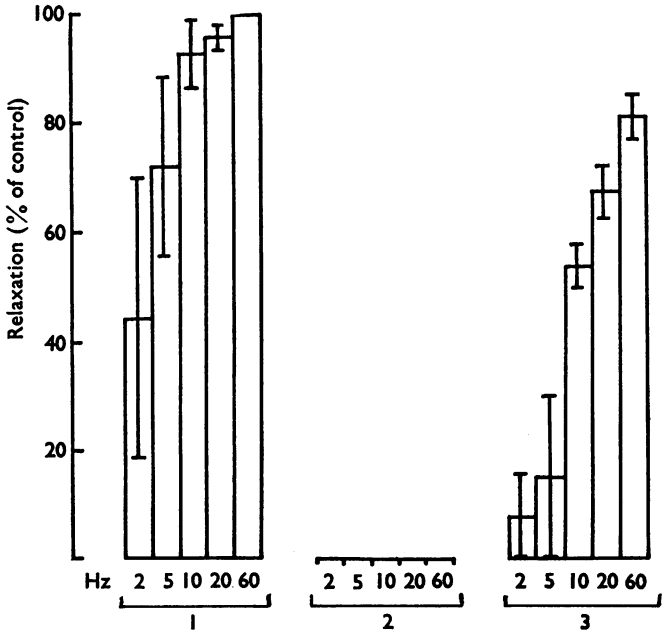

FIG. 4. Responses of Finkleman preparations to periarterial nerve stimulation at various frequencies. The responses are expressed as % of control response. Panel 1 shows control responses and panel 2 shows responses after exposure of the preparations to guanethidine ($3{\cdot}3 \times 10^{-6}$M) for 10 minutes. Responses shown in panel 3 were elicited after exposing the preparations to noradrenaline ($1{\cdot}2 \times 10^{-3}$M) followed immediately by guanethidine ($3{\cdot}3 \times 10^{-6}$M) for 10 min followed by wash-outs (eight–nine) until the tone and pendular movements returned. Vertical lines indicate standard errors.

Deprivation of potassium

In five preparations the control mean inhibitory responses to nerve stimulation at frequencies 5, 10 and 20 Hz were 54·1 ± 9·5%, 78·7 ± 6·6% and 93·5 ± 4·8% of the maximal responses, respectively. Guanethidine totally blocked responses to all the frequencies of nerve stimulation. In three other preparations made from the ilea of the same rabbits the mean responses at frequencies 5, 10 and 20 Hz after exposing the tissue to potassium-free medium for 20 min and subsequent washing with normal solution were 65·6 ± 11·1%, 75·8 ± 3·1%, 90·2 ± 8·8% of the control responses, respectively. In potassium-free medium the tissue was relaxed. The pendular movements returned after several washes in normal solution. The adrenergic neurone blocking action of guanethidine was neither prevented (five experiments) nor reversed by exposing the preparations to potassium-free medium (one experiment).

Deprivation of calcium

In five preparations the control mean inhibitory responses to nerve stimulation at frequencies 2, 5, 10, 20 and 60 Hz were 43·3 ± 14·9%, 94·1 ± 25%, 76·4 ± 13·2%, 93·3 ± 6·7% and 100 ± 0·0% of the maximal responses, respectively. Guanethidine completely blocked the responses to all the frequencies of nerve stimulation. In four other preparations made from the ilea of the same rabbits, the mean responses at frequencies 2, 5, 10 and 20 Hz after exposing the tissue to calcium-free solution and sodium edetate treatment ($2{\cdot}67 \times 10^{-4}$M for 20 min) followed by several wash-outs with normal McEwen's solution were 46·1 ± 23·4%, 96·7 ± 61·9%, 143·1 ± 54·5% and 172·1 ± 46·5% of the initial maximal responses, respectively. In the presence of calcium-free medium and sodium edetate treatment, the tissue completely lost its tone and pendular movements. The tone and pendular movements returned after several wash-outs with normal McEwen's solution. The adrenergic neurone blocking action of guanethidine was neither prevented (five experiments) nor reversed (one experiment) by exposing the preparations to calcium-free medium and sodium edetate treatment.

Mersalyl

In four preparations the control mean inhibitory responses to nerve stimulation at frequencies 2, 5, 10, 20 and 60 Hz were 54·7 ± 8·6%, 74·8 ± 8·5%, 81·7 ± 13·20%, 90·3 ± 12% and 100 ± 0·0% of the maximal responses, respectively. Guanethidine completely blocked the responses at frequencies 2, 5, 10 and 20 Hz. The mean response was 26·9 ± 0·0% of the maximal response at frequency 60 Hz.

In four other preparations made from the ilea of the same rabbits the mean responses at frequencies 2, 5, 10, 20 and 60 Hz after exposing the tissue to mersalyl ($0{\cdot}6 \times 10^{-7}$M) for 10 min were 20·9 ± 0·0%, 23·2 ± 0·5%, 25·8 ± 1·70%, 37·2 ± 3·3% and 66·2 ± 0·0% of the maximal responses, respectively. With mersalyl the tissue became slightly relaxed and the pendular movements were sluggish. The effect of mersalyl persisted for over 2 hours. The adrenergic neurone blocking action of guanethidine was neither prevented (four experiments) nor reversed (four experiments) by exposure of the tissue to mersalyl ($0{\cdot}6 \times 10^{-7}$M).

High calcium

The results are presented in Fig. 5.

In five control preparations, guanethidine produced almost complete adrenergic neurone blockade.

In four other preparations made from ilea of the same rabbits after exposing the tissue to calcium ($5{\cdot}0 \times 10^{-2}$M) for 10 min the tissue went into spasm and the pendular movements ceased. Responses to nerve stimulation were blocked (50%) at frequency 2 Hz; at other frequencies the responses were not affected. After several washes with normal solution the tissue regained the pendular movements and the original tone.

In four experiments calcium ($5{\cdot}0 \times 10^{-2}$M) was placed in the bath for 10 minutes. This was followed by exposing the tissue to guanethidine. After several washes, responses were re-elicited. Adrenergic neurone blockade due to guanethidine was largely prevented.

After eliciting responses of five control preparations following exposure to guanethidine, the tissue was treated with calcium ($5{\cdot}0 \times 10^{-2}$M for 20 min), washed and the responses to different frequencies were re-elicited. The adrenergic neurone blocking action of guanethidine was slightly reversed.

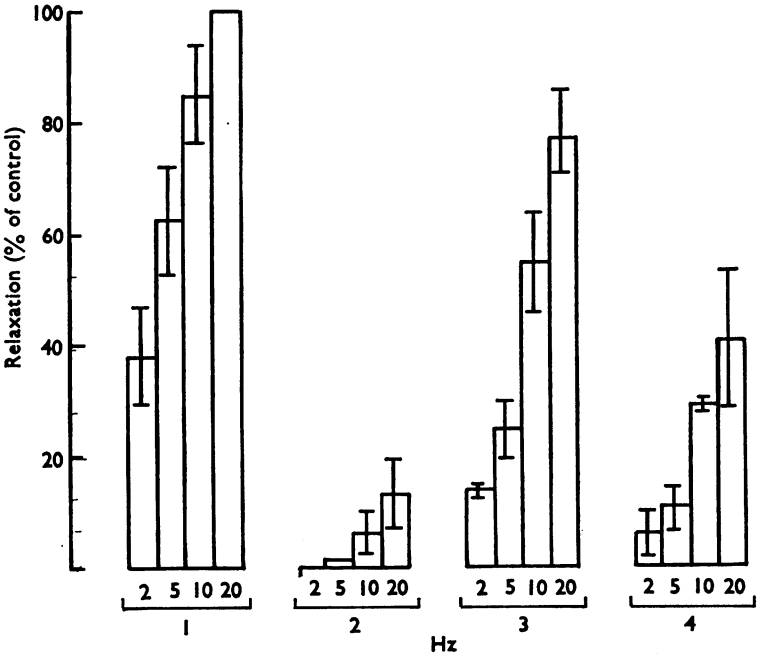

FIG. 5. Responses of Finkleman preparations to periarterial nerve stimulation at various frequencies. The responses are expressed as % of the control response. Panel 1 shows control responses and panel 2 shows responses after exposure of the preparations to guanethidine ($3{\cdot}3 \times 10^{-6}$M) for 10 minutes. Responses shown in panel 3 were elicited after exposing the preparations to calcium (5×10^{-2}M) for 10 min followed by exposure to guanethidine ($3{\cdot}3 \times 10^{-6}$M) for 10 min in the presence of high calcium and several wash-outs. After eliciting responses shown in panel 2, the preparations were treated with calcium (5×10^{-2}M) for 20 min, washed and the responses re-elicited (panel 4). Vertical lines indicate standard errors.

Effect of presence and absence of nerve stimulation on the neurone blocking action of guanethidine

In some of the experiments described so far the tissue was incubated with guanethidine when it was unresponsive to nerve stimulation and when no noradrenaline was being released. Preparations were, therefore, set up in duplicate to study the effect of the presence or absence of nerve stimulation on the uptake of guanethidine. In two out of three experiments, the presence or absence of stimulation was inconsequential ; that is guanethidine produced complete neuronal block in both the types of preparations. In the third experiment there was complete block of responses to nerve stimulation in the preparation not subjected to stimulation during incubation and wash-outs, but the preparation subjected to stimulation during incubation and wash-out, though unresponsive at lower frequencies was fully responsive at a frequency of 20 Hz.

Discussion

Interference with energy metabolism reduces the uptake and retention of noradrenaline in the isolated tissues (Dengler, Michaelson, Spiegel & Titus, 1962 ; Green & Miller, 1966 ; Wakade & Furchgott, 1968). Since energy-dependent active processes are temperature sensitive, the effect of low temperature (15° and 10° C) was studied on the adrenergic neurone blocking action of guanethidine. The observations indicated that at low temperature the adrenergic neurone blocking action of guanethidine was completely prevented. However, if adrenergic neurone blockade was induced first, lowering the temperature subsequently had no effect. The effect of low temperature must be on the uptake of guanethidine and not on the blockade of release of noradrenaline since the latter action would occur after the entry of guanethidine into adrenergic neurones.

The mechanism by which energy is utilized for the active process of uptake is not clear. The mechanism suggested by Kirpekar & Wakade (1968) for the uptake of noradrenaline could also explain the guanethidine uptake. It may be that ATP, generated by either glycolysis or oxidative phosphorylation is somehow utilized as a source of energy for ' pumping ' guanethidine or noradrenaline across the neuronal membrane. The other possibility may be that guanethidine, like noradrenaline, is complexed with a carrier having great affinity for these amines when sodium concentration is high and the potassium concentration is low. Such an ion-coupled carrier system could produce an influx of guanethidine against the concentration gradient when extracellular sodium is much higher than intracellular sodium. The sodium and potassium gradient upon which this mechanism depends is maintained by the sodium pump (associated with sodium and potassium ATPase) in the membrane which in turn is maintained by the energy derived from metabolism. This type of ion-coupled transport has been studied for noradrenaline (Garrahan & Glynn, 1967 ; Crane, 1967 ; Kirpekar & Wakade, 1968).

Recent studies indicate the requirement of sodium for (i) the uptake and storage of noradrenaline by sympathetic nerve endings (Iversen & Kravitz, 1966 ; Bogdanski & Brodie, 1969 ; Gillis & Paton, 1967 ; Horst, Kopin & Ramey, 1968 ; Kirpekar & Wakade, 1968 ; Tissari, Schönhöfer, Bogdanski & Brodie, 1969) (ii) the cholinergic transmission in sympathetic ganglia (Birks & MacIntosh, 1961), and (iii) the transport of glucose across intestinal epithelial cells (Riklis & Quastel, 1958; Crane,

1962). Thus the requirement of sodium may not be unique to the active transport of noradrenaline.

If it is postulated that guanethidine uptake by the sympathetic nerve endings is also a sodium dependent active process, and that the transport mechanism involves a sodium-complexed carrier mechanism, then the procedures or drugs which interfere with the sodium-potassium gradient or permeability to sodium of the neuronal membrane should affect the uptake of guanethidine. The low concentration of external sodium ion in the tissue, the drugs which block the permeability of sodium across the neuronal membrane and the drugs which inhibit the sodium-potassium dependent ATPase essential for the sodium pump controlling the sodium-potassium gradient across the membrane should all block the transport of guanethidine across the neuronal membrane.

The criteria outlined above were met in this investigation. The deprivation of sodium from the external medium for 30 min prevented the adrenergic neurone blocking action of guanethidine. Tetrodotoxin which selectively blocks the increase in sodium conductance with little or no change in the potassium conductance mechanism in neuronal membrane (Kao, 1966 ; Cheymol & Bourillet, 1966), was used to block the permeability of sodium across the neuronal membrane. Tetrodotoxin completely prevented the neuronal blocking action of guanethidine. It appears that the sodium ion plays an obligatory role in the active transport of guanethidine across the sympathetic neuronal membrane and the mechanism seems to be similar to that of noradrenaline uptake into the sympathetic neuronal endings.

The possibility that sulphydryl binding sites present in the sodium-potassium dependent ATPase may be involved in the uptake of guanethidine could be excluded by the lack of effect of mersalyl on the adrenergic neurone blockade of guanethidine.

The actions of other ions such as potassium and calcium on the neuronal blocking action of guanethidine were also studied and the observations indicated that guanethidine uptake was unaffected in potassium-free as well as calcium-free McEwen's solutions. Thus it appears that external calcium and potassium ions are not essential for the uptake of guanethidine by the adrenergic nerve endings. It was observed that the inhibitory responses to adrenergic nerve stimulation were blocked in calcium-free solution. These observations are closely similar to those of Kirpekar & Wakade (1968) on the uptake and release of noradrenaline by the adrenergic nerve endings.

If the site and mechanism of uptake of noradrenaline and guanethidine on the adrenergic neuronal membrane are the same, then simultaneous exposure to both should interfere with the adrenergic neurone blocking action of guanethidine. This is indeed what happened. However, noradrenaline added 5 min before exposure to guanethidine failed to exert a preventive action suggesting a quick disappearance of noradrenaline from the tissue.

Calcium ($5{\cdot}0 \times 10^{-2}$M) antagonized the action of guanethidine when added before or after the effect of guanethidine had been established. The prevention of action of guanethidine may be due to the high concentration of calcium interfering with the accessibility of guanethidine to its site of action. This suggestion is in support of the findings of Boullin, Costa & Brodie (1966) who showed that high calcium concentrations inhibit the uptake of ^{14}C-guanethidine. The unblocking effect of calcium could be a result of calcium entering into the nerve endings and competing with guanethidine for the same binding sites. Kirpekar, Wakade, Dixon & Prat,

(1969) have suggested that on nerve stimulation, noradrenaline release from this site is made easier by the displacement of guanethidine by calcium leading to a restoration of the transmission process. Thus it can be said that the sites at which calcium ions act to trigger noradrenaline release are the same sites with which guanethidine combines to inhibit noradrenaline release.

It is a pleasure to acknowledge the gifts of guanethidine by Ciba, Basle, and of noradrenaline by A. V. Mody of Unichem Laboratories, Bombay. This work was supported by a research grant from the Council of Scientific and Industrial Research, New Delhi.

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(*Received June* 8, 1970)