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REDUCTION OF ^{14}C-GUANETHIDINE LEVELS IN RAT HEART AND DIAPHRAGM BY EXCESS CALCIUM

BY

D. J. BOULLIN

From the Department of Pharmacology and Therapeutics, St. Thomas's Hospital Medical School, London

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There appear to be at least two types of binding of guanethidine in tissues (Brodie, Chang & Costa, 1965 ; Chang, Costa & Brodie, 1965). One of these is considered to be specific. It occurs in tissues with an adrenergic innervation and is antagonized by amphetamine, which also antagonizes the pharmacological and catecholamine-depleting actions of guanethidine (Day & Rand, 1963 : Matsumoto & Horita, 1963). The other is non-specific. It is found in skeletal muscle and is unaffected by drugs which influence adrenergic mechanisms (Chang *et al.*, 1965).

Guanethidine is released by electrical stimulation of post-ganglionic sympathetic neurones, and may possibly function as a false transmitter or inactive substitute for noradrenaline (Boullin, Costa & Brodie, 1966 ; Boullin, 1966b). As calcium is generally required for the release of the cholinergic and adrenergic neuro-hormones (Harvey & MacIntosh, 1940 ; Douglas & Rubin, 1961, 1963 ; Burn & Gibbons, 1964, 1965 ; Boullin, 1966a) and for the uptake of exogenous noradrenaline (Titus & Dengler, 1966), it seemed of interest to determine whether calcium also played a part in the uptake of guanethidine *in vitro*, and to compare its action in a tissue with many adrenergic nerve fibres, such as the heart, with that in a tissue like skeletal muscle, where adrenergic neurones are sparse and catecholamine levels low (Sedvall, 1964).

METHODS

Slices (0.5 mm thick) of ventricular muscle taken from male Sprague-Dawley rats weighing 180–200 g and triangular strips of diaphragm (about 5 mm wide at the base) taken from 120–150 g animals were used. Tissues (20–30 mg) were incubated at 37° C in 10 ml. Locke solution having the following composition (mM): NaCl 154, KCl 5.6, $NaHCO_3$ 6.0, glucose 11, and $CaCl_2$ 2.2. In some experiments $CaCl_2$ was increased to 10 or 20 mM (tonicity was maintained by removal of NaCl). The medium was normally gassed with 5% carbon dioxide in oxygen or nitrogen, when the pH was 7. In other experiments pure nitrogen was used and then the pH was 8.4.

After 10 min incubation in a metabolic shaker, 2 μg/ml. ^{14}C-guanethidine sulphate, specific activity 0.78 mC/mM (a gift of Dr. C. I. Furst, Ciba Laboratories Ltd., Horsham, Sussex) was added to the incubation flasks. Slices were removed from the flasks after timed intervals (15 to 180 min). They were blotted dry, weighed and homogenized in 3 ml. 0.4N perchloric acid. After centrifugation (2,000 rev/min for 5 min) 1 ml. of supernatant was assayed for total tissue radioactivity by liquid scintillation spectrometry. At the end of incubation 1 ml. of the incubation medium was also assayed for radioactivity. Results are expressed as the ratio of cpm/g tissue to cpm/ml. in the incubation medium at the beginning of experiment (T/M).

RESULTS

In the first group of experiments, heart slices were incubated in media containing 2.2, 10 and 20 mM calcium under aerobic conditions. The accumulation of guanethidine in slices was rapid at first, then proceeded more slowly until steady-state conditions were attained after 2 to 3 hr. The rate of uptake was fast until the ratio of radioactivity in tissue to that in the medium (T/M) was about 1. This value was reached in about 12 min (Table 1). The initial uptake was not altered by changes in the calcium concentration. However, subsequent uptake occurred more slowly and was depressed when the calcium concentration was increased to 10 or 20 mM (Fig. 1a). Excess cation also depressed the T/M ratios at the steady-state. In 2.2 mM calcium the T/M ratio at equilibrium was 2.8; it was reduced to 2.1 by 10 mM calcium and to 1.8 by 20 mM calcium.

TABLE 1

KINETICS OF UPTAKE OF ^{14}C-GUANETHIDINE IN ISOLATED RAT TISSUES

Number of experiments indicated in parentheses

Tissue	Experimental conditions	Fig. No.	Time to attain T/M ratio of 1 (min)	T/M ratio at steady-state calcium concentration (mM)		
				2·2	10	20
Heart	Aerobic pH 7 (12)	1a	12	2·8†	2·1	1·8†
Heart	Anaerobic pH 7 (6)	1b	18	2·3†	1·8	1·6†
Diaphragm	Aerobic pH 7 (6)	2a	24	2·1*	1·8*	1·8*
Diaphragm	Anaerobic pH 7 (6)	2b	24	1·7*	1·5	1·3*
Heart	Anaerobic pH 8·4 (4)	3	7	4·5†	4·0	2·8†
Diaphragm	Anaerobic pH 8·4 (4)	3	20	Not attained in 3 hr		

* Values significantly different ($P<0·01$). † Values significantly different ($P<0·001$).

Schanker & Morrison (1965) found that uptake of guanethidine into slices of rat heart was depressed by 40% when nitrogen was used in place of oxygen. Therefore it was of interest to determine whether the effect of calcium on uptake was modified under anaerobic conditions. In these experiments the incubation medium was gassed with 5% carbon dioxide in nitrogen, rather than pure nitrogen, in order to maintain the pH at 7. Table 1 shows that the initial rate of uptake, indicated by the time required to reach a T/M ratio of 1, was decreased by about 50% in the absence of oxygen. Figure 1b shows an overall reduction of uptake under anaerobic conditions, though the depressant action of excess calcium itself was little changed.

As histochemical studies show that the heart has a massive adrenergic innervation (Norberg & Hamberger, 1964), an attempt was made to determine whether the effects of calcium were associated with an action of guanethidine on adrenergic neurones by

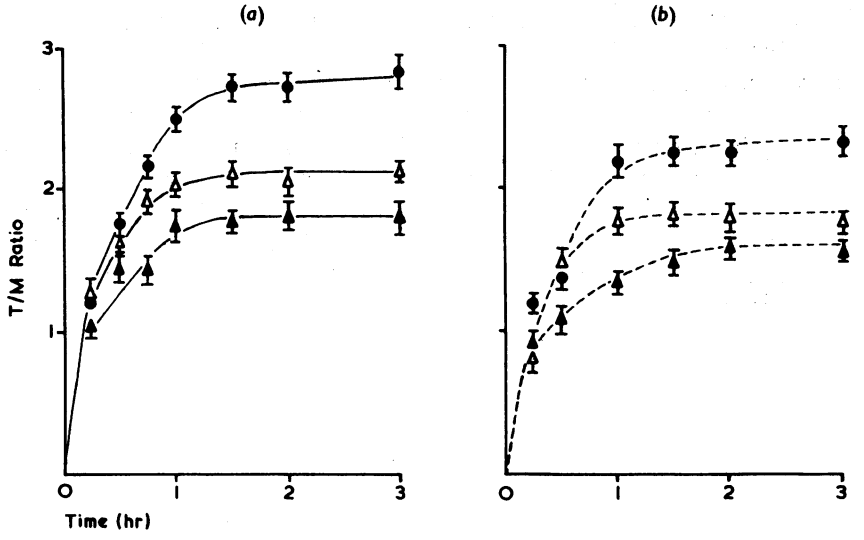

Fig. 1. Effect of calcium on uptake of 2 μg/ml. ^{14}C-guanethidine into heart ventricle slices at pH 7 under aerobic conditions (solid lines, left) and anaerobic conditions (dotted lines, right). Data are expressed as the ratio of concentration of guanethidine in tissue to concentration in incubation medium (T/M) plotted against incubation time. Solutions containing 10 mM (Δ—Δ) and 20 mM (▲—▲) calcium depress uptake compared to that seen in 2.2 mM calcium (●—●). Each point is the mean (±SE) of four to 12 determinations.

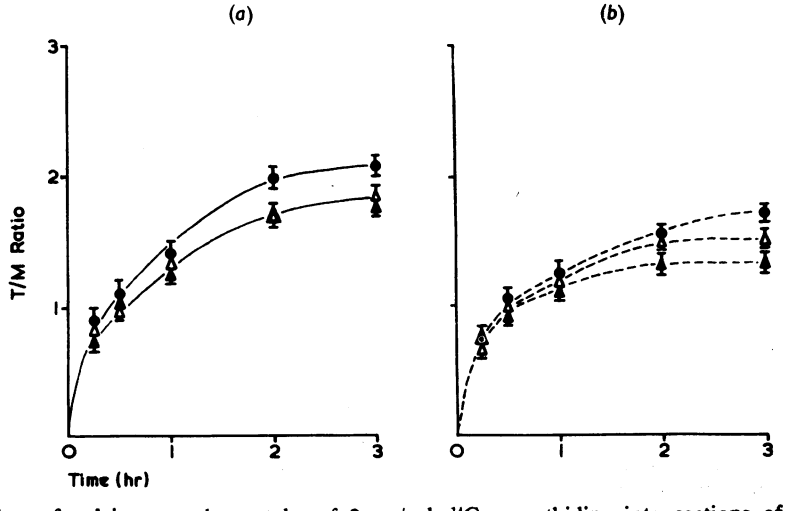

Fig. 2. Effect of calcium on the uptake of 2 μg/ml. ^{14}C-guanethidine into sections of diaphragm at pH 7 under aerobic conditions (solid lines, left) and anaerobic conditions (dotted lines, right). Legend as Fig. 1. Overall uptake is depressed under anaerobic conditions (right), but the small depressant effect of 10 and 20 mM calcium remains unimpaired. Each point is the mean of six determinations.

comparing the effects of calcium on the uptake of guanethidine in the heart and the diaphragm. Figures 2a and b show the pattern of accumulation of guanethidine into diaphragm sections under aerobic and anaerobic conditions. Initial uptake occurred at a slower rate than in cardiac tissue; the time required to reach a T/M ratio of 1 was twice that required by heart muscle, was not changed by removal of oxygen (Table 1), and was not affected by excess calcium. The drug level at the steady-state was reduced by excess calcium but the depression was less than in the heart. Whereas in the cardiac muscle the T/M ratio was reduced from 2.8 to 2.1 by an increase in the calcium concentration from 2.2 to 10 mM, in the diaphragm the reduction under these conditions was from 2.1 to 1.8. In addition, there was no further decrease when the calcium concentration was doubled

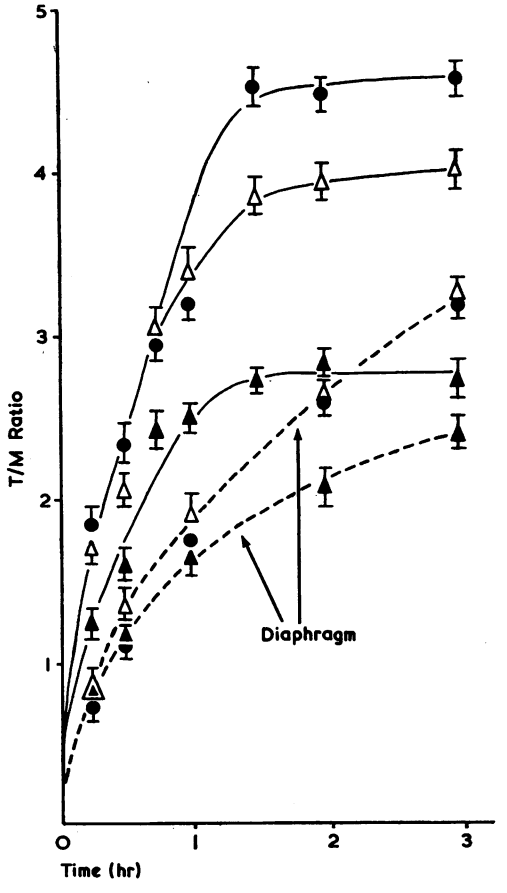

Fig. 3. Effect of calcium on uptake of 2 μg/ml. ^{14}C-guanethidine into heart (solid lines) and diaphragm (dotted lines) under anaerobic conditions (pure nitrogen) at pH 8.4. Legend as Fig. 1. Overall uptake in the heart is increased and the depressant action of 10 and 20 mM calcium enhanced compared to responses seen under aerobic conditions at pH 7 (see Fig. 1). In the diaphragm the rate of uptake is diminished, but the degree of uptake is enhanced (compare with Fig. 2). Note that steady-state conditions are not obtained after 3 hr incubation. Each point is the mean of four determinations.

to 20 mM. From these results it may also be seen that during anaerobic incubation the depressant action of 10 and 20 mM calcium was less in the diaphragm than in the heart.

As guanethidine is a base that is normally highly ionized in solution, and as uptake of organic compounds is commonly accentuated by an increase in the proportion of un-ionized molecules (Brodie, 1964), some experiments were carried out under anaerobic conditions using pure nitrogen. As the pH in these experiments was 8.4 more guanethidine was present in a less highly ionized form (see Discussion). It was found that guanethidine was concentrated in the heart to a much greater extent than at physiological pH (Fig. 3). Initial uptake was very rapid ; a T/M ratio of 1 was reached in only 7 min, compared with 12–18 min under the other experimental conditions (Table 1). Furthermore, uptake continued at a fast rate until eventually a much higher T/M ratio was reached at the steady-state (T/M 4.5, Table 1). Nevertheless, 10 and 20 mM calcium continued to exert a powerful depressant effect on uptake. In contrast, experiments on the uptake of guanethidine in the diaphragm at pH 8.4 showed a different pattern of accumulation. The inital rate of uptake was only slightly increased and steady-state values were not obtained even after 3 hr incubation.

DISCUSSION

In the present experiments, the uptake of guanethidine into tissues was modified by oxygen, pH and calcium. Schanker & Morrison (1965) showed that removal of oxygen depressed uptake in the rat heart. The present experiments with normal calcium concentration confirm this by showing that removal of oxygen reduced the initial rate of uptake and the T/M ratio at the steady-state. These experiments support the view of Schanker & Morrison (1965) that uptake of guanethidine into heart occurred by an active process requiring energy, in addition to any uptake that took place by diffusion. In the diaphragm anaerobic incubation also depressed uptake at the steady-state but the initial rate was not affected. These results, and those obtained by increasing the pH from 7 to 8.4, suggest that specific uptake was minimal in the diaphragm. The increase in alkalinity caused a big increase in the initial rate of uptake in the heart, but had very little effect in the diaphragm. The latter observation was surprising. In solution guanethidine is present entirely in an ionized form, but the type and degree of ionization of guanethidine is critically dependent upon pH as the compound possesses two positive charges. The first charge, associated with the ring nitrogen, has a pKa (pKa_1) of 8.7. The guanidine portion of the molecule is much more basic, with a pKa (pKa_2) greater than 11. At pH 7 used in the majority of the experiments described here, 98% of guanethidine is present in the di-ionized form. At pH 7.4, used by Schanker & Morrison (1965), 95% is di-ionized, but at pH 8.4 only 66% is in the doubly charged form. (This information was kindly supplied by Mr. C. MacMartin and Dr. C. I. Furst, Ciba Laboratories Ltd., Horsham, Sussex.) These differences in the degree of ionization explain most of the reported observations on the uptake of guanethidine in tissues. For example, Schanker & Morrison (1965) found a T/M ratio of 5.1 for uptake into rat heart slices at pH 7.4 under aerobic conditions when 5% guanethidine was presumably present as the mono-ionized molecule. The comparable T/M ratio obtained in the present work was 2.8 but here only 3% guanethidine was mono-ionized. Similar remarks also apply to the greatly enhanced uptake obtained in cardiac tissue at pH 8.4 when 34%

was mono-ionized. However, the failure of the increase in pH to affect the uptake of drug in the diaphragm to any degree, suggests that the tissue levels recorded were largely due to accumulation of drug by passive diffusion, or adsorption at extracellular sites.

Apart from the influence of oxygen and pH, calcium concentration had a profound effect on guanethidine uptake by the heart.

As the depressant effect of 10 and 20 mM calcium was independent of oxygen, and as excess calcium did not influence the initial rate of uptake into the tissues, the cation does not appear to have produced an action on uptake by an energy-dependent process. Costa, Chang & Brodie (1964) and Bogdanski & Boullin (unpublished) have found guanethidine in the microsomal fraction of rat heart that is rich in synaptic vesicles (Potter & Axelrod, 1963 ; Potter, 1966), and it is possible that calcium reduced uptake of guanethidine by an action leading to reduced intracellular storage. Uptake of guanethidine in the diaphragm was only slightly diminished by excess calcium, and this agrees with the finding of Chang *et al.* (1965) that only a small amount of specific binding occurred in skeletal muscle.

If calcium changed the levels of guanethidine in synaptic vesicles in adrenergic nerves, it may have done so by either or both the following direct mechanisms: decrease in guanethidine binding, or increase in guanethidine release. It is not possible to say from these experiments which of these explanations is correct. Indeed, it is difficult to devise experiments that will give an unequivocal answer. From a theoretical point of view tissue levels at any given time may indicate a dynamic state where a drug is being taken up, stored, released and also taken back into store.

However, there is reason for supposing that calcium reduces guanethidine levels by releasing the drug from synaptic vesicles. Philippu & Schümann (1962) and Schümann & Philippu (1963) have shown that calcium ions can release noradrenaline from isolated bovine adrenal medullary granules on a dose-dependent basis. In view of the known pattern of distribution of guanethidine in adrenergic nerves (see above) and its release by nerve stimulation (Boullin, Costa & Brodie, 1966 ; Boullin, 1966b) the cation may also release guanethidine stored in adrenergic nerve granules.

SUMMARY

1. The effects of calcium on the uptake of ^{14}C-guanethidine by isolated rat tissues were investigated by incubation of slices of ventricle and strips of diaphragm in Locke solution containing 2.2, 10 and 20 mM calcium, together with 2 ug/ml. ^{14}C-guanethidine under aerobic or anaerobic conditions.

2. In 2.2 mM calcium at pH 7 with oxygen, guanethidine accumulated in heart and diaphragm until steady-state conditions were attained. Ten or 20 mM calcium reduced the drug levels at the steady-state but did not affect the initial rate of uptake ; the depressant effect of excess calcium was greater in the heart than in the diaphragm.

3. In both tissues uptake was reduced in the absence of oxygen, but the depressant effect of calcium was unaltered.

4. In the heart at pH 8.4 under anaerobic conditions, the initial rate of uptake and the levels of guanethidine at the steady-state were both increased, and the effects of 10 and 20 mM calcium were also greater.

5. In the diaphragm under these conditions, guanethidine uptake was slow, and steady-state conditions were not attained after 3 hr incubation; the effect of calcium was much less than in the heart.

6. The conclusion drawn from the data is that excess calcium reduced the uptake of guanethidine by an indirect action, possibly by releasing guanethidine from intracellular storage sites in adrenergic nerves.

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