The Effect of Butacaine on Adenine Nucleotide Binding and Translocation in Rat Liver Mitochondria

By DAVID R. H. FAYLE, GREGORY J. BARRITT* and FYFE L. BYGRAVE
Department of Biochemistry, Faculty of Science, The Australian National University, Canberra, A.C.T., Australia

(Received 14 January 1975)

1. The effect of the local anaesthetic, butacaine, on adenine nucleotide binding and translocation in rat liver mitochondria partially depleted of their adenine nucleotide content was investigated. 2. The range of butacaine concentrations that inhibit adenine nucleotide translocation and the extent of the inhibition are similar to the values obtained for native mitochondria. 3. Butacaine does not alter either the total number of atractyloside-sensitive binding sites of depleted mitochondria, or the affinity of these sites for ADP or ATP under conditions where a partial inhibition of the rate of adenine nucleotide translocation is observed. 4. The data are consistent with an effect of butacaine on the process by which adenine nucleotides are transported across the mitochondrial inner membrane rather than on the binding of adenine nucleotides to sites on the adenine nucleotide carrier. 5. The results are briefly discussed in relation to the use of local anaesthetics in investigations of the mechanism of adenine nucleotide translocation.

Studies in this laboratory have shown that local anaesthetics, of which butacaine is the most potent, alter the rate of adenine nucleotide translocation across the inner membrane of rat liver mitochondria. A stimulation of translocation is observed at low concentrations of both adenine nucleotide and anaesthetic, whereas the presence of higher concentrations of these leads to an inhibition of translocation (Spencer & Bygrave, 1974).

This report describes the results of experiments designed to define further the effect of butacaine on adenine nucleotide translocation. It is shown that in rat liver mitochondria partially depleted of their adenine nucleotide content, butacaine does not alter the binding of ADP or ATP to atractyloside-sensitive sites under conditions where the rate of adenine nucleotide translocation is inhibited. These results are discussed in relation to the mechanism(s) by which butacaine inhibits adenine nucleotide translocation, and the use of local anaesthetics as a specific means of studying the mechanism of translocation.

Experimental

Mitochondria

Mitochondria were isolated from the livers of 200 g male rats (Wistar albino), and their protein concentration and respiratory properties determined as described by Reed & Bygrave (1974).

* Present address: School of Medicine, The Flinders University of South Australia, Bedford Park, South Australia 5042.

Depletion of endogenous adenine nucleotides

Partial depletion of the adenine nucleotide content of mitochondria was achieved by incubation in a medium containing 50 mM-sucrose, 50 mM-K₂HPO₄ adjusted to pH 7.4 with KOH, 5 mM-MgCl₂ and 0.25 mg of mitochondrial protein/ml as described by Erdelt *et al.* (1972). After depletion, the mitochondria were washed once in 250 mM-sucrose containing 10 mM-Hepes* (pH 7.4) and 2 mM-EGTA and finally suspended in 250 mM-sucrose containing 5 mM-Hepes (pH 7.0).

Binding of adenine nucleotides to atractyloside-sensitive binding sites

The binding of ADP or ATP to atractyloside-sensitive sites on depleted mitochondria was measured at 0°C in a medium that contained (in a final volume of 0.4 ml) 250 mM-sucrose, 5 mM-Hepes, 5 mM-Mes (pH 7.0), 2 mM-EGTA, 1 mM-adenine 3'-monophosphate, 2 mg of depleted mitochondria/ml and ADP or ATP and butacaine at the concentrations indicated. For the measurement of ATP binding, oligomycin (12 µg/ml) was included in the medium (Weidemann *et al.*, 1970). The specific radioactivity of exogenous adenine nucleotides was corrected for dilution by unlabelled endogenous adenine nucleotides (Morris, 1971). For each determination of the amount of adenine nucleotide bound to atractyloside-

* Abbreviations: Hepes, 2-(*N*-2-hydroxyethyl)piperazine-*N'*-yl)ethanesulphonic acid; Mes, 2-(*N*-morpholino)ethanesulphonic acid; EGTA, ethanedioxybis-(ethylamine)tetra-acetic acid.

sensitive sites, the mean values and the means $\pm$ s.d. were determined from the measured radioactivity in the three parallel binding samples (cf. Weidemann *et al.*, 1970) by standard procedures of statistical analysis.

Rate of adenine nucleotide translocation

The rate of adenine nucleotide translocation was measured at 0°C in a medium identical with that used for the measurement of adenine nucleotide binding by forward exchange as described by Spencer & Bygrave (1972). The standard deviation of values obtained for the rate of adenine nucleotide translocation was estimated by measuring the rate of translocation under a fixed set of conditions in a number of independent assays; s.d. was $\pm 6\%$ of the mean value.

Analytical methods

ATP, and ADP and AMP were assayed as described by Lamprecht & Trautschold (1965) and Adam (1965) respectively. Adenine nucleotides were separated chromatographically as described by Morrison (1968).

Chemicals

[^{14}C]ADP and [^{14}C]ATP were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Butacaine sulphate was a gift from Abbot Australasia Pty. Ltd., Carlton, N.S.W., Australia. All other reagents were of analytical grade.

Results

Inhibition of adenine nucleotide translocation in depleted mitochondria

Accurate measurement of the binding of adenine nucleotides to atractyloside-sensitive binding sites on mitochondria by the method of Weidemann *et al.* (1970) can be made only if the concentration of endogenous adenine nucleotides is low. Rat liver mitochondria that had been partially depleted of their endogenous adenine nucleotides by treatment with potassium phosphate buffer (see the Experimental section) were found to have low acceptor control ratios, and much smaller pools of total and exchangeable adenine nucleotides when compared with native mitochondria (Table 1). The rate of ADP translocation in depleted mitochondria is about 50% of that observed in native mitochondria. Since the properties of depleted mitochondria differ from those of native mitochondria (Table 1 and Weidemann *et al.*, 1970) it was important to establish that the effect of butacaine on adenine nucleotide translocation in depleted mitochondria is

similar to that observed in native mitochondria. The rate of ADP translocation was measured in both native and depleted mitochondria in the presence of increasing concentrations of butacaine (50–450 μM). No difference was observed in either the maximum inhibition (68%) or the concentration of butacaine (76 μM) that gives half-maximum inhibition (cf. Spencer & Bygrave, 1974). This result indicates that butacaine has similar effects on adenine nucleotide translocation in depleted and native mitochondria.

Effect of butacaine on adenine nucleotide binding at high concentrations of adenine nucleotide

Maximum inhibition by butacaine of adenine nucleotide translocation in native rat liver mitochondria is observed at high concentrations (100–400 μM) of adenine nucleotides (Spencer & Bygrave, 1974). The effect of butacaine on the binding of ADP to atractyloside-sensitive sites on depleted mitochondria was therefore tested at the highest concentration of adenine nucleotide (100 μM) at which accurate estimations of the amount of binding can be made. Fig. 1(a) shows the effect of butacaine on the amount of ADP bound to non-specific sites, the sum of the amounts of ADP bound to non-specific sites and translocated at 2 min and the sum of the amounts of ADP bound to non-specific and atractyloside-sensitive sites and translocated at 2 min. Buta-

Table 1. *Comparison of the adenine nucleotide pools and rate of ADP translocation in native mitochondria and mitochondria partially depleted of their endogenous nucleotides*

Mitochondria were partially depleted of their endogenous adenine nucleotides, and their adenine nucleotide content and acceptor control ratios (see Reed & Bygrave, 1974) determined as described in the Experimental section. Rates of ADP translocation were measured at 0°C in a medium that contained 250 mM-sucrose, 5 mM-Hepes, 5 mM-Mes (pH 7.0), 2 mM-EDTA, 1 mM-adenosine 3'-phosphate, 100 μM -ADP and 2.5 mg of mitochondrial protein/ml. The exchangeable pool size was determined by measuring the amount of exogenous [^{14}C]ADP that had exchanged after 15 min.

	Mitochondria	
	Native	Depleted
Acceptor control ratio	4.5–7.5	1.2–3.1
Adenine nucleotide content (nmol/mg of protein)		
AMP	4.5–5.7	0.5
ADP	5.8–6.2	2.3
ATP	4.9–5.1	1.4
Total pool size	14.7–16.6	4.1
Exchangeable pool size	10.8–11.1	3.6
Rate of ADP translocation (nmol/min per mg)	5.5	2.4

caine had no significant effect on the amount of ADP bound to either atractyloside-sensitive (Fig. 1*b*) or non-specific (Fig. 1*a*) sites. By comparison the local anaesthetic markedly inhibits the rate of ADP translocation (Fig. 1*c*) as well as the amount of ADP translocated at 2 min (Fig. 1*a*). In particular, at 100 μM -ADP, where ADP translocation is inhibited by almost 50% (cf. Spencer & Bygrave, 1974), butacaine has no effect on the binding of ADP to atractyloside-sensitive sites (Fig. 1*b*).

Butacaine also has no effect on the binding of ATP to atractyloside-sensitive sites (Fig. 2*a*) at 100 μM -ATP under conditions where a significant inhibition of the rate of ATP translocation is observed (Fig. 2*b*). These data, which were obtained at sufficient concentrations of either ADP or ATP to saturate binding, indicate that butacaine does not alter the total number of atractyloside-sensitive binding sites on the mitochondrial inner membrane.

Effect of butacaine on the affinity of the adenine nucleotide translocase for ADP or ATP

The effect of butacaine on the binding of adenine nucleotides to atractyloside-sensitive sites was investigated at low concentrations of adenine nucleotide to determine whether butacaine alters the affinity of the translocase for adenine nucleotides. Butacaine did not effect the amount of ADP or ATP bound to atractyloside-sensitive sites at 3 μM -

ADP or -ATP when the concentration of butacaine was varied from 30 to 500 μM (results not shown).

The amount of ADP bound to atractyloside-sensitive binding sites was measured over a range of ADP concentrations in the presence and absence of a fixed concentration (100 μM) of butacaine. No significant difference between the data obtained in the presence or the absence of butacaine was observed (Fig. 3*a*). Similar results were obtained for ATP (Fig. 3*b*), although the range of ATP concentrations tested was less extensive. Thus it is concluded that butacaine does not alter the affinity of the adenine nucleotide translocase for ADP or ATP.

Discussion

The influence of butacaine on the rate of adenine nucleotide translocation could involve an effect on (a) the binding of adenine nucleotides to the atractyloside-sensitive binding sites on the translocase protein, (b) the translocation of adenine nucleotides across the mitochondrial inner membrane, (c) their release from the carrier protein or (d) an effect on a combination of these processes. The present results show that in depleted mitochondria, butacaine does not alter either the total number of atractyloside-sensitive binding sites, or the affinity of these sites for adenine nucleotides under conditions where the rate of adenine nucleotide translocation is inhibited. Moreover, the binding of

Fig. 1. Effect of butacaine on the binding and translocation of ADP in depleted rat liver mitochondria at 100 μM -ADP

(a) Amount of ADP associated with the mitochondria (see the Experimental section), measured at 0°C when atractyloside (50 μM) was added before the addition of ADP (■), 2 min after ADP addition (△), or when no atractyloside was added (▲). (b) Amount of ADP bound to atractyloside-sensitive sites, calculated from the data in (a) as described in the Experimental section. (c) Rate of ADP translocation in the presence of butacaine expressed as a percentage of the rate in the absence of butacaine. In each case, the incubation medium contained, in a final volume of 0.4 ml, 250 mM-sucrose, 5 mM-Hepes, 5 mM-Mes (pH 7.0), 2 mM-EDTA, 1 mM-adenosine 3'-phosphate, 1.5–2 mg of depleted mitochondria/ml, 100 μM -ADP and butacaine at the concentrations indicated. The amount of ADP associated with the mitochondria or bound to atractyloside-sensitive sites, and rates of ADP translocation were determined as described in the Experimental section. Values for s.d. were estimated as described in the Experimental section. The data represent one of a number of experiments which gave similar results.

Fig. 2. Effect of butacaine on the binding of ATP to atractyloside-sensitive binding sites (a) and on the rate of ATP translocation (b) in depleted mitochondria at $100\mu\text{M}$ -ATP

The experimental procedures and incubation conditions were as described in Fig. 1, with the replacement of ADP by $100\mu\text{M}$ -ATP and the inclusion of oligomycin ($12\mu\text{g/ml}$) in the incubation medium.

Fig. 3. Effect of butacaine on the binding of ADP (a) and ATP (b) to atractyloside-sensitive binding sites in depleted mitochondria at various concentrations of adenine nucleotide

The amount of adenine nucleotide bound was determined (see the Experimental section) under the conditions described in Fig. 1 in the presence (●) and the absence (○) of $100\mu\text{M}$ -butacaine. ADP or ATP was present at the concentrations indicated and oligomycin ($12\mu\text{g/ml}$) was included in the incubation medium when ATP was present.

adenine nucleotides to atractyloside-sensitive sites measured under the conditions described here represents binding to carrier sites that face both sides of the inner mitochondrial membrane (Klingenberg *et al.*, 1973). Therefore it is concluded that butacaine affects the process by which adenine nucleotides are translocated through the mitochondrial inner membrane, rather than adenine nucleotide binding to, or release from, sites on the adenine nucleotide

translocase. Comparison of the inhibition of adenine nucleotide translocation by butacaine in native and depleted mitochondria indicates that the effect (and hence mechanism of action) of butacaine on this process in native mitochondria is similar to its effect on depleted mitochondria.

The effect of butacaine on mitochondria appears to be relatively specific for adenine nucleotide translocation. Other studies in this laboratory show,

for example, that a number of activities associated with respiratory-chain phosphorylation are unaffected by this local anaesthetic (G. J. Barritt & F. L. Bygrave, unpublished results). Together with the lack of effect of butacaine on adenine nucleotide binding, these observations are consistent with the idea that butacaine inhibits adenine nucleotide translocation by its interaction with phospholipids, which form the phospholipid environment of the translocase, leading to an alteration in the rate of the translocation process (Spencer & Bygrave, 1972, 1974). The mechanism(s) by which butacaine interacts with these phospholipids is at present unknown, although it is likely to involve both electrostatic and hydrophobic interactions (Ritchie & Greengard, 1966; Seeman, 1972; Cerbon, 1972; Butler *et al.*, 1973). Perhaps more, importantly the present observations suggest that butacaine and related compounds can be used as specific inhibitors in physicochemical investigations of the mechanism of the process by which adenine nucleotides are translocated through the inner mitochondrial membrane.

We are grateful to Mr. D. C. Chant and Mr. G. R. Knowles for the development of procedures for the statistical analysis of the binding data and to Mrs. F. A. Doy and Mr. R. F. W. Thorne for the measurement of adenine nucleotide pool sizes in native rat liver mitochondria.

References

- Adam, H. (1965) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), pp. 573–577, Academic Press, New York
- Butler, K. W., Schneider, H. & Smith, I. C. P. (1973) *Arch. Biochem. Biophys.* **154**, 548–554
- Cerbon, J. (1972) *Biochim. Biophys. Acta* **290**, 51–57
- Erdelt, H., Weidemann, M. J., Buchholz, M. & Klingenberg, M. (1972) *Eur. J. Biochem.* **30**, 107–122
- Klingenberg, M., Scherer, B., Stengel-Rutkowski, L., Bucholz, M. & Grebe, K. (1973) in *Mechanisms in Bioenergetics* (Azzone, G. F., Ernster, L., Papa, S., Quagliariello, E. & Siliprandi, N., eds.), pp. 257–284, Academic Press, New York
- Lamprecht, W. & Trautschold, I. (1965) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), pp. 543–551, Academic Press, New York
- Morris, R. (1971) B.Sc. Honours Thesis, The Australian National University
- Morrison, J. F. (1968) *Anal. Biochem.* **24**, 106–111
- Reed, K. C. & Bygrave, F. L. (1974) *Biochem. J.* **138**, 239–252
- Ritchie, J. M. & Greengard, P. (1966) *Annu. Rev. Pharmacol.* **6**, 405–430
- Seeman, P. (1972) *Pharmacol. Rev.* **24**, 583–655
- Spencer, T. & Bygrave, F. L. (1972) *Biochem. J.* **129**, 355–365
- Spencer, T. L. & Bygrave, F. L. (1974) *Biochem. J.* **140**, 413–422
- Weidemann, M. J., Erdelt, H. & Klingenberg, M. (1970) *Eur. J. Biochem.* **16**, 313–335