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EFFECTS OF GUANETHIDINE ON ELECTRON TRANSPORT AND PROTON MOVEMENTS IN RAT HEART, BRAIN AND LIVER MITOCHONDRIA

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Abstract—Guanethidine at 5–25 mM concentrations was found to induce up to 79% inhibition of ADP-stimulated (state III) oxygen consumption in isolated rat heart, brain or liver mitochondria, when the added substrate was glutamate or succinate, but the inhibition was considerably lower (24% or less) when respiration was supported by ascorbate plus tetramethylphenylenediamine (TMPD). Comparable results were seen regarding ADP-stimulated proton uptake, where even greater inhibition (up to 94% with glutamate or succinate, but not ascorbate plus TMPD) was found. Similar but somewhat less marked effects were also seen in resting (state IV) respiration and on the acceptor control ratio (state III/state IV respiration). 2,4-Dinitrophenol was unable to relieve guanethidine-induced inhibition of electron transport. These results indicate that guanethidine inhibits primarily mitochondrial electron transport itself, and that the site where such inhibition is more marked is located in the span between ubiquinone and cytochrome *c* of the respiratory chain. It is, therefore, suggested that active guanethidine uptake by noradrenergic neurons can lead to a high drug concentration in their cytoplasm and hence to mitochondrial alterations that can contribute to the pharmacological effect of this drug. Our results demonstrate the interaction between guanethidine and the electron transport chain of mitochondria derived from different tissues and, therefore, support this hypothesis.

Guanethidine is known to specifically block stimulus-induced noradrenaline (NA)§ release from noradrenergic neurons by a mechanism that is independent of depletion of NA stores [1–4]. A number of findings suggest that guanethidine and other adrenergic neuron blockers have an intracellular site of action. Thus, their blocking effects on NA release can be prevented by cocaine, desipramine, *d*-amphetamine and other inhibitors of the NA uptake 1 system, present in the plasma membrane of noradrenergic neurons [5–10]. The active uptake of guanethidine, bethanidine, debrisoquine, guanoxan and bretylium into sympathetic neurons has also been demonstrated [9, 11–14], and, for guanethidine, evidence showing its distribution into both synaptic vesicles and an extravesicular compartment has been shown [3, 15, 16].

It has been postulated recently [17] that guanethidine and other adrenergic neuron blockers inhibit NA release because they activate a hyperpolarizing (i.e. inhibitory) calcium-activated potassium conductance in the plasma membrane of noradrenergic neurons. This would be due to calcium release from an intraneuronal reservoir, possibly mitochondria.

Morphological studies have shown that guanethidine can induce severe and early mitochondrial damage in sympathetic neurons [18, 19]. No biochemical studies on the effect of guanethidine on mitochondria derived from nervous tissue have been performed, and only a study of the effects of adrenergic neuron blockers on isolated liver mitochondria has been made [20]. To test whether the effects of guanethidine on mitochondrial function are of a general nature and, thus, applicable also to sympathetic neurons, we have now studied the effects of guanethidine on resting and on ADP-stimulated respiration and proton translocation in mitochondria derived from rat brain, heart and liver, using substrates that donate electrons to different sites of the respiratory chain.

MATERIALS AND METHODS

Preparation of mitochondria. Female Wistar rats weighing about 300 g, and fasted overnight, were used throughout. Heart mitochondria were prepared exactly as described by Vercesi *et al.* [21]. Liver mitochondria were obtained by a modification of the procedure of Schneider and Hogeboom [22], as described by Gil *et al.* [23]. Brain mitochondria were purified by the following procedure. Rats were killed by decapitation, and their brains were removed, cleaned of meningeal membranes, and washed two to three times with cold homogenization medium [0.3 M mannitol; 1 mM ethyleneglycol-bis (β-aminoethylether) *N,N'*-tetraacetic acid (EGTA); 5 mM Tris–HCl pH 7.4; and 1 mg/ml of bovine serum albu-

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§ Abbreviations: NA, noradrenaline; DNP, dinitrophenol; TMPD, tetramethylphenylenediamine; and HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid.

min]. The cerebral cortex was freed from white matter, as far as possible, and cut into small pieces. These were weighed, washed several times with cold homogenization medium, and then homogenized gently (three up and down strokes at about 80 rpm) with 9 vol. of the same solution in a loosely fitting Teflon–glass homogenizer. The homogenate was centrifuged at 600 *g* for 10 min in a Sorvall RC2-B centrifuge, to remove nuclei and unbroken cells. The supernatant fraction was then centrifuged at 10,000 *g* for 10 min. The resulting supernatant fraction as well as the pellet white fluffy upper layer were carefully aspirated and discarded. The brownish pellet was then resuspended in one-third of the original volume of homogenization medium, and centrifuged again at 10,000 *g* for 10 min. The crude mitochondrial pellet was then resuspended in one-sixth of the original volume of homogenization medium and placed on top of a discontinuous Ficoll gradient, with 5-ml layers of 7.5, 10, 15 and 20% (w/v) Ficoll in homogenization solution, preformed in a 34-ml centrifuge tube. This was then centrifuged in an SW 25.1 swinging bucket rotor in a Beckman L2-65B ultracentrifuge at 25,000 rpm for 25 min. The pellet obtained was resuspended in 0.3 M mannitol, pH 7.4, and washed by centrifuging at 10,000 *g* for 10 min. This latter pellet was resuspended and centrifuged again under the same conditions. Finally, the mitochondrial pellet was resuspended in the same medium at a protein concentration of 50 mg/ml. Protein was measured according to Lowry *et al.* [24]. The entire procedure was carried out at 0–5°.

Measurement of oxygen consumption and H^+ movements. Oxygen uptake was measured polarographically by means of a Clark electrode, and H^+ movements were measured simultaneously with oxygen consumption, by means of a pH-sensitive combination electrode as described by Ferreira and Gil [25]. The 2-ml reaction medium had the following composition: (a) Heart mitochondria [21]: 130 mM KCl, 3 mM HEPES buffer; 2 mM KH_2PO_4, with the pH of the mixture adjusted to pH 7.2 with 0.1 N KOH; and 1.5 to 3 mg mitochondria protein. (b) Liver mitochondria [26]: 117 mM LiCl; 13 mM KCl; 3 mM HEPES buffer; 2 mM KH_2PO_4, pH adjusted to 7.2 with 0.1 N KOH, and 2.5 to 5 mg mitochondrial protein. (c) Brain mitochondria: 75 mM KCl; 3 mM HEPES buffer; 2 mM KH_2PO_4, with pH adjusted to 7.4 with 0.1 N KOH; 0.5 mg/ml of beef serum albumin; and 2.5 to 5 mg mitochondrial protein. The substrates added were: 2 mM glutamate; 2.5 mM succinate or 5 mM ascorbate plus 0.5 mM TMPD. When indicated 200–400 nmoles of ADP was added as phosphate acceptor. The incubation mixture was equilibrated with mitochondria at 25° for 5 min, and then the rates of oxygen consumption and H^+ movements were measured. When guanethidine was added, the pH was readjusted with 0.1 N KOH. Each experiment was performed three to five times.

Materials. Guanethidine sulfate was a gift of CIBA-Geigy. All other reagents were obtained from the Sigma Chemical Co.

RESULTS

In heart mitochondria, guanethidine induced marked changes in mitochondrial function when glutamate or succinate was used as substrate, but lesser ones when respiration was supported by ascorbate plus TMPD. Thus, in the presence of glutamate (Fig. 1, a and b), guanethidine inhibited resting (state IV) respiration, and, especially, ADP-stimulated (state III) respiration and ADP-induced medium alkalinization. Similar results were seen when succinate was used as substrate (Fig. 1, c and d), while, in the presence of ascorbate plus TMPD, all these drug effects were much attenuated (Fig. 1, e and f).

When the effects of different guanethidine concentrations were studied (Fig. 2), it was seen that ADP-stimulated (state III) H^+ uptake (i.e. medium alkalinization) was the mitochondrial function most sensitive to the drug, where up to 94% inhibition was attained (Fig. 2b), and that also large (up to

Table 1. Effect of guanethidine on dinitrophenol-stimulated respiration of heart mitochondria*

Substrate and additions	Oxygen consumption (ng atoms/mg protein/min)	% Inhibition
Glutamate	27.1	
Glutamate + Gua	20.8	23
Glutamate + DNP	131.7	
Glutamate + DNP + Gua	37.5	71.5
Succinate	58.8	
Succinate + Gua	19.7	
Succinate + DNP	160.4	
Succinate + DNP + Gua	21.4	86.6
Ascorbate + TMPD	96.0	
Ascorbate + TMPD + Gua	95.0	
Ascorbate + TMPD + DNP	133.0	
Ascorbate + TMPD + DNP + Gua	96.0	27.8

* The test system contained, in a 2-ml volume, 130 mM KCl, 3 mM HEPES buffer, 2 mM KH_2PO_4 (with pH of the mixture adjusted to 7.2 with 0.1 N KOH), 3 mg of mitochondrial protein, and the substrates: 2 mM glutamate, 2.5 mM succinate, or 5 mM ascorbate plus 0.5 mM tetramethylphenylenediamine (TMPD). When present, guanethidine (Gua) was 25 mM, and 2,4-dinitrophenol (DNP) 0.05 mM.

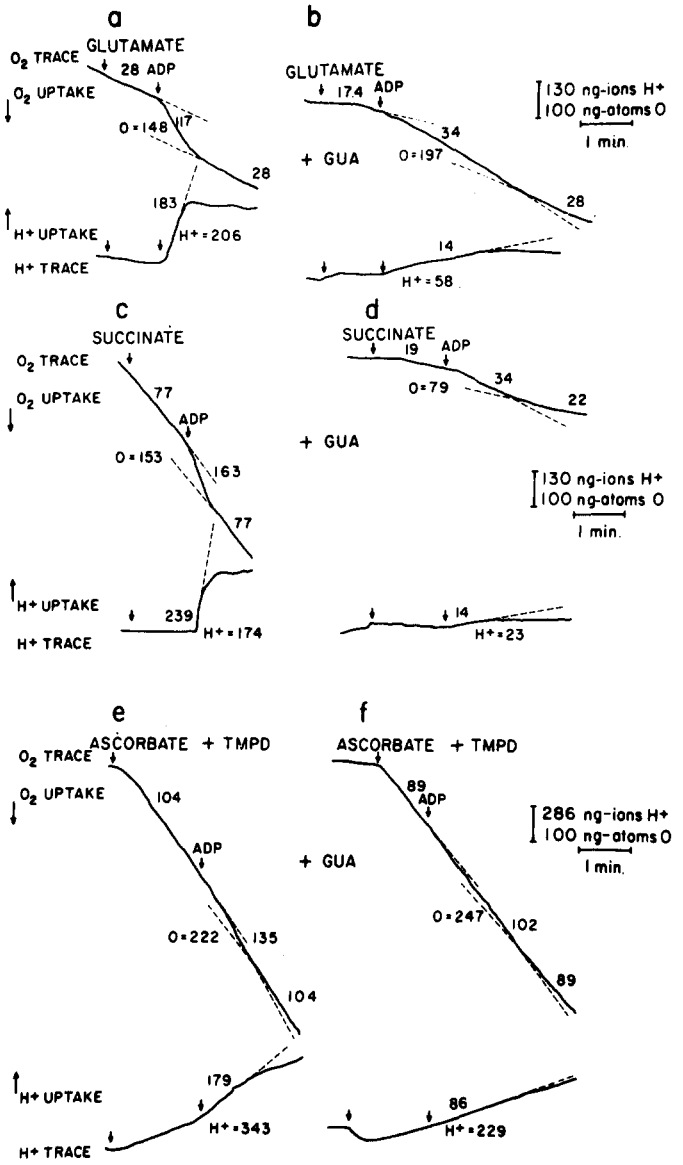

Fig. 1. Effects of guanethidine on the rates of oxygen and H^+ uptake for glutamate, succinate or ascorbate oxidation in rat heart mitochondria. In a and b, 2 mM glutamate was used as substrate; in c and d, 2.5 mM succinate; and in e and f, 5 mM ascorbate plus 0.5 mM tetramethylphenylenediamine (TMPD). In b, d and f, 25 mM guanethidine was also present from the beginning. The upper trace represents the oxygen concentration in the medium, and the numbers placed at intervals above such curve indicate the rate of oxygen uptake in ng atoms/mg protein/min. The numbers close to the symbol O (e.g. O = 148 in a) indicate the extra oxygen consumption (in ng atoms) induced by addition of ADP. The time of addition of ADP (400 nmoles in a, b; 300 in c, d; and 200 in e, f) is indicated by arrows. The lower curves represent the H^+ trace. The numbers placed above the curve indicate the rate of H^+ uptake (ng ion/mg protein/min) following the addition of ADP, while those below the curves (e.g. H^+ = 206 in a) represent the ADP-induced H^+ uptake (in ng atoms). Oxygen, H^+ and time calibrations are placed at the right of each pair of figures. In each experiment oxygen uptake and H^+ changes were measured simultaneously. For other details, see Methods.

79%) inhibitions in state III respiration could be induced (Fig. 2a), while the acceptor control ratio (state III/state IV respiration) was the least sensitive parameter, and the only one in which differences, regarding drug sensitivity, between glutamate and succinate could be seen (Fig. 2c). In all cases, much lower inhibition was seen when the substrate was ascorbate plus TMPD.

DNP-stimulated respiration (Table 1), was also greatly inhibited by 25 mM guanethidine. As in other cases, the inhibition attained depended on which substrate was used.

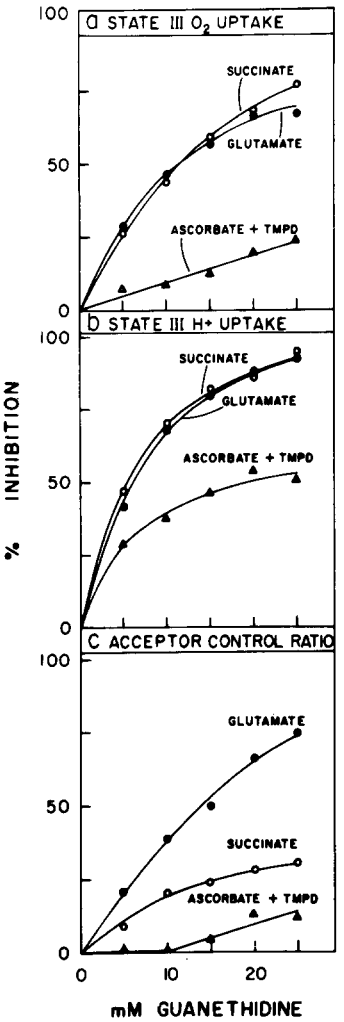

Fig. 2. Influence of guanethidine concentration on inhibition of ADP-stimulated oxygen and H^+ uptakes, and acceptor control ratio in rat heart mitochondria. The inhibitory effects of different guanethidine (free base) concentrations of ADP-stimulated (state III) oxygen uptake (a), and H^+ uptake (b), and on the acceptor control (state III/state IV respiration) ratio (c) were obtained from experiments like those of Fig. 1, in which the drug concentration varied betweem 5 and 25 mM. Each point is the mean of three to five independent experiments. The substrates used are indicated close to each curve. Acceptor control ratios in the absence of guanethidine were 4.2, 2.1 and 1.3 for the oxidation of glutamate, succinate and ascorbate + TMPD respectively. Other conditions were as in Fig. 1.

A comparison of guanethidine effects on mitochondria obtained from rat heart, brain or liver (Fig. 3) indicated that, aside from a small difference in sensitivity of acceptor control ratio of liver mitochondria, drug effects were essentially identical on mitochondria obtained from these different sources.

DISCUSSION

The finding that guanethidine predominantly inhibited mitochondrial electron transport when respiration was supported by glutamate or succinate, but much less when the substrate was ascorbate, indicates that the predominant site of action of guanethidine, at least at the concentrations used here, must be located somewhere between ubiquinone and cytochrome *c*. The fact that DNP did not relieve the guanethidine-induced inhibition of oxygen consumption also indicates that the primary effect of guanethidine is on the electron transport chain, *per se*, and not a secondary consequence of inhibition of oxidative phosphorylation, as is the case with oligomycin. These effects of guanethidine are different from those of guanidine or its derivatives, that predominantly block the NAD-ubiquinone span of the respiratory chain, and also inhibit the F_0F_1-ATPase [27–29].

The present results confirm, in a general manner, those of Malmquist and Oates [20], obtained in liver mitochondria, and extend them to mitochondria obtained from other tissues. However, in order to inhibit electron transport we have used higher guanethidine concentrations. Thus, these authors reported that the IC_{50} for the effect of guanethidine on state III respiration is 0.9 mM, while we found it to be 10 mM. Furthermore, they localized the predominant site of drug action at phosphorylation site I, while in the present work inhibition was suggested to occur between ubiquinone and cytochrome *c*. These differences can possibly be explained by the large differences in experimental conditions between both groups, i.e. the previous report studied the effect of much lower drug concentrations, that could be observed only after much longer latency; also incubation media and ADP concentration differed markedly in both studies.

ADP-stimulated mitochondrial H^+ uptake is known to occur through the F_0 F_1-ATPase, and the driving force for it is both the inwardly directed H^+ gradient and the electrical potential difference across the inner mitochondrial membrane (inside negative). Both the H^+ and the electrical potential gradients are generated by mitochondrial electron transport. The finding that guanethidine markedly affects ADP-stimulated H^+ uptake is an indirect indication that the drug has decreased the driving force for such uptake (i.e. the H^+ and/or electrical potential gradients), probably by interfering with electron transport.

Since guanethidine affects similarly mitochondria obtained from different tissues, including those derived from nervous tissue, it seems plausible to think that mitochondria present in sympathetic axons may be similarly affected. Under *in vivo* conditions, however, guanethidine and other sympathetic neuron blockers are actively transported into sympathetic neurons [11–13] through the NA uptake-1 system [5–9], and millimolar drug concentrations are attained in the cytoplasm of these neurons [30]. This shall selectively inhibit the mitochondria of these cells, particularly in the axonal varicosities, where a high surface to volume ratio exists, relative to the cell soma. Such selective inhibition, as shown in this paper, is not due to a special sensitivity of mitochondria in sympathetic neurons but, rather, to the high intracellular drug concentrations reached in this neuronal type. The present findings, therefore, are

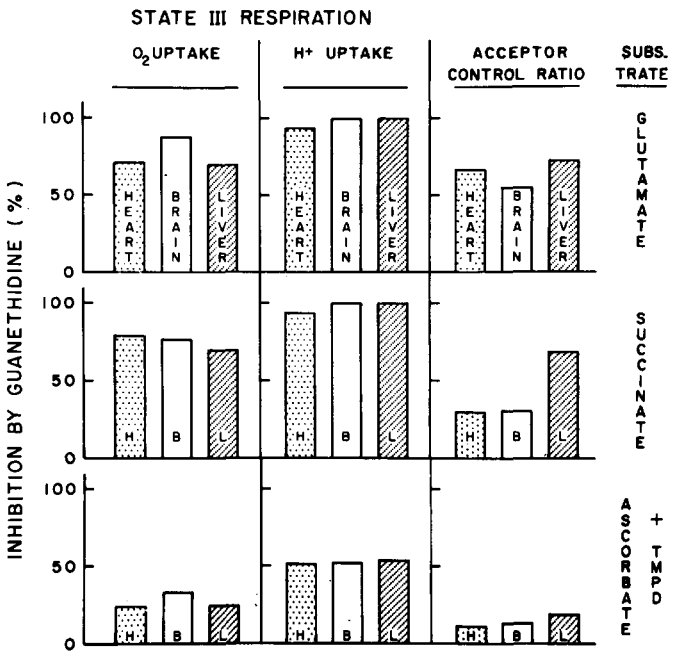

Fig. 3. Inhibitory effects of guanethidine on heart, brain and liver mitochondria. The inhibitory effects of 25 mM guanethidine on state III oxygen and H^+ uptake, and on acceptor control ratio in mitochondria obtained from rat heart, brain or liver are compared. The substrate used is indicated on the right-hand side of the figure. Results are from five experiments with heart and three each with brain or liver mitochondria. With brain mitochondria, control state III respiration (in ng atoms 0/mg protein/min) was 60.4 with glutamate, 80.5 with succinate, and 137.5 with ascorbate plus TMPD. State III H^+ uptake (ng ion H^+/mg protein/min) was 99.7, 99.2 and 63.2 for glutamate, succinate, and ascorbate plus TMPD, respectively, while the acceptor control ratio in the absence of guanethidine was 2.3, 1.6 and 1.55 for glutamate, succinate, and ascorbate plus TMPD. Liver mitochondria had the following control values: state III oxygen uptake (ng atom/mg protein/min): 83.9 (glutamate), 124.9 (succinate) and 221.8 (ascorbate plus TMPD); state III H^+ uptake (ng ion/mg protein/min): 136.2, 148.6 and 266.4 for glutamate, succinate, and ascorbate plus TMPD respectively. The acceptor control ratio for the same substrates was 3.73, 3.2 and 1.3 respectively.

compatible with the hypothesis of an intraneuronal, extravesicular site of action for guanethidine [16, 17].

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