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EFFECTS OF ADRENERGIC NEURON-BLOCKING GUANIDINE DERIVATIVES ON MITOCHONDRIAL METABOLISM*

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Abstract—The effect of four adrenergic neuron-blocking drugs, guanethidine, bethanidine, debrisoquin and guanoxan, on mitochondrial metabolism has been investigated. These drugs were found to inhibit oxidative phosphorylation, in accord with data available on various other guanidine derivatives. The possible significance of this drug effect for the blockade *in vivo* of adrenergic neurons is discussed.

GUANETHIDINE and the related group of guanidines which block adrenergic neurons are believed to interfere with the process whereby the action potential causes release of norepinephrine (NE) from the adrenergic nerve terminals. Most of these drugs also cause some degree of depletion of NE from the nerve-ending stores. Since the degree of depletion varies considerably with different drugs and since the time course of depletion is different from that of blockade of transmitter release, the depletion is probably not an essential part of the blocking mechanism.

The first drug with this action to be studied in detail, including clinical use as an antihypertensive agent, was bretylium, a quarternary ammonium compound.[1, 2] However, the majority of adrenergic neuron blockers of present interest are guanidines with various cyclic substituents, the prototype being guanethidine.[3] (For a review of chemical structures and possible mechanisms of action, see Boura and Green.[4])

Since the original demonstration by Hollunger[5] that guanidine and the hypoglycemic drug Synthalin A (decamethylenediguanidine) are inhibitors of the energy transfer process in mitochondria, a number of guanidine derivatives, including the currently used hypoglycemic drug DBI (phenylethylbiguanide), have been studied in this regard.[6–13] With few exceptions, all these compounds have been shown to have the same influence on mitochondrial metabolism, although with considerable differences in potency. They also differ in their sites of action; each compound studied acts preferentially on only one of the three phosphorylation sites along the electron transport chain.[11]

A characteristic property of the adrenergic neuron-blocking drugs is that they are selectively taken up into these neurons, reaching high local concentrations.[4] If these agents were to block oxidative phosphorylation, this effect would be localized in

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adrenergic neurons. Accordingly, their action on oxidative phosphorylation in isolated mitochondria was investigated. In the present communication, data obtained with guanethidine, bethanidine, debrisoquin and guanoxan are presented. The structures of these agents are shown in Fig. 1.

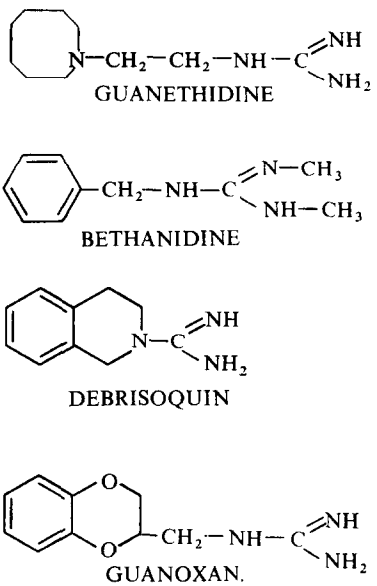

FIG. 1. Structure of the adrenergic neuron -blocking guanidines studied.

MATERIALS AND METHODS

Drugs and reagents. Guanethidine sulfate was obtained from CIBA Pharmaceutical Co., Summit, N.J.; bethanidine sulfate and bretylium *p*-toluenesulfonate from Burroughs Wellcome & Co., Inc., Tuckahoe, N.Y.; debrisoquin sulfate from Hoffmann-La Roche, Inc., Nutley, N.J.; and guanoxan sulfate from Charles Pfizer & Co., Inc., Groton, Conn. Synthalin A (decamethylenediguanidine dihydrochloride) was provided by Schering A. G., Berlin, Germany.

Mitochondrial substrates were purchased from Calbiochem, ADP from Sigma Chemical Co. and 2,4-dinitropenol (DNP) as well as guanidine hydrochloride from Eastman Organic Chemicals.

Rat liver mitochondria were prepared according to Schneider,[14] except that no attempt was made to recover those mitochondria that sedimented with the nuclear fraction. Homogenization and washing were done with 0·25 M sucrose–1 mM EDTA. The mitochondria were stored at 0° in mannitol–sucrose–Tris according to Chance and Hagihara,[15] omitting the EDTA. Experiments were concluded within 4 hr after obtaining the mitochondria.

Protein was determined according to Lowry *et al.*[16] with bovine serum albumin as standard.

Mitochondrial respiration was measured with an oxygen polarograph (Oxygraph, Gilson Medical Electronics). The incubation conditions were largely those of Pressman.[6] The medium contained sucrose, 250 mM; KCl, 25 mM; $MgCl_2$, 5 mM;

Tris-HCl, pH 7·4, 17 mM; Na phosphate, pH 7·4, 5 mM. NAD-linked respiration was studied with glutamate plus malate, 3 mM each, as substrates. Succinate was likewise used in 3 mM concentration. ADP was added to a final concentration of 1·5 mM. Total volume was 2·0 ml. The medium was air-saturated and its initial oxygen concentration was taken to be 240 μM.[17] The experiments were performed at room temperature, varying between 25° and 26°. The final concentration of mitochondrial protein was 0·4–0·8 mg/ml.

Phosphorylation was measured with the same incubation system, but in test tubes in a 25° water bath, with shaking. As with the respiration experiments (see Results) the complete reaction mixture, except ADP, was preincubated for 5 min before starting phosphorylation by addition of ADP. Ten min thereafter, a 1-ml aliquot was blown into 1 ml of ice-cold 5% (w/v) trichloroacetic acid. After the mixture was centrifuged in the cold, a 0·5-ml aliquot of the supernatant was taken for determination of inorganic phosphate according to Lowry and Lopez.[18] The extent of phosphorylation was obtained as the difference between the amount of inorganic phosphate present immediately after ADP addition (measured in separate control tubes) and that present after completed incubation. Additional controls showed that, as expected, only trace amounts of inorganic phosphate disappeared during the preincubation, whether a drug was present or not. All drug concentrations given are the final concentrations in the incubation systems.

RESULTS

In accord with results of previous investigators, it was found that the inhibition of oxygen uptake caused by guanidines had a gradual onset. Guanidine-induced inhibition was reversed by the addition of DNP, though slowly and incompletely. In contrast, the inhibition caused by Synthalin was unaffected by DNP.

The same pattern of inhibition was seen with the four adrenergic neuron-blocking guanidines tested. However, the very slow onset of inhibition obtained with the drug concentrations selected made it difficult to assess the inhibitory potency by this technique, as each experiment can only be run for a few minutes. Only with the most potent of the drugs, debrisoquin, could a marked inhibition with relatively rapid onset be observed (Fig. 2); this inhibition was partially reversed by DNP. Fig. 2 shows that when rapid respiration was started by the addition of DNP rather than ADP, the subsequent addition of debrisoquin did not inhibit oxygen uptake, confirming that, with the concentration used, this compound does not influence mitochondrial electron transport *per se*.

Quantitative data on inhibitory potency were obtained by a variation of the technique. The mitochondria were incubated in the polarograph reaction vessel for 5 min with the drug and with a complete medium except for ADP. Active (state 3) respiration was then started by addition of ADP. In this way, advantage could be taken of the fact that guanidine compounds establish their effect on the energy transfer process most effectively during the slow (state 4) respiration obtained in the absence of phosphate acceptor, as was noted and discussed by Chappell.[7] Not only did preincubation enhance the inhibitory effect of the drugs, but in addition, the oxygen uptake rate after ADP addition in most cases was constant throughout each experiment, making it easy to measure the degree of inhibition by comparison with separate

control experiments in which mitochondria were similarly preincubated in the absence of drug. (Since the drugs were used as sulfates, controls with suitable concentrations of sodium sulfate were included; no inhibition was observed.) In the few cases where the respiration rate showed a slight continuous decrease, the rate at 3 min after ADP addition was selected.

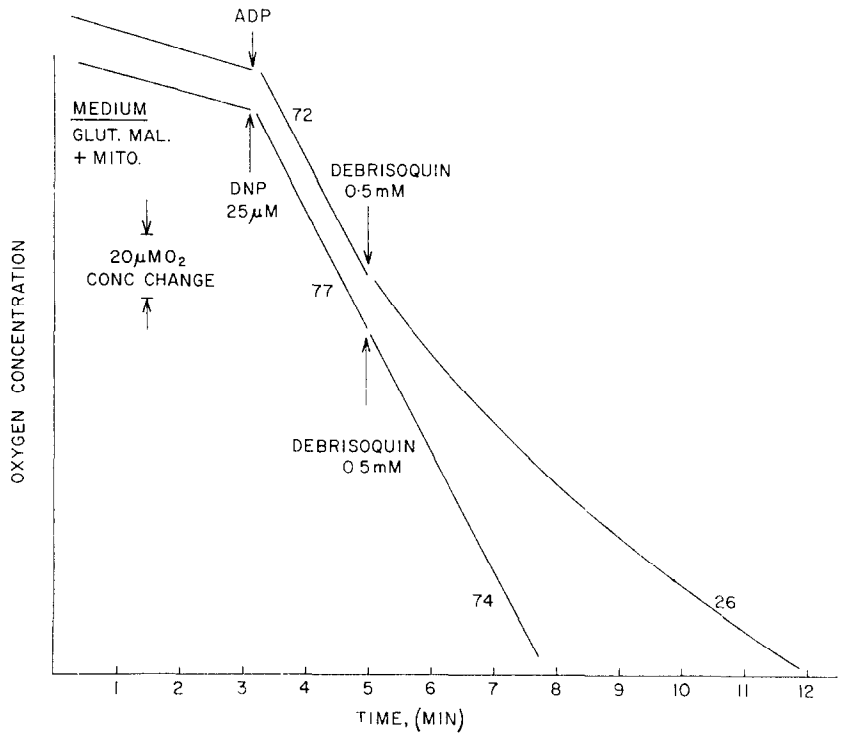

FIG. 2. Effect of debrisoquin on phosphorylating (upper tracing) and uncoupled (lower tracing) respiration. The figures at the tracings indicate respiration rate in mμmoles O_2 consumed per min.

The degree of inhibition was expressed as per cent reduction in state 3 respiration rate. The drugs as a rule had no effect on the slow state 4 rate; in a few cases a slight decrease was observed, whereas stimulation i.e. an uncoupling effect, never occurred. Since the state 3 rate was not corrected by subtraction of the state 4 rate (see Chance and Williams[17] for discussion), it follows that the calculated inhibition could not attain 100 per cent. Rather, maximal inhibition meant that the addition of ADP caused no acceleration of respiration. This was equivalent to 85–90 per cent inhibition, corresponding to a respiratory control index (ratio state 3 : state 4 rate) varying between 7 and 10 with different mitochondrial preparations.

A typical set of experiments with debrisoquin is shown in Fig. 3. Similar tracings were obtained with the three other drugs, but these were less potent. Fig. 4 shows the relationship between drug concentration and extent of respiratory inhibition. As can be seen, the semilogarithmic representation gives a relationship approximating a straight line with all compounds. The 50 per cent inhibitory concentrations derived from these lines are as follows: debrisoquin, 70 μM; guanoxan, 440 μM; guanethidine,

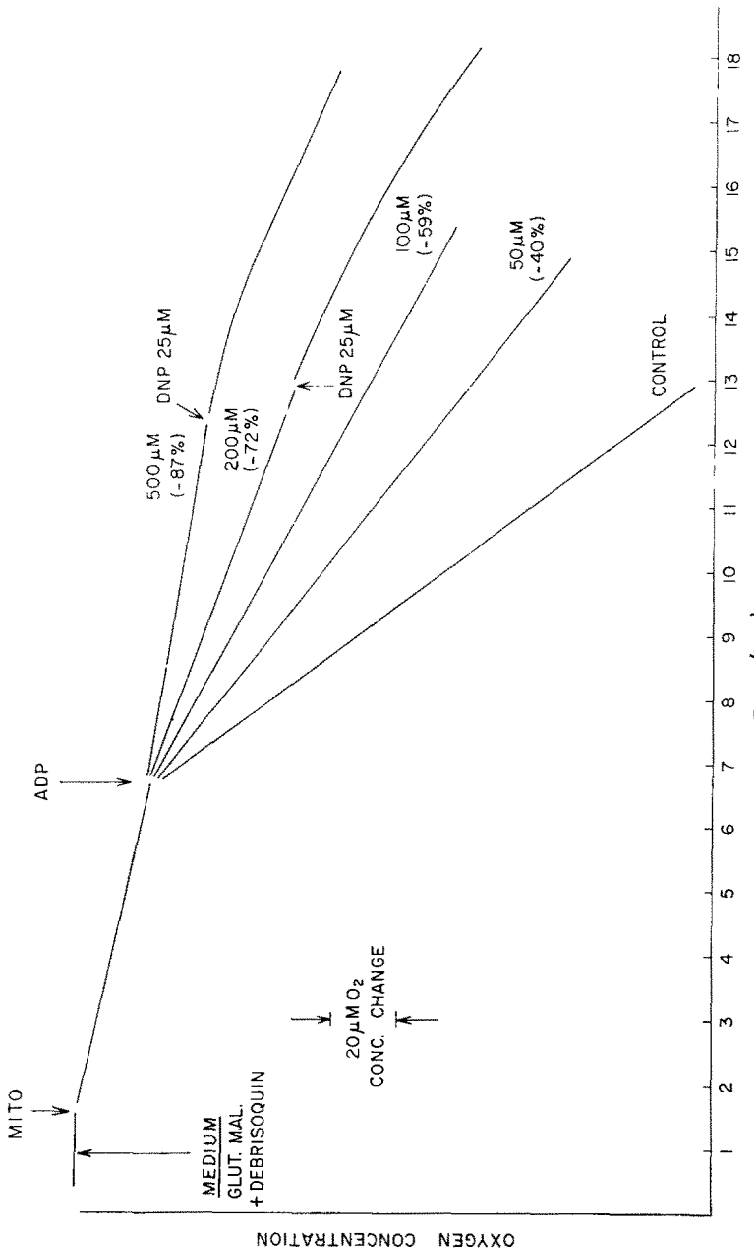

FIG. 3. Titration of effect of debrisoquin on phosphorylating respiration as observed after 5 min of preincubation of mitochondria with drug before ADP addition. Superimposed tracings from successive experiments with mitochondria obtained from the same preparation. Since the differences in respiration rate during the preincubation period were minimal, these parts of the tracings have been drawn together as a single line. Above each of the four debrisoquin curves, the drug concentration and the percentage inhibition of respiration have been indicated. In addition, the two uppermost curves show the partial reversal of the inhibition obtained by addition of DNP.

900 μM; bethanidine, 1·7 mM; and guanidine 2·2 mM. It is obvious that debrisoquin is much more potent than the other drugs; Synthalin, however, was even more potent, half-maximal inhibition being obtained at about 20 μM. The least potent drug was bethanidine, which did not significantly differ from guanidine. However, it is probable that the differences observed, to some extent, reflect differences in rate of penetration to the site of action; thus, the results obtained should not be taken as indicating true relative potencies as metabolic inhibitors. With all drugs, the addition of DNP (25 μM) resulted in a partial, sometimes nearly complete, reversal of the inhibition.

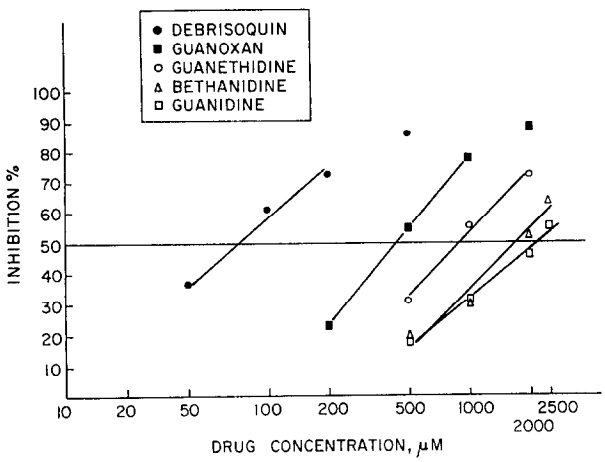

FIG. 4. Relationship between drug concentrations and extent of inhibition of mitochondrial oxygen uptake. Each point represents the average result of 2–6 experiments.

In spite of the necessity for using a large number of separate mitochondrial preparations, the reproducibility of the drug effects was satisfactory; the range of percentage inhibition values did not exceed $\pm$ 20 per cent of the means.

TABLE 1. EFFECT OF DRUGS ON SUCCINATE VS. GLUTAMATE-MALATE OXIDATION*

Drug	Concn (mM)	Inhibition (%)	
		Glutamate-malate	Succinate
Debrisoquin	0·1	59, 65, 59, 64, 63, 51, 67	28
Guanoxan	0·5	51, 53, 61	25
Guanethidine	1·0	51, 59	0
Bethanidine	2·0	45, 50, 61	9
Guanidine	2·0	40, 48, 49	22

* Figures represent inhibition of state 3 respiration obtained in individual experiments, expressed as per cent reduction from the respiration rate in the absence of drug. Mitochondria were preincubated with the drugs for 5 min, as described in text.

A limited number of experiments were performed with succinate as substrate. In all cases less inhibition of respiration was noted than with glutamate-malate (Table 1). The interpretation is made somewhat difficult by the well known fact that succinate oxidation is characterized by a less efficient respiratory control than is NAD-linked

respiration,[19] i.e. the state 4 rate is higher and the respiratory control index lower. However, it may tentatively be concluded that the drugs studied affect predominantly phosphorylation site I (NAD site), as with alkylguanidines, but in contrast to phenylethylbiguanide and Synthalin.[11]

Because bretylium was the forerunner of the guanidine-type of adrenergic blockers and seems to cause an essentially pure blockade of physiological transmitter release without concomitant NE depletion,[4] it was of interest to investigate that drug in the present system. It proved to have an effect similar to that of the other drugs, but differed in that its effect was established even more slowly, giving a progressively declining state 3 rate after the usual preincubation. In a typical experiment, 2·5 mM bretylium resulted in an inhibition increasing from 34 to 50 per cent during 7 min of state 3 respiration (control experiments showed that the *p*-toluenesulfonate ion was without effect). Longer preincubation periods gave higher degrees of inhibition. These characteristics of brethylium made comparison with the other drugs difficult and further studies with bretylium were not undertaken.

The phosphorylation experiments were included in order to establish the drug effects by an additional method. It is apparent from Table 2 that, as expected, the

TABLE 2. EFFECT OF DRUGS ON DISAPPEARANCE OF INORGANIC PHOSPHATE DURING OXIDATIVE PHOSPHORYLATION*

Drug	Concn (mM)	P_i consumed (μmoles)	Inhibition (% of P_i consumption	P value (difference from control)	No. of experiments
Control		2·56			10
Debrisoquin	0·1	0·90	65	$< 0·05$	6
Guanoxan	0·5	0·78	70	$< 0·05$	5
Guanethidine	1·0	0·76	70	$< 0·05$	5
Bethanidine	2·0	0·72	72	$< 0·05$	5
Guanidine	2·0	0·80	69	$< 0·05$	6

* Glutamate and malate were used as substrates for mitochondrial oxidation. For other experimental details, see Methods. Drug effect is expressed as average percentage reduction in the amount of inorganic phosphate disappearing during incubation.

inhibitory potency of the agents in these experiments parallels their efficiency as inhibitors of phosphorylating respiration. Inhibition of the incorporation of inorganic phosphate by all of these compounds was significant at the 0·5 per cent level of probability. The fact that the efficiency of inhibition seems somewhat higher in the phosphorylation experiments is obviously related to the pre-ADP incubation period where, as mentioned above, a drug-resistant respiration occurred, whereas the disappearance of inorganic phosphate was insignificant or nil during the preincubation.

DISCUSSION

Because some degree of energy transfer inhibition has been observed with all previously studied nonacidic guanidine derivatives, it was not surprising to find inhibition by the present series of guanidines. For any consideration of a possible relationship between this biochemical effect and the actions *in vivo*, the selective uptake of these drugs into adrenergic neurons is highly relevant. Detailed tissue

distribution studies, including nervous tissue, have been reported only with bretylium[20] and bethanidine.[21] In cats, administration of single doses well below the toxic range resulted in concentrations in adrenergic ganglia of 13–66 μM (per liter of tissue water) with bethanidine and 290–990 μM with bretylium.[21] Drug concentrations in adrenergic nerves were similar to those of ganglia with bethanidine, but somewhat lower with bretylium. For guanethidine, comparable data are not available, but gross tissue distribution studies suggest that this drug likewise localizes extensively in adrenergic nervous tissue.[22] Additional studies on guanethidine have demonstrated a mechanism for active specific uptake of the drug into tissue with rich adrenergic innervation, e.g. heart. Drugs such as desipramine and amphetamine, which prevent the uptake of guanethidine, will antagonize the neuron blockade produced by guanethidine; this is further evidence that concentration of the drug within adrenergic neurons is essential for its selective blocking action.[22–25] The mechanism responsible for the uptake of guanethidine may be identical to the catecholamine "pump" that exists in adrenergic nerve endings.

For obvious reasons, data on drug concentrations in the actual nerve terminals are not available, but this part of the neuron probably accumulates drug concentrations that are at least as high as those found in sympathetic ganglion cells and their axons.[4]

The highly selective localization of the adrenergic neuron blockers in their target structures prompts a consideration of the possibility that their biochemical mechanisms of action may completely lack tissue specificity, and that the specificity of action *in vivo* may result only from the drug distribution; concentrations of drug in cells other than adrenergic neurons remain too low to cause any significant biochemical effects.[21, 26, 27] The absence of tissue specificity is illustrated by the fact that doses exceeding those required for adrenergic neuron blockade will also interfere with transmission at the cholinergic neuromuscular junction (reviewed by Boura and Green[4]).

At present, it is possible only to speculate on the nature of this general action which produces neuron blockade. Because topical application of these drugs to adrenergic neurons will block conduction, they have been assigned a local anesthetic action.[4] Although blockade of conduction does grossly resemble the action of local anesthetics, there is no specific information which indicates that these drugs produce the same characteristic electrophysiologic effect as local anesthetics or even that the effect is primarily on the neuron membrane. In any case, a primary effect of these drugs on neuron membranes is one mechanism which could account for neuron blockade.

Alternatively, blockade of neurotransmitter release could result from impaired mitochondrial energy metabolism within the neuron terminal. Heretofore, in consideration of adrenergic neuron function, the mitochondria present in neuron terminals have received attention primarily in relation to monoamine oxidase, which is known to be localized predominantly in these organelles. This enzyme is inhibited by several of the adrenergic neuron blockers.[28, 29] However, the main function of mitochondria, the synthesis of high energy phosphate, can hardly be irrelevant for the normal function of the adrenergic neuron and its neuro-effector junctions.

The ATP requirement for maintenance of the ion transport mechanisms that ensure a normal membrane potential is well known.[30] It may become a limiting requirement when the nerve is depolarized repeatedly, demanding an accelerated sodium transport to restore the ion gradients. Greengard[31] emphasizes the great

differences between myelinated and nonmyelineated nerve fibers, the latter being much more prone to deplete their stores of high energy phosphate (ATP and creatine phosphate) during electrical stimulation. In addition to their lack of an isolating myelin sheath, nonmyelinated fibers tend to have a smaller diameter, giving a higher surface : volume ratio. The latter factor is relevant in that it causes larger ion concentration changes within the nerve for each action potential.

Specific information regarding the requirement for metabolic energy for the function of synaptic and neuro-effector junctions is largely lacking. Eccles[32] points out that the conspicious presence of mitochondria in nerve terminals indicates a high level of metabolic activity in these structures. Neuron terminals can be expected to have a higher energy requirements than axons, not only because they are highly branched delicate filaments, but also because of their specialized functions of uptake and storage of neurotransmitter.

A significant inhibition of oxidative phosphorylation within a neuron would reduce ATP production markedly, in that little ATP is likely to be formed by alternate pathways. Glycolysis is an insignificant energy source in nervous tissue, accounting for less than 3 per cent of all ATP production in brain.[33] Likewise, the substrate level phosphorylation associated with the oxidation of α-ketoglutarate can hardly be a major energy source, particularly if, as seems likely, the Krebs cycle transformations are impaired as a consequence of the inhibition of the terminal oxidation process. Most processes are qualitatively similar in the mitochondria of neurons and liver cells; a quantitative comparison of the observed inhibition in liver mitochondria with that in mitochondria from neurons will be of interest, however.

Alteration of neuron function by an inhibitor of ATP production has been demonstrated by Beani *et al.*,[34] who produced neuromuscular block in rat and guinea pig diaphragm preparations with DNP. The block resulted from a subnormal acetylcholine release on nerve stimulation; concomitantly, a reduction of the stores of ATP and acetylcholine was observed.

Because adrenergic neuron-blocking drugs are concentrated within their target organs, it is conceivable that local levels *in vivo* may be sufficient to inhibit oxidative phosphorylation. Although evidence linking the observed inhibition of oxidative phosphorylation *in vitro* with blockade of adrenergic transmission *in vivo* is currently lacking, the possibility of such a relationship clearly warrants further investigation.

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