Biochemical Pharmacology, Vol. 28, pp. 1017–1022.
Pergamon Press Ltd. 1979. Printed in Great Britain.

INHIBITION BY LOCAL ANAESTHETICS OF ANION TRANSPORT IN ISOLATED RAT HEART MITOCHONDRIA

GREGORY J. BARRITT

Clinical Biochemistry Unit, Flinders University School of Medicine, Flinders Medical Centre, Bedford Park, South Australia, 5042

(*Received* 7 *August* 1978; *accepted* 5 *October* 1978)

Abstract— (1) The effects of nupercaine and other local anaesthetics on anion transporters in isolated rat heart mitochondria were investigated at temperatures of 25° or 30°. The concentrations of nupercaine which inhibited by 50 per cent the rates of pyruvate-stimulated oxygen utilisation in (a) the presence and (b) the absence of α-cyano-3-hydroxycinnamate, (c) ADP-stimulated oxygen utilisation in the presence of atractyloside, (d) succinate-stimulated oxygen utilisation in the presence of phenylsuccinate (e) phosphate-induced swelling were (a) 0.02, (b) 0.4, (c) 0.3, (d) 1.1 and (e) 0.6 mM, respectively.

(2) Low concentrations of nupercaine (10–50 μM) increased by about 10 per cent the rates of ADP- and succinate-stimulated oxygen utilisation measured at 30° in the presence of atractyloside and phenylsuccinate, respectively.

(3) Inhibition by nupercaine of pyruvate-stimulated oxygen utilisation in the presence of α-cyano-3-hydroxycinnamate was found to be non-competitive with respect to pyruvate.

(4) Butacaine and tetracaine inhibited pyruvate-stimulated oxygen utilisation, but were less effective than nupercaine. Procaine and xylocaine had little effect.

(5) [2-^{14}C]Pyruvate uptake was inhibited by nupercaine with 50% inhibition given by 0.2 and 0.6 mM nupercaine at 25° and 4° respectively.

(6) The concentrations of nupercaine which inhibited pyruvate-stimulated oxygen utilisation in the presence of α-cyano-3-hydroxycinnamate had no effect on the rate of mitochondrial swelling or the acceptor control ratio of the mitochondria.

(7) It is concluded that the pyruvate transporter is markedly more susceptible to the inhibitory effects of nupercaine than are the adenine nucleotide, dicarboxylate or phosphate ion transporters.

Anion transporters located in the inner membrane of heart mitochondria include those for pyruvate, adenine nucleotides, dicarboxylic acids and phosphate ions [1]. It is assumed that both a specific carrier protein and its association with neighbouring phospholipids are necessary for transport systems of this type [2]. Proteins which are presumed to catalyse the transport of adenine nucleotides and phosphate ions have recently been isolated from the inner membrane of heart mitochondria [3, 4]. However, little is known about the nature of the phospholipid environment which surrounds these and other carrier proteins.

Previous studies in this laboratory have provided evidence which indicates that in rat heart mitochondria there is some interaction between the dicarboxylic acid transporter and other anion transporters in the mitochondrial inner membrane [10]. The aim of the present experiments was to investigate further the relationship between different anion transporters in heart mitochondria using local anaesthetics as tools to probe the inner membrane. Local anaesthetics which contain benzene and tertiary amine moieties have been shown to interact with the phospholipid components of biological membranes [5, 18]. We have previously shown that adenine nucleotide transport in mitochondria isolated from rat liver is inhibited by low concentrations of butacaine [6–8], and evidence which is consistent with an interaction of butacaine with membrane phospholipids was presented [7, 8].

The fluidity of mammalian mitochondrial membranes changes markedly with changes in temperature [15]. Thus the membrane lipids are considered to be predominantly in a fluid or "liquid-crystalline" phase at temperatures above 23° and in a solid or "gel" phase at temperatures below 8° [15]. Most measurements of mitochondrial anion transport are conducted at temperatures below 10° [1]. Since it was considered important to study the effects of local anaesthetics on anion transporters under conditions in which the membrane lipids are in the fluid phase (i.e. at temperatures above 23°), it was necessary to develop methods for the estimation of rates of anion transport at these temperatures. This has been done by measuring anion-dependent oxygen utilisation in the presence of low concentrations of a specific inhibitor of the transport of a given anion. Under these conditions, it is assumed that anion transport is the rate-limiting step in the reaction sequence.

The results show that at 30° a significant inhibition of pyruvate transport is observed in the presence of low concentrations of nupercaine. Much higher concentrations of the local anaesthetic are required to inhibit the transport of adenine nucleotides, dicarboxylic acids and phosphate ions.

MATERIALS AND METHODS

Isolation of mitochondria. The source of rats and the methods used for the isolation of mitochondria and

determination of acceptor control ratios (the ratio of the rate of ADP-stimulated oxygen consumption to the rate observed after depletion of a limited amount of exogenous ADP [9]) and mitochondrial protein content were as described previously [10]. In order to reduce the amount of blood associated with the heart tissue, hearts were removed from rats while the animals were anaesthetised with ether [11]. Values of 4–7 in the presence of 10 mM glutamate and 5 mM malate, and 5–10 in the presence of 2 mM pyruvate and 0.5 mM malate were routinely obtained for acceptor control ratios of the mitochondria.

Mitochondrial respiration. Rates of oxygen utilisation were measured at 30° in a Clark oxygen electrode (Rank Brothers, Bottisham, Cambridge, England). All reaction media (2.0 ml total volume) contained 125 mM KCl, 20 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (Hepes*), pH 7.4, and 2 mM potassium phosphate (pH 7.4). Additional components were as follows: (a) pyruvate-stimulated oxygen utilisation: 2.5 μM carbonyl cyanide m-chlorophenyl hydrazone, 500 μM malate, 0.4 mg mitochondrial protein per ml, the indicated concentrations of inhibitors and 2 mM pyruvate, added 2 min after addition of the mitochondria; (b) succinate-stimulated oxygen utilisation: 2.5 μM carbonyl cyanide m-chlorophenyl hydrazone, 5 mg/ml rotenone, 0.5 mg mitochondrial protein per ml, the indicated concentrations of inhibitors and 5 mM succinate added 2 min after addition of the mitochondria; (c) ADP-stimulated oxygen utilisation: 5 mM malate, 10 mM glutamate, 0.5 mg mitochondrial protein per ml, the indicated concentrations of inhibitors and 0.75 mM ADP added 3 min after addition of the mitochondria; and (d) NADH oxidation: 2.5 μM carbonyl cyanide *m*-chlorophenyl hydroazone, 0.4 mg mitochondrial protein per ml, the indicated concentrations of nupercaine and 0.25 mM NADH added 2 min after the mitochondria.

[2-^{14}C]pyruvate and [U-^{14}C]malate uptake. Initial rates of [2-^{14}C]pyruvate uptake were measured at 25° or 4° as indicated, by a modification of the "inhibitor-stop" method of Halestrap [12]. The reactions were conducted in plastic centrifuge tubes (1.5 ml capacity). Each reaction mixture contained, in a final volume of 0.20 ml, 125 mM KCl, 20 mM Hepes, pH 7.4, 1.0 mM sodium arsenite, 5 μg/ml rotenone, 1 μg/ml antimycin A, 3 mM ascorbic acid, 0.05 mM tetramethylphenylenediamine, 0.25 mM [2-^{14}C]pyruvate (0.07 μCi), nupercaine at the concentrations indicated and 1–1.5 mg mitochondrial protein per ml. All components except the mitochondria and [2-^{14}C]pyruvate were equilibrated at the required temperature before addition of the mitochondria. After 2 min, [2-^{14}C]pyruvate was added and the reaction stopped 10 (25°) or 60 sec (4°) later by the addition of 0.01 ml of 40 mM α-cyano-3-hydroxycinnamate. The tubes were centrifuged at 7000 *g* (Eppendorf Microfuge 3200) at 4° for 2 min, the supernatants removed by aspiration and the pellets washed once with 0.5 ml of the reaction medium from which the mitochondria and [2-^{14}C]pyruvate were omitted. The amount of radioactivity present in the pellets was determined as described previously [13]. The amount of [2-^{14}C]pyruvate, expressed as nmol per mg mitochondrial protein, associated with the mitochondria was calculated from the amount of radioactivity in the mitochondrial pellet and the specific activity of the [2-^{14}C]pyruvate. The amount of [2-^{14}C]pyruvate present in the fluid outside the matrix or absorbed to the mitochondria [16] was estimated in reactions in which α-cyano-3-hydroxycinnamate was added before [2-^{14}C]pyruvate. The difference between the amount of [2-^{14}C]pyruvate associated with the mitochondria in the absence of α-cyano-3-hydroxycinnamate and that associated with the organelles in reactions in which the inhibitor was added before [2-^{14}C]pyruvate, was defined as [2-^{14}C]-pyruvate uptake (i.e. α-cyano-3-hydroxycinnamatesensitive [2-^{14}C]pyruvate uptake [16]).

Initial rates of [U-^{14}C]malate uptake were measured as described previously [10]. The incubation medium contained 250 mM sucrose, 10 mM 2-amino-2-hydroxymethyl-propane-1,3-diol (Tris)–HCl (pH 7.5), 10 mM EDTA, 1 mM arsenite, 5 μg/ml rotenone, 50 μM [U-^{14}C] malate (0.2 μCi), mitochondria (1 mg protein per ml) and the additions shown in Fig. 6.

Mitochondrial swelling. The energy-linked accumulation of phosphate ions was measured at 25° by the method of Brierley *et al.* [14]. The medium contained 100 mM potassium phosphate (pH 7.4), 5 mM succinate (pH 7.4) and 0.25 mg per ml mitochondrial protein. The initial rate of swelling was estimated from the rate of decrease in apparent extinction at 546 nm measured using a Ziess PM2 D2 spectrophotometer (Carl Zeiss, Oberkochen, West Germany).

Chemicals. The local anaesthetics, which were obtained from the sources described by Spencer and Bygrave [6], were dissolved in water and the pH adjusted to about 7.4 with KOH. α-Cyano-3-hydroxycinnamate was obtained from the Aldrich Chemical Company, Gillingham, Dorset, U.K.; 2-phenylsuccinate from K and K laboratories, Plainview, New York; atractyloside from the Sigma Chemical Company, Missouri and [2-^{14}C]pyruvic acid and [U-^{14}C]malic acid from the Radiochemical Centre, Amersham, Bucks., U.K. All other reagents were of the highest grade available.

RESULTS

Pyruvate oxidation and [2-^{14}C]pyruvate uptake. The rate of pyruvate-stimulated oxygen utilisation in the presence of α-cyano-3-hydroxycinnamate was used as a measure of pyruvate transport across the inner mitochondrial membrane at 30°. The concentration of α-cyano-3-hydroxycinnamate, an inhibitor which is specific for the pyruvate transporter [12, 16], was chosen to give about 50% inhibition of the rate of oxygen utilisation (Fig. 1a and b). Under these conditions, the transport of pyruvate across the inner membrane should be the rate-limiting step in the sequence of reactions which lead from pyruvate entry into the mitochondria to oxygen utilisation. The addition of nupercaine (10 μM) caused a significant inhibition of pyruvate-stimulated oxygen utilisation (Fig. 1c). Measurement of the rate of pyruvate-stimulated oxygen utilisation in the presence of 1.5 μM α-cyano-3-hydroxycinnamate and increasing concentrations of nupercaine showed that 50 per cent inhibition of oxygen utilisation

* Hepes-4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

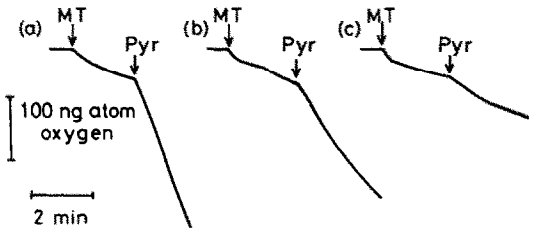

Fig. 1. The effects of α-cyano-3-hydroxycinnamate and nupercaine on pyruvate-stimulated oxygen utilisation at 30°. Pyruvate oxidation was measured as described in Materials and Methods. Pyruvate (Pyr) was added 2 min after addition of the mitochondria (MT). The concentrations of the inhibitors, which were present before the addition of mitochondria, were: none (a), 1.5 μM α-cyano-3-hydroxycinnamate (b) and 1.5 μM α-cyano-3-hydroxycinnamate plus 10 μM nupercaine (c).

is obtained at 0.02 mM nupercaine (Fig. 2a). In the absence of α-cyano-3-hydroxycinnamate, the concentration of nupercaine which gives 50 per cent inhibition of pyruvate-stimulated oxygen utilisation is 0.4 mM (Fig. 2b).

The inhibition by nupercaine of pyruvate-stimulated oxygen utilisation in the presence of α-cyano-3-hydroxycinnamate was found to be non-competitive with respect to pyruvate (Fig. 3). Pyruvate-stimulated oxygen utilisation in the presence of 1.5 μM α-cyano-3-hydroxycinnamate was less sensitive to inhibition by butacaine and tetracaine with 50 per cent inhibition observed at 0.04 and 0.5 mM of these agents respectively. Procaine and xylocaine had very little effect when tested at concentrations between 0.1 and 1.0 mM.

Nupercaine inhibited the initial rate of [2-^{14}C]pyruvate uptake (Fig. 4a and b). Concentrations of 0.2 and 0.6 mM nupercaine (the average of values obtained from three separate experiments) gave 50 per cent inhibition at 25° and 4° respectively.

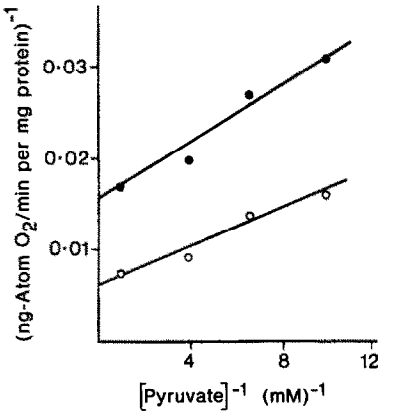

Fig. 3. The reciprocal of the rate of pyruvate-stimulated oxygen utilisation (measured in the presence of 1.5 μM α-cyano-3-hydroxycinnamate) plotted as a function of the reciprocal of the pyruvate concentration in the presence (●) and absence (○) of 20 μM nupercaine. The rate of pyruvate-stimulated oxygen utilisation was measured at varying concentrations of pyruvate under the conditions described in the legend of Fig. 1. The data shown are those of one of three experiments which gave similar results.

Transport of dicarboxylic acids, ADP and phosphate ions. The effects of nupercaine on succinate-stimulated oxygen utilisation in the presence of phenylsuccinate, a specific inhibitor of dicarboxylic acid transport [17], and ADP-stimulated oxygen utilisation in the presence of atractyloside, an inhibitor of adenine nucleotide translocation [1], were investigated. The concentrations of phenylsuccinate and atractyloside were adjusted to give 50 per cent inhibition of the rate of oxygen utilisation so that the movement of succinate or ADP across the mitochondrial membrane should be the rate-limiting step in the reaction sequence. The concentrations of nupercaine which give 50 per cent inhibition

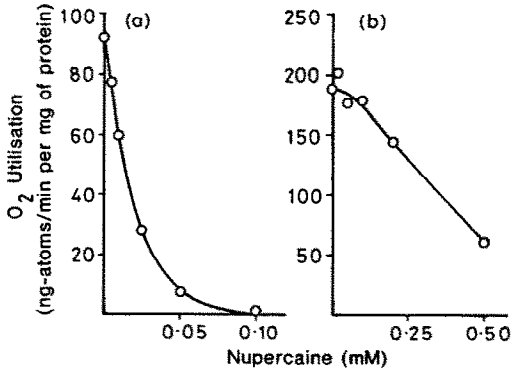

Fig. 2. Effect of increasing concentrations of nupercaine on the rate of pyruvate-stimulated oxygen utilisation measured in the presence (a) and absence (b) of 1.5 μM α-cyano-3-hydroxycinnamate. Rates of pyruvate-stimulated oxygen utilisation were measured in the presence of the indicated concentrations of nupercaine under the conditions described in the legend of Fig. 1. The data shown are the results of one of four (a) and two (b) experiments which gave similar results.

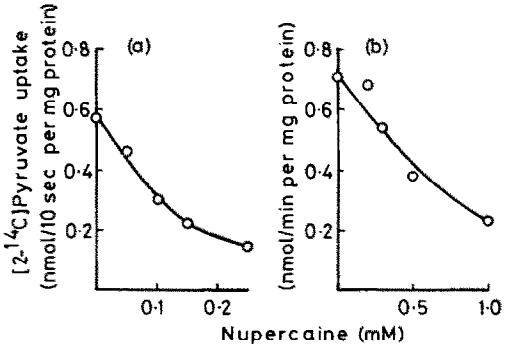

Fig. 4. The effect of increasing concentrations of nupercaine on the initial rate of [2-^{14}C]pyruvate uptake measured at 25° (a) and 4° (b). The composition of the reaction media and measurement of [2-^{14}C]pyruvate uptake were as described in Materials and Methods. The data shown are those for one of three experiments which gave similar results.

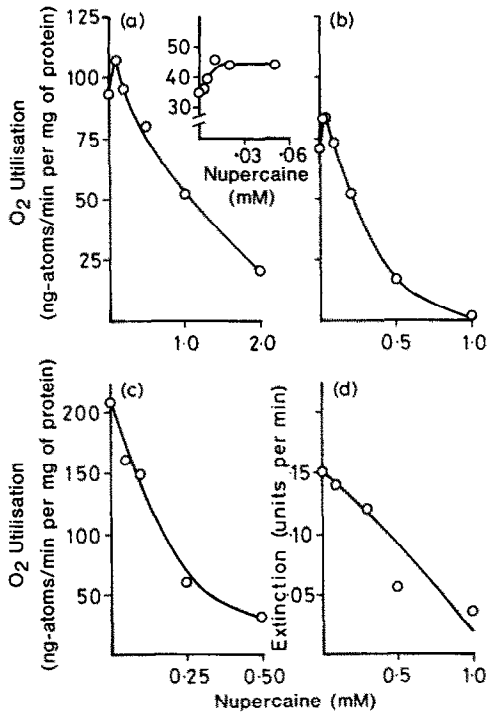

Fig. 5. The effects of increasing concentrations of nupercaine on (a) the rate of succinate-stimulated oxygen utilisation measured at 30° in the presence of 2 mM phenylsuccinate, (b) ADP-stimulated oxygen utilisation measured at 30° in the presence of 3.5 μM atractyloside and (c) NADH-stimulated oxygen utilisation by sonicated mitochondria measured at 30°. The inset (a) shows the effect of low concentrations of nupercaine on the rate of succinate-stimulated oxygen utilisation in the presence of 3.8 mM phenylsuccinate. The composition of the reaction media and procedure for the measurement of oxygen utilisation are described in Materials and Methods. Sonicated mitochondria were prepared by sonicating a suspension of mitochondria in isolation medium (15 mg protein per ml) for 10 sec at 0° using a Branson B-12 Sonifier (Branson Sonic Power Co., Danbury, Conneticut, U.S.A.). (d) The effect of nupercaine on the rate of swelling of mitochondria incubated at 25° in the presence of 100 mM potassium phosphate and 5 mM succinate. The composition of the reaction medium and procedure used to measure mitochondrial swelling are described in Materials and Methods. The data shown are the results of one of four (a), three (b) and two (c and d) experiments which gave similar results.

of succinate- and ADP-stimulated oxygen utilisation were found to be 1.1 and 0.3 mM respectively (Fig. 5a and b). In each case, a small stimulation of oxygen utilisation was observed at much lower concentrations of nupercaine (Fig. 5a and inset, Fig. 5b). The presence of 1.5 μM α-cyano-3-hydroxycinnamate did not modify the inhibitory effects of nupercaine. Nupercaine inhibited (i) the oxidation of NADH in sonicated mitochondria (Fig. 5c) and (ii) the rate of phosphate-induced swelling, measured in the presence of succinate (Fig. 5d). The concentrations of the local anaesthetic which inhibited these processes by 50 per cent were 0.2 and 0.6 mM respectively.

The effects of nupercaine on the movement of dicarboxylic acids across the mitochondrial membrane were also studied by investigating its effects on [U-^{14}C]malate uptake. The initial rate of [U-^{14}C]malate uptake at 4° was inhibited 50 per cent by 0.6 mM nupercaine (Fig. 6a). Smaller effects were exerted by tetracaine, butacaine and procaine (Figs. 6b–d).

Mitochondrial swelling. At 25°, 0.05 mM nupercaine had no effect on the rate of swelling as monitored by the decrease in apparent absorbance at 546 nm (Fig. 7a, b). Concentrations of 0.2 and 0.5 mM nupercaine caused an increase in the rate of swelling over the first 2 min, but this effect was not evident at 1 mM nupercaine (Figs. 7c–e). At 2 mM nupercaine, an enhanced rate of swelling was observed over the whole time period investigated (Fig. 7f). A similar trend was observed at 30° under conditions used to measure the effects of nupercaine on pyruvate-stimulated oxygen utilisation in the absence of α-cyano-3-hydroxycinnamate (data not shown).

Concentrations of nupercaine below 0.05 mM had no effect on the acceptor control ratios (the ratio of the rate of oxygen utilisation in the presence of a limited amount of ADP to that observed after depletion of ADP) measured at 25° in a medium which contained 125 mM KCl, 20 mM Hepes (pH 7.4), 2 mM potassium phosphate (pH 7.4), 10 mM glutamate and 5 mM malate. Higher concentrations of nupercaine caused a progressive decrease in the value of the acceptor control ratio from 2.8 in the absence of nupercaine to 2.2 in the presence of 0.5 mM nupercaine.

DISCUSSION

The most striking effect of nupercaine is the inhibition by low concentrations of this agent of pyruvate-stimulated oxygen utilisation measured in the presence of α-cyano-3-hydroxycinnamate. At these concentrations of nupercaine, no change was observed in the integrity of the mitochondria as monitored by the rate

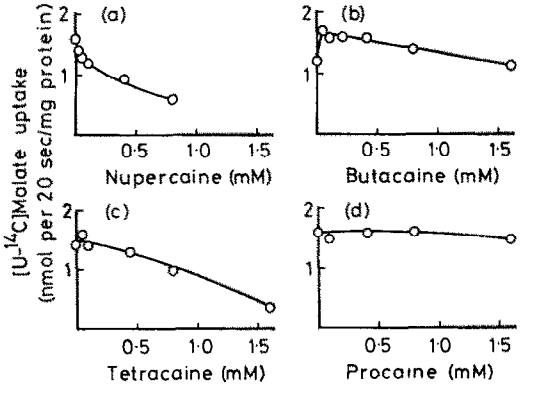

Fig. 6. The effects of nupercaine (a), butacaine (b), tetracaine (c) and procaine (d) on the initial rate of [U-^{14}C]malate uptake at 4°. The composition of the reaction medium and procedure used to measure [U-^{14}C]malate uptake are described in Materials and Methods. The data are the results of one of two experiments which gave similar results.

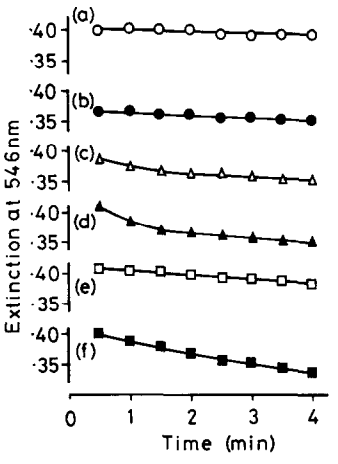

Fig. 7. The effect of nupercaine on the swelling of mitochondria at 25°. Each reaction mixture contained, in a final volume of 3.0 ml; 125 mM KCl, 20 mM Hepes (pH 7.4), 1.0 mM sodium arsenite, 5 μg/ml rotenone, 1 μg/ml antimycin A, 3 mM ascorbic acid, 0.05 mM tetramethylphenylenediamine, 0.25 mg per ml mitochondrial protein and nupercaine at concentrations of 0 (a), 0.05 (b), 0.2 (c), 0.5 (d), 1.0 (e) and 2.0 mM (f). Mitochondrial swelling was measured as described in Materials and Methods after addition of the mitochondria at 0 min. The data shown are the results of one of four experiments which gave similar results.

of mitochondrial swelling and the acceptor control ratio. Higher concentrations of nupercaine (about 0.4 mM) were required to cause significant inhibition of pyruvate-stimulated oxygen utilisation in the absence of α-cyano-3-hydroxycinnamate, or of [2-^{14}C]pyruvate uptake. One explanation for these results is that α-cyano-3-hydroxycinnamate confers a change in the pyruvate transporter which renders it more sensitive to inhibition by nupercaine. This effect would be specific for the pyruvate transporter, as no change in the susceptibility of ADP- or succinate-stimulated oxygen utilisation was observed in the presence of α-cyano-3-hydroxycinnamate. A second explanation is that, in the absence of α-cyano-3-hydroxycinnamate, the movement of pyruvate across the inner membrane is not a rate-limiting step for either pyruvate oxidation or [2-^{14}C]pyruvate uptake (cf. Pande and Parvin [16]). The observations that (a) the inhibitory effect of nupercaine on pyruvate oxidation is most pronounced in the presence of α-cyano-3-hydroxycinnamate, and (b) much higher concentrations of nupercaine are required to inhibit NADH oxidation in sonicated mitochondria, indicate that the effects seen on pyruvate oxidation in the presence of α-cyano-3-hydroxycinnamate reflect the action of nupercaine on the pyruvate transporter rather than on subsequent metabolism of pyruvate.

The concentration of nupercaine (about 0.1 mM) which completely inhibits pyruvate oxidation in the presence of α-cyano-3-hydroxycinnamate had little or no inhibitory effect on the rates of succinate- and ADP-stimulated oxygen utilisation and phosphate-induced swelling. Therefore it is concluded that the dicarboxylic acid, adenine nucleotide and phosphate transporters are markedly less susceptible to inhibition by nupercaine than the pyruvate transporter. Furthermore, since succinate- and ADP-stimulated oxygen utilisation were measured under conditions in which the transport of succinate and ADP, respectively, was the rate-limiting step, it is concluded that the concentrations of nupercaine which give 50 per cent inhibition of the dicarboxylate and adenine nucleotide transporters are about 1.1 and 0.3 mM, respectively. In the case of the dicarboxylic acid transporter, the inhibitory effects of nupercaine were confirmed by demonstrating an inhibition of [U-^{14}C]malate uptake at 4°.

The effects of local anaesthetics, of the type employed here, on biological membranes appear to be mediated predominantly through an interaction of the drugs with membrane phospholipids [5, 18]. The present results do not exclude a direct interaction between nupercaine and an α-cyano-3-hydroxycinnamate-pyruvate carrier protein complex. However, the observed stimulation of succinate- and ADP-stimulated oxygen utilisation by concentrations of nupercaine which are similar to those which inhibit pyruvate-stimulated oxygen utilisation in the presence of α-cyano-3-hydroxycinnamate, is consistent with an effect of nupercaine on membrane phospholipids which, in turn, affects anion transporters in different ways.

Acknowledgements—This work was conducted with the skilled technical assistance of Mr. Paul Spiel, and was supported by grant G 1120 from the National Heart Foundation of Australia.

REFERENCES

1. A. J. Meijer and K. van Dam, *Biochim. biophys. Acta* **346**, 213 (1974).
2. S. J. Singer, *J. Supramol. Struct.* **6**, 313 (1977).
3. D. H. Boxer, J. Feckl and M. Klingenberg, *FEBS Lett.* **73**, 43 (1977).
4. R. K. Banerjee, H. G. Shertzer, B. I. Kanner and E. Racker, *Biochem. biophys. Res. Commun.* **75**, 772 (1977).
5. P. Seeman, *Pharmac. Rev.* **24**, 583 (1972).
6. T. L. Spencer and F. L. Bygrave, *Biochem. J.* **140**, 413 (1974).
7. D. R. H. Fayle, G. J. Barritt and F. L. Bygrave, *Biochem. J.* **148**, 527 (1975).
8. M. Crompton, G. J. Barritt, J. H. Bradbury and F. L. Bygrave, *Biochem. Pharmac.* **25**, 2461 (1976).
9. R. W. Estabrook, *Meth. Enzym.* **10**, 41 (1967).
10. G. J. Barritt, *Int. J. Biochem.* **9**, 317 (1978).
11. A. L. Kerbey, P. J. Randle, R. H. Cooper, S. Whitehouse, H. T. Pask and R. M. Denton, *Biochem. J.* **154**, 327 (1976).
12. A. P. Halestrap, *Biochem. J.* **148**, 85 (1975).
13. M. Foldes and G. J. Barritt, *J. biol. Chem.* **252**, 5372 (1977).
14. G. P. Brierley, M. Jurkowitz, K. M. Scott and A. J. Merola, *Archs Biochem. Biophys.* **147**, 545 (1971).
15. J. K. Raison and E. J. McMurchie, *Biochim. biophys. Acta* **363**, 135 (1974).
16. S. V. Pande and R. Parvin, *J. biol. Chem.* **253**, 1565 (1978).
17. F. E. Sluse, C. M. Sluse-Goffart, C. Duyckaerts and C. Liebecq, *Eur. J. Biochem.* **56**, 1 (1975).
18. M. B. Feinstein, M. Volpi, S. Perrie, A. Makriyannis and R. I. Sha'afi, *Molec. Pharmac.* **13**, 840 (1977).