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EFFECT OF BARBITURATES ON THE CALCIUM-INDUCED LOSS OF RESPIRATORY CONTROL IN BEEF HEART MITOCHONDRIA*

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Abstract—The addition of barbiturates (pentobarbital, Amytal, thiopental or thiamylal) to beef heart mitochondria that accumulated Ca^{2+} resulted in the loss of respiratory control. The effect of barbiturates was dependent on Ca^{2+} uptake, as the respiratory control was not lost when barbiturates were added after Sr^{2+} uptake. Barbiturates potentiated the calcium-induced configurational change of mitochondria (aggregated to orthodox) as determined by light-scattering measurements. These results indicate that barbiturates potentiate the calcium-induced membrane transition, which involves a calcium specific induction of non-specific permeability of the mitochondrial inner membrane to small molecules and ions [D.R. Hunter, R. A. Haworth and J. H. Southard, *J. biol. Chem.* **251**, 5069 (1976)].

The effects of barbiturates on the functions of mitochondria have been studied by a number of investigators. Aldridge and Parker [1] showed that both oxy- and thiobarbiturates inhibit respiration while only thiobarbiturates uncouple oxidative phosphorylation. Chance and Hollunger [2] demonstrated that Amytal and thiopental, respectively, induced increased reduction and oxidation of pyridine nucleotides. More recently, Weiss *et al.* [3] showed Amytal- or pentobarbital-induced reduction of pyridine nucleotides in perfused rat heart. Little is known, however, about the effects of barbiturates on the uptake and release of Ca^{2+} by mitochondria. In preliminary experiments, we found that barbiturates are not specific inhibitors of Ca^{2+} uptake as determined by their effect on calcium-stimulated respiration of beef heart mitochondria. On the other hand, we noted that respiratory control was lost when a barbiturate was added to mitochondria that accumulated Ca^{2+}. The characterization of this phenomenon is the subject of this communication.

MATERIALS AND METHODS

Heavy beef heart mitochondria were isolated according to the method of Hatefi and Lester [4]. As isolated, over 90 per cent of the heavy beef heart mitochondria are in the aggregated configuration [5]. Respiration was measured polarographically using a Beckman oxygen analyzer at 30°. The basal reaction mixture (4 ml) contained 250 mM sucrose; 10 mM Tris–HCl (pH 7.4), 2.5 μM rotenone; 12.5 mM succinate and 1.25 mg of mitochondrial protein per ml. With fourteen preparations of heavy beef heart mitochondria, the rates of respiration in the presence and in the absence of an uncoupler (1 μM mClCCP)§ were, respectively, 0.34 ± 0.04 and 0.08 ± 0.01 μatoms[O]/min·mg (respiratory control ratio, 4.4 ± 0.5). Protein concentration was determined by the biuret method [6]. Light scattering of a mitochondrial suspension was measured as described by Hunter and Haworth [7]. The measurement of calcium-induced permeability of the mitochondrial inner membrane (calcium-induced transition) under non-energized conditions was carried out as described elsewhere [8]. The method involves the measurement of the rate of shrinkage of hypotonically swollen mitochondria by polyethylene glycol (mol. wt 1500), the rate of shrinkage being dependent on the permeability of the inner membrane to low molecular weight solutes trapped in the matrix space (see Ref. 8 for detail). Pentobarbital (sodium salt) and thiopental (sodium salt) were obtained from Abbott Laboratories, North Chicago, IL, and Amytal (amobarbital, sodium salt) and thiamylal (sodium salt) were obtained from Eli Lilly, Indianapolis, IN, and Parke-Davis, Detroit, MI, respectively.

RESULTS AND DISCUSSION

The addition of Ca^{2+} to an aerobic suspension of mitochondria in the presence of a substrate results in the stimulation of respiration concomitant with the uptake of Ca^{2+} [9]. The rate of respiration slows down (respiratory control) as the uptake of Ca^{2+} declines. When a barbiturate was added at this point, the rate of respiration gradually increased, and with sufficiently high concentrations of barbiturates

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§ *m*ClCCP: carbonylcyanide *m*-chlorophenylhydrazone.

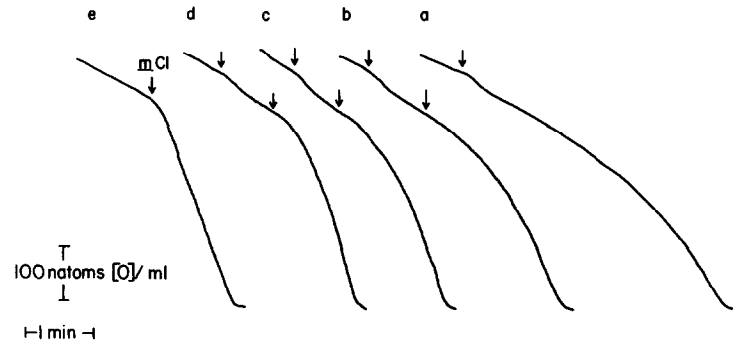

Fig. 1. Effect of Amytal on the calcium-induced loss or respiratory control. Mitochondria were incubated with succinate in the presence of rotenone. In trace a, the arrow indicates the addition of $CaCl_2$ (250 μM), and in traces b–d, the first arrow indicates the addition of $CaCl_2$ (250 μM) and the second, the addition of Amytal at the following concentrations: (b) 0.5 mM; (c) 1 mM; and (d) 2 mM. In trace e, the arrow indicates the addition of *m*ClCCP (1 μM).

reached a maximum rate comparable to that obtained in the presence of an uncoupler. Figure 1 shows the effect of Amytal on the time course of respiration after the Ca^{2+} uptake. The characteristic effect of Amytal was seen more clearly when the rates of respiration were plotted against time (Fig. 2). Similar results were obtained with other barbiturates; an effect comparable to 2 mM Amytal was obtained with 2 mM pentobarbital*, 0.25 mM thiopental or 0.25 mM thiamylal. The effect of barbiturates was specific for Ca^{2+}, as the respiratory control was not lost when a barbiturate was added to mitochondria after Sr^{2+} uptake. A concentration (4 mM) of pentobarbital twice as high as that which showed a maximum effect after Ca^{2+} uptake did not induce the loss of respiratory control after Sr^{2+} uptake (Fig. 3). Similar results were obtained when Amytal, thiamylal, or thiopental was used instead of pentobarbital.

The time-dependent loss of respiratory control after Ca^{2+} uptake and the inability of Sr^{2+} to replace Ca^{2+} strongly suggest that the barbiturates potentiated the calcium-induced membrane transition reported by Hunter *et al.* [5]. An alternative possibility is that the loss of respiratory control was due to specific Ca^{2+} cycling as in the case of the release of respiratory control caused by the addition of the divalent cation ionophore A23187 [10]. In this case, respiratory control should be restored by inhibiting Ca^{2+} uptake with ruthenium red or lanthanum [11, 12]. If the loss of respiratory control is due to the calcium-induced transition, ruthenium red or lanthanum should not restore respiratory control. As can be seen in Fig. 4, a concentration of ruthenium red which was sufficient to inhibit Ca^{2+} uptake completely when added prior to

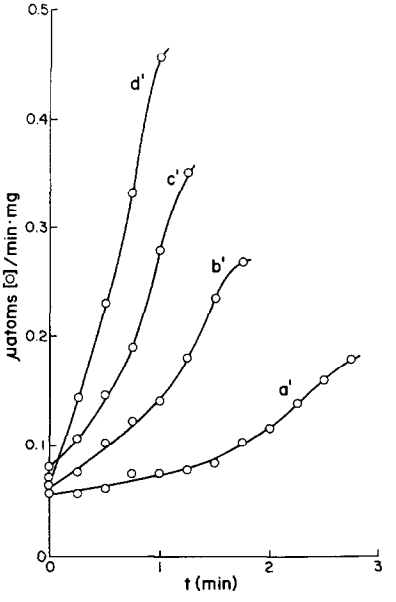

Fig. 2. Effect of Amytal on the time course of the increase in the rate of respiration after Ca^{2+} uptake. The plots were obtained from the slopes of the traces in Fig. 1. Curves a'–d' correspond to the traces a–d in Fig. 1, and $t = 0$ in Fig. 2 corresponds to the time of Amytal addition or, in the case of the curve a', 45 sec after the Ca^{2+} addition.

Ca^{2+} addition was not effective in preventing or reversing the loss of respiratory control when added after Ca^{2+} uptake had taken place.

It has been shown by Hunter and Haworth [7] that light-scattering changes of a mitochondrial suspension accurately reflect the aggregated-to-orthodox configurational change. Figure 5 shows that the addition of pentobarbital (2 mM) resulted in a 3-fold increase in the rate of the light-scattering change of a mitochondrial suspension following Ca^{2+} uptake. Similar results were obtained with the other barbiturates used in this study. The plasma concentration of pentobarbital can be as high as 0.3 mM when the drug is used to limit the deleterious effect of

* Pentobarbital was supplied as a solution (0.2 M) in 40% propylene glycol plus 10% ethanol. In a control experiment, up to 0.8% propylene glycol plus 0.2% ethanol (concentrations that were attained when 4 mM pentobarbital was added) had no effect on the calcium-induced loss of respiratory control.

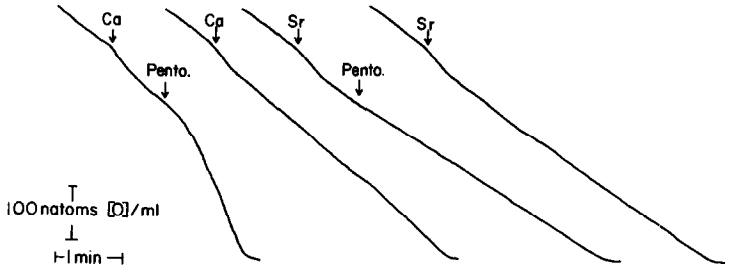

Fig. 3. Inability of Sr^{2+} to replace Ca^{2+}. The concentration of Ca^{2+} and Sr^{2+} was 250 μM. Pento = pentobarbital (4 mM).

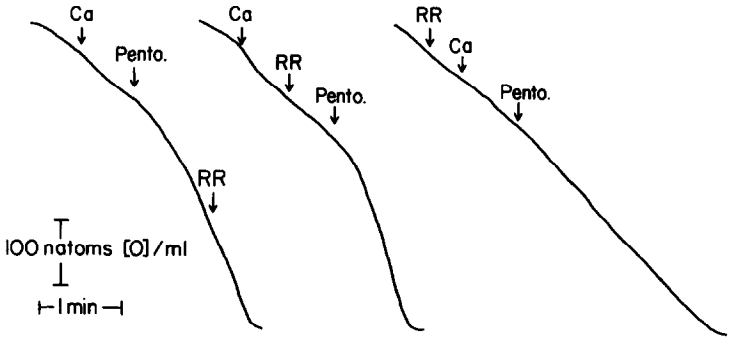

Fig. 4. Effect of ruthenium red on the calcium-induced loss of respiratory control. Key: Ca, $CaCl_2$ (250 μM); RR, ruthenium red (5 μM); and Pento, pentobarbital (2 mM).

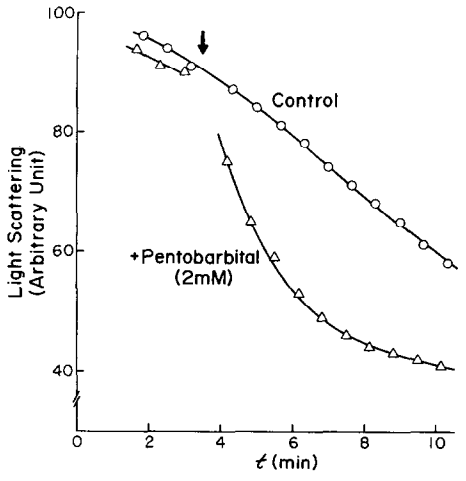

Fig. 5. Effect of pentobarbital on calcium-induced light-scattering change. The reaction mixture (3 ml) contained 250 mM sucrose, 10 mM Tris–HCl (pH 7.4), 3.3 μM rotenone, 1.67 mM succinate and 0.5 mg of mitochondrial protein per ml. The concentrations of mitochondria and Ca^{2+} lower than those used in the respiration assay were used to allow an accurate determination of the rate of light-scattering changes. At $t = 0$, $CaCl_2$ (50 μM) was added, and after 3.5 min (arrow) pentobarbital (2 mM) was added to one of the two cuvettes while the other served as the control.

cerebral ischemia [13]. For a myocardial depressant effect, Dresel *et al.* [14] showed that the concentration of pentobarbital required for half-maximal inhibition of isometric contraction of cat papillary muscle was about 0.2 mM, while from the data of Weiss *et al.* [3] it can be seen that about 1 mM pentobarbital or Amytal was required for the half-maximal inhibition of left ventricular pressure or heart rate of perfused rat heart. Thus the concentrations of barbiturates shown in this study to potentiate the calcium-induced transition are within an order of magnitude of the concentrations in experiments with intact myocardium. It is important to note that, with oxybarbiturates, the loss of respiratory control was observed only after accumulation of Ca^{2+} by mitochondria. In the absence of Ca^{2+} accumulation, the effect of oxybarbiturates is the inhibition of respiration and, consequently, the reduction of pyridine nucleotides [2, 3]. The potentiation of the calcium-induced transition by barbiturates described in this paper is likely to occur *in vivo* under pathological conditions when there is an abnormal accumulation of Ca^{2+} by mitochondria.

Four mechanisms are known to protect mitochondria from the calcium-induced transition; these are NADH, Mg^{2+}, ADP and energization [7]. Since a number of thiobarbiturates uncouple oxidative phosphorylation [1], and thiopental is known to cause oxidation of pyridine nucleotides [2], it seems likely that thiobarbiturates potentiate the calcium-induced

transition by interfering with protection by NADH and energization. On the other hand, the potentiation of the calcium-induced transition by oxybarbiturates could not involve the removal of protection by NADH or by energization, since oxybarbiturates have little uncoupling action [1] and cause reduction of pyridine nucleotides [2, 3]. In fact, the results in Fig. 6 show that 2 mM Amytal potentiated the calcium-induced transition by increasing the affinity of binding site(s) for Ca^{2+} even under non-energized conditions. Barbiturates are anionic compounds with a hydrophobic moiety, and they are 'anesthetics' in the broad sense of the term [15]. A characteristic of anesthetics is interaction with biological membranes, which modifies their structure and functions, and certain neutral anesthetics are known to enhance Ca^{2+} binding to nerve [16] and red cell [17] membrane. It appears very likely that barbiturates enhance Ca^{2+} binding to the mitochondrial inner membrane and potentiate the calcium-induced transition by virtue of their effects on membrane structure. In this regard, it should be noted that Grist and Baum [12] reported that a neutral anesthetic, halothane, potentiated calcium-induced loss of respiratory control in rat liver mitochondria.

Finally, since calcium-induced changes in permeability are not limited to mitochondria but are known to occur also in red blood cells [18], nerve [19–21] and cardiac plasma membrane [22], it is possible that barbiturates may affect the calcium-induced permeability changes in biological membranes other than mitochondria. In this respect, the present study may be regarded as a study of the effect of barbiturates on mitochondria as a model biological membrane which can be readily isolated in large quantity.

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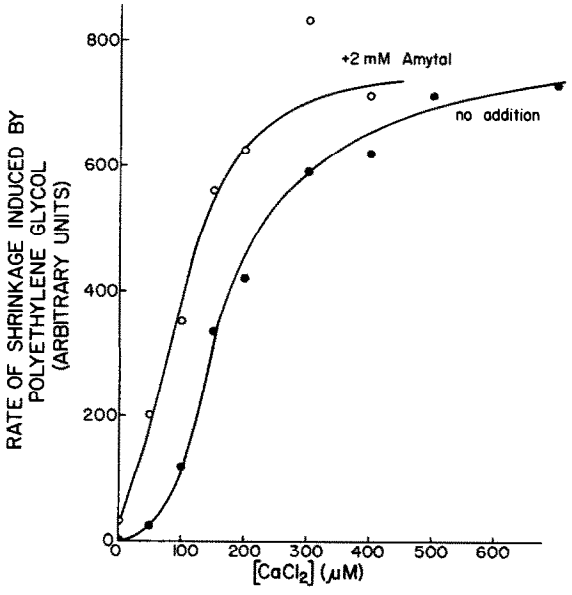

Fig. 6. Potentiation of calcium-induced permeability increase by Amytal under the non-energized condition. The transition was induced in heavy beef heart mitochondria under hypotonic conditions by incubation in the presence of 1 mM arsenate, as described earlier [8]. Three milligrams of such treated mitochondria were suspended in 2.7 ml of medium which was 5 mM in K^+-morpholinopropanesulfonic acid (K^+-MOPS), 25 mM in K^+-cacodylate, and 50 mM in KCl, pH 6.8, at 30°. The suspension was mixed after the addition of 2.5 μg A23187 and varying concentrations of $CaCl_2$, and 2 mM Amytal. The identical reaction mixture with no Amytal served as the control. The pH was readjusted to 6.8 with cacodylic acid, and the calcium-induced increase in the permeability of the inner membrane was measured from the rate of shrinkage of mitochondria caused by the addition of 3.5% polyethylene glycol (mol. wt 1500). The rate of shrinkage was estimated from the rate of change in the light-scattering signal, as described previously [8].

REFERENCES

1. W. N. Aldridge and V. H. Parker, *Biochem. J.* **76**, 47 (1960).
2. B. Chance and G. Hollunger, *J. biol. Chem.* **278**, 418 (1963).
3. J. P. Weiss, C. H. Barlow and B. Chance, *Biochem. Pharmac.* **27**, 1510 (1978).
4. Y. Hatefi and R. L. Lester, *Biochem. biophys. Acta* **27**, 83 (1958).
5. D. R. Hunter, R. A. Haworth and J. H. Southard, *J. biol. Chem.* **251**, 5069 (1976).
6. A. G. Gornall, C. J. Bardawill and M. M. Davis, *J. biol. Chem.* **177**, 751 (1949).
7. D. R. Hunter and R. A. Haworth, *Archs Biochem. Biophys.* **195**, 453 (1979).
8. R. A. Haworth and D. R. Hunter, *Archs Biochem. Biophys.* **195**, 460 (1979).
9. C. S. Rossi and A. L. Lehninger, *J. biol. Chem.* **239**, 3971 (1964).
10. P. W. Reed and H. A. Lardy, *J. biol. Chem.* **247**, 6970 (1972).
11. E. Carafoli, K. Malmström, M. Capano, E. Sigel and M. Crompton, in *Calcium Transport in Contraction and Secretion* (Eds. E. Carafoli, F. Clementi, W. Drabikowski and A. Margreth), p. 53. American Elsevier, New York (1975).
12. E. M. Grist and H. Baum, *Eur. J. Biochem.* **57**, 617 (1975).
13. J. T. Hoff, A. L. Smith, H. L. Hankinson and S. L. Nielson, *Stroke* **6**, 28 (1975).
14. P. E. Dresel, R. W. Ellett and L. Schulter, *Life Sci.* **9**, 759 (1970).
15. P. Seeman, *Pharmac. Rev.* **24**, 583 (1972).
16. S. Ehrenpreis, *J. Cell. comp. Physiol.* **66**, 159 (1965).
17. P. Seeman, M. Chau, M. Goldberg, T. Saks and L. Sax, *Biochim. biophys. Acta* **225**, 185 (1971).
18. R. Whittam, *Nature, Lond.* **219**, 610 (1968).
19. R. W. Meech, *Comp. Biochem. Physiol.* **42A**, 493 (1972).
20. K. Krnjevic and A. Lisiewicz, *J. Physiol., Lond.* **225**, 363 (1972).
21. R. W. Meech, *J. Physiol., Lond.* **237**, 259 (1974).
22. G. Isenberg, *Nature, Lond.* **253**, 273 (1975).