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Inhibition by Local Anaesthetics of Adenine Nucleotide Translocation in Rat Liver Mitochondria

By TERRY L. SPENCER and FYFE L. BYGRAVE
Department of Biochemistry, Faculty of Science, Australian National University, Canberra, A.C.T. 2600, Australia

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1. The mechanism of adenine nucleotide translocation in mitochondria isolated from rat liver was further examined by using the local anaesthetics procaine, butacaine, nupercaine and tetracaine as perturbators of lipid–protein interactions. Each of these compounds inhibited translocation of ADP and of ATP; butacaine was the most effective with 50% inhibition occurring at 30μM for 200μM-ATP and at 10μM for 200μM-ADP. The degree of inhibition by butacaine of both adenine nucleotides was dependent on the concentration of adenine nucleotide present; with low concentrations of adenine nucleotide, low concentrations of butacaine-stimulated translocation, but at high concentrations (greater than 50μM) low concentrations of butacaine inhibited translocation. Butacaine increased the affinity of the translocase for ATP to a value which approached that of ADP. 2. Higher concentrations of nupercaine and of tetracaine were required to inhibit translocation of both nucleotides; 50% inhibition of ATP translocation occurred at concentrations of 0.5mM and 0.8mM of these compounds respectively. The pattern of inhibition of ADP translocation by nupercaine and tetracaine was more complex than that of ATP; at very low concentrations (less than 250μM) inhibition ensued, followed by a return to almost original rates at 1mM. At higher concentrations inhibition of ADP translocation resulted. 3. That portion of ATP translocation stimulated by Ca^{2+} was preferentially inhibited by each of the local anaesthetics tested. In contrast, inhibition by the anaesthetics of ADP translocation was prevented by low concentrations of Ca^{2+}. 4. The data provide further support for our hypothesis that lipid–protein interactions are important determinants in the activity of the adenine nucleotide translocase in mitochondria.

Stimulation by Ca^{2+} of adenine nucleotide translocation, and particularly that of ATP, in rat liver mitochondria is now well documented (Spencer & Bygrave, 1971, 1972*a*,*b*, 1973; Meisner, 1971). Investigations in this laboratory into the mechanism of this stimulation have led us to propose that the Ca^{2+} effect involves an interaction of the ion with membrane phospholipids located in the environment of the translocase. Such an interaction is envisaged as inducing changes in the phospholipid molecule, which are then relayed to the protein carrier and ultimately result in a modification of adenine nucleotide translocation. Evidence supporting this concept has been presented (Spencer & Bygrave, 1972*a*).

Local anaesthetics are known to induce profound changes in the structure and function of excitable and non-excitable biomembranes (reviewed by Seeman, 1966, 1972), including those of mitochondria (see Johnson *et al.*, 1973). At low concentrations they are able to stabilize membranes, whereas at high concentrations they can induce disorder in membrane structure. These changes in membrane 'fluidity' probably reflect interaction of the anaesthetics with membrane phospholipids and particularly with Ca^{2+}-binding sites on the membrane (Seeman, 1972). Studies using phospholipid bilayer membranes provide further support for this conclusion (see Papahadjopoulos, 1972).

With this background in mind we have undertaken a study of the effect of various local anaesthetics on adenine nucleotide translocation in rat liver mitochondria. Data in this paper show that tetracaine, nupercaine, procaine and especially butacaine inhibit the translocation of adenine nucleotides. It is also shown that each of these anaesthetics antagonizes the stimulatory action of Ca^{2+} on ATP translocation. In this respect inhibition by the anaesthetics correlates approximately with their potency in inhibiting nerve cell conduction. Our data support the contention (Spencer & Bygrave, 1972*a*,*b*) that lipid–protein interactions are important determinants in the activity of the adenine nucleotide translocase.

Experimental

Preparation of mitochondria from rat liver and measurement of translocase activity was carried out as described previously, by using the back-exchange technique (see Pfaff & Klingenberg, 1968) at a temperature of 4°C (Spencer & Bygrave, 1972*a*). The incubation medium contained 200mM-sucrose, 2mM-Hepes [2-(*N*-2-hydroxyethylpiperazin-*N'*-yl)-ethanesulphonic acid]-KOH (pH7.4) and 0.4mg of mitochondrial protein in a total volume of 0.25ml. Mitochondria were preincubated for 2min with the anaesthetic; the reaction was initiated by the addition of an appropriate amount of adenine nucleotide and Ca^{2+} (when present) and terminated after an incubation period of 20s by the addition of atractyloside at a final concentration of 60μM.

Protein was determined by using a modified biuret method which corrects for turbidity of the sample (Szarkowska & Klingenberg, 1963).

All reagents, radioactive nucleotides and enzymes required for the determination of translocation activity were obtained and used as described previously (Spencer & Bygrave, 1972*a*). Local anaesthetics were obtained from the following sources: tetracaine, Glaxo Australia Pty, Ltd., Boronia, Vic., Australia; nupercaine hydrochloride, Ciba Pharmaceuticals, Crows Nest, N.S.W., Australia; butacaine sulphate, Abbott Australia Pty, Ltd., Carlton, N.S.W., Australia; procaine hydrochloride, Drug Houses of Australia Ltd., Camperdown, N.S.W., Australia. Each anaesthetic was made up in water and the pH adjusted to approx. 7.4 with KOH. The structures of these compounds are shown in Fig. 1.

The kinetic constants for inhibition or stimulation by local anaesthetics were determined by plotting the reciprocal of the percentage inhibition or stimulation against the reciprocal of the effector concentration.

Results

Influence of local anaesthetics on ATP translocation

Data in Fig. 2 show the effect of increasing concentrations of the local anaesthetics on the translocation of ATP (200μM) in the absence and presence of 200μM Ca^{2+}. Concentrations of procaine up to 6mM have little effect on the translocation of ATP itself. However, that portion of the translocase stimulated by Ca^{2+} is progressively inhibited as the concentration of procaine increases. This is seen more clearly in the insert to Fig. 2(*a*), where the degree of stimulation by Ca^{2+} of ATP translocation (expressed as the relative activation ratio, $R_{act.}$) is shown as a function of procaine concentration.

ATP translocation in both the absence and presence of Ca^{2+} is particularly sensitive to butacaine (Fig. 2*b*). In this experiment the K_i was of the order of 30μM in each case. Again the Ca^{2+}-stimulated portion is progressively inhibited by increasing concentrations of the local anaesthetic (see insert to Fig. 2*b*).

Both nupercaine and tetracaine at concentrations of 0.5mM and 0.8mM respectively (Figs. 2*c* and 2*d*)

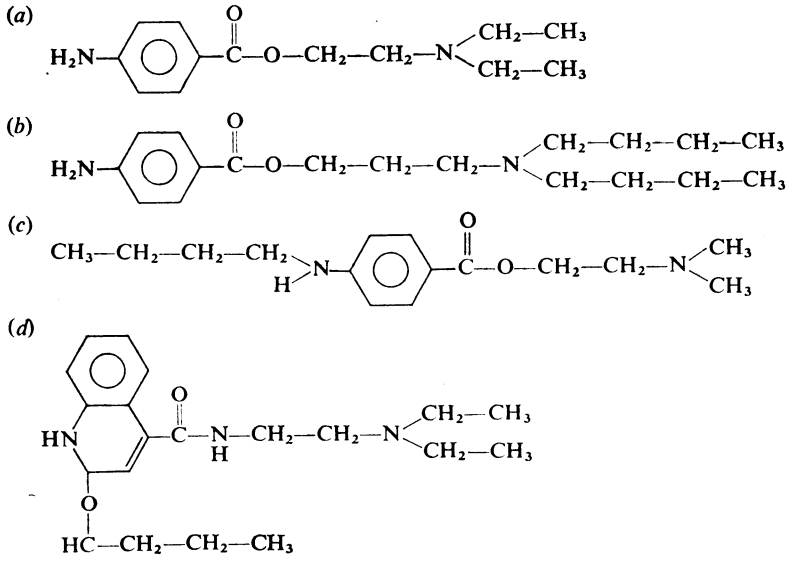

Fig. 1. *Structure of local anaesthetics used in this study*
a, Procaine; *b*, butacaine; *c*, nupercaine; *d*, tetracaine.

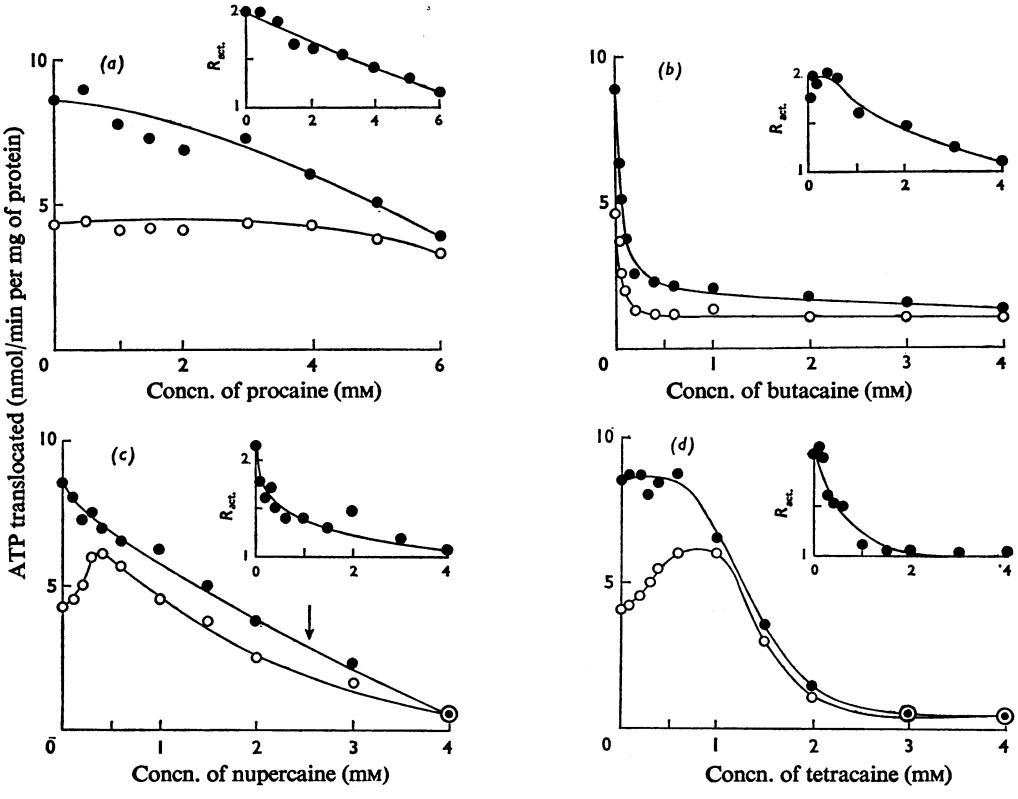

Fig. 2. *Effect of local anaesthetics on the translocation of ATP in the absence and presence of Ca^{2+}*

Incubations were carried out as described in the Experimental section with 200 μM-ATP and 200 μM-Ca^{2+} present as indicated. The concentrations of anaesthetics were varied as shown. ●, Ca^{2+} present; ○, no Ca^{2+}. (*a*) procaine; (*b*) butacaine; (*c*) nupercaine; (*d*) tetracaine. Data in inserts to each figure indicate the relative activation by Ca^{2+} ($R_{act.}$) of ATP translocation plotted as a function of anaesthetic concentration.

stimulate ATP translocation by about 30% in the absence of Ca^{2+}. At high concentrations, translocation is inhibited, being more sensitive to nupercaine than to tetracaine. In the presence of Ca^{2+} the stimulation of ATP translocation by low concentrations of these anaesthetics is not seen. Rather, with tetracaine there is little effect up to about 0.5mM, but thereafter the activity approaches that found in the absence of Ca^{2+}. With nupercaine there is a steady decline in the activity, which also approaches that observed in the absence of Ca^{2+}.

The data contained in the inserts to Fig. 2 show that the concentrations of anaesthetic required to decrease the stimulation by Ca^{2+} of ATP translocation by 50% are 200μM, 250μM, 1.7mM and 3.3mM for nupercaine, tetracaine, butacaine and procaine respectively. The point indicated with an arrow shows the concentration of nupercaine that induces lysis of the mitochondria, as reflected by leakage of endogenous adenine nucleotides, and increased swelling of the mitochondria. Under the conditions used in these experiments lysis of the mitochondria occurred only with nupercaine, and then only when the concentration approached 3mM.

Influence of local anaesthetics on ADP translocation

The next series of experiments (Fig. 3) were carried out in a system identical with that in Fig. 2 except that translocation of ADP was studied. As found with ATP translocation, butacaine is the most potent of the anaesthetics in inhibiting ADP translocation. The K_i is approx. 10μM under the conditions of the experiment both with and without added Ca^{2+} (Fig. 3*b*).

The effects of nupercaine and tetracaine on ADP translocation, especially in the absence of Ca^{2+}, are more complex than those seen with ATP translocation (Figs. 3*c* and 3*d*). Quite low concentrations (approx.

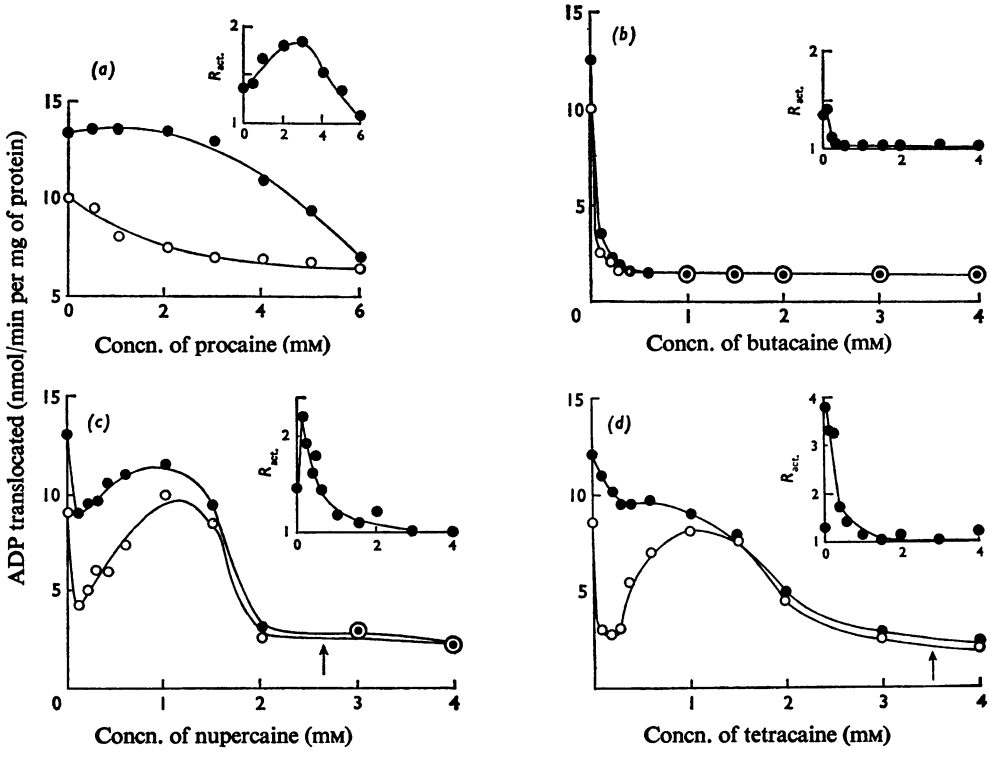

Fig. 3. *Effect of local anaesthetics on the translocation of ADP in the absence and presence of* Ca^{2+}

The experiments were identical with those shown in Fig. 2 except that 200μM-ADP was present., ●, Ca^{2+} present; ○, Ca^{2+} absent. (*a*) Procaine; (*b*) butacaine; (*c*) nupercaine; (*d*) tetracaine. Data in inserts to each figure indicate the relative activation by Ca^{2+} ($R_{act.}$) of ATP translocation as a function of anaesthetic concentration.

250μM) of each of these local anaesthetics inhibit ADP translocation by about 50%, but as the anaesthetic concentration is increased to about 1mM the rate of translocation is gradually restored to the control value. Further increase in anaesthetic concentration then produces a progressive inhibition of the translocation. Maximal inhibition (about 70%) occurs between 2 and 3mM. As with ATP, added Ca^{2+} dampens the response of ADP translocation to the effects of nupercaine and tetracaine, resulting in a relatively large enhancement of the activation by Ca^{2+} (see inserts to Figs. 3*c* and 3*d*). This is seen especially with tetracaine, where at a concentration of 100μM the activation ratio approaches 4.

The response of ADP translocation to procaine is also more complex than that found with ATP (Fig. 3*a*). In the absence of Ca^{2+}, translocation is slightly decreased by about 2mM-procaine, but in the presence of Ca^{2+} concentrations of up to about 3mM have little effect. At 5mM-procaine, ADP translocation is inhibited by about 30%.

The concentrations of anaesthetics required to bring about a 50% decrease in Ca^{2+}-stimulated ADP translocation are 1.1mM, 1.3mM, 150μM and 5.7mM for nupercaine, tetracaine, butacaine and procaine respectively. Thus apart from butacaine, the Ca^{2+}-stimulated portion of ATP translocation is more sensitive to inhibition by these anaesthetics than is the Ca^{2+}-stimulated portion of ADP translocation.

Effect of butacaine concentration on translocation of various concentrations of ATP

In view of the potent effect of butacaine on the translocation of both ATP and ADP, it was decided to investigate the effects of this anaesthetic more fully. Data in Fig. 4 show the effect of butacaine concentration on the translocation rate of different concentrations of ATP, varied in the range 10–400μM. Stimulation of ATP translocation is observed when the concentrations of both added ATP and of

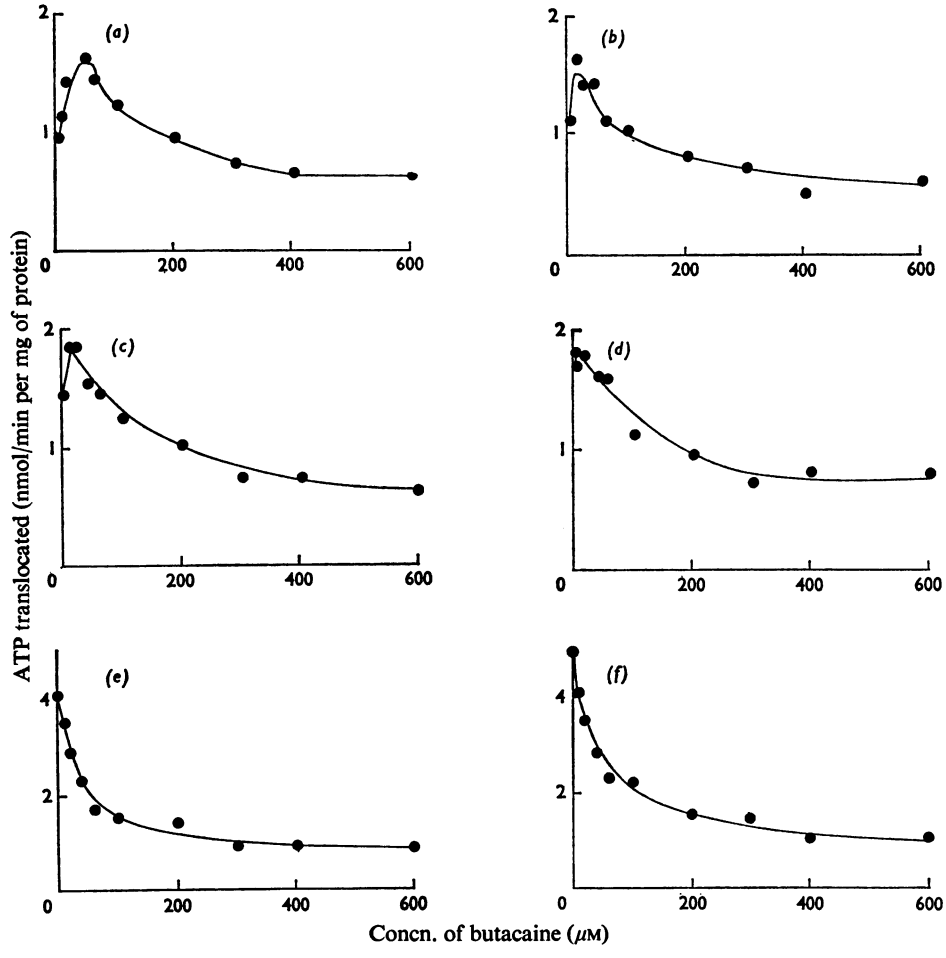

Fig. 4. *Effect of butacaine concentration on the translocation of different concentrations of ATP*

Each concentration of ATP (as indicated) was independently examined as a function of butacaine concentration. Figs. 4(*a*)–(*f*) are data obtained with 10, 20, 50, 100, 200 and 400μM-ATP respectively.

Table 1. *Effect of ATP concentration on the kinetic parameters for butacaine inhibition and stimulation of ATP translocation*

Translocation of ATP at the concentrations shown was determined as a function of butacaine concentration (see Fig. 4). Maximal inhibition (%) and stimulation (%) and K_i and K_a values were determined from the data contained in Fig. 4 by the use of double-reciprocal plots.

Concn. of ATP (μM)	Maximal stimulation (%)	K_a (μM)	Maximal inhibition (%)	K_i (μM)
10	78	15	30	280
20	50	6	50	200
50	30	4	55	165
100	—	—	60	80
200	—	—	80	30
400	—	—	85	28

butacaine are low, up to about 50μM of each compound. The degree of stimulation decreases with increasing ATP concentrations from 78% at 10μM-ATP to zero at 100μM-ATP (see Table 1); the respective concentrations of butacaine producing half-maximal stimulation (K_a) also decrease from 15μM-butacaine at 10μM-ATP to 4μM-butacaine at 50μM-ATP. Inhibition observed with higher butacaine concentrations is not complete and reaches a basal value of approx. 0.6–0.8nmol/min per mg of protein and is virtually independent of the ATP concentration used. Consequently, the maximal percentage inhibition of ATP translocation increases from 30% at 10μM-ATP to 85% at 400μM-ATP. Moreover, as shown in Table 1, the butacaine concentration needed to give 50% of maximal

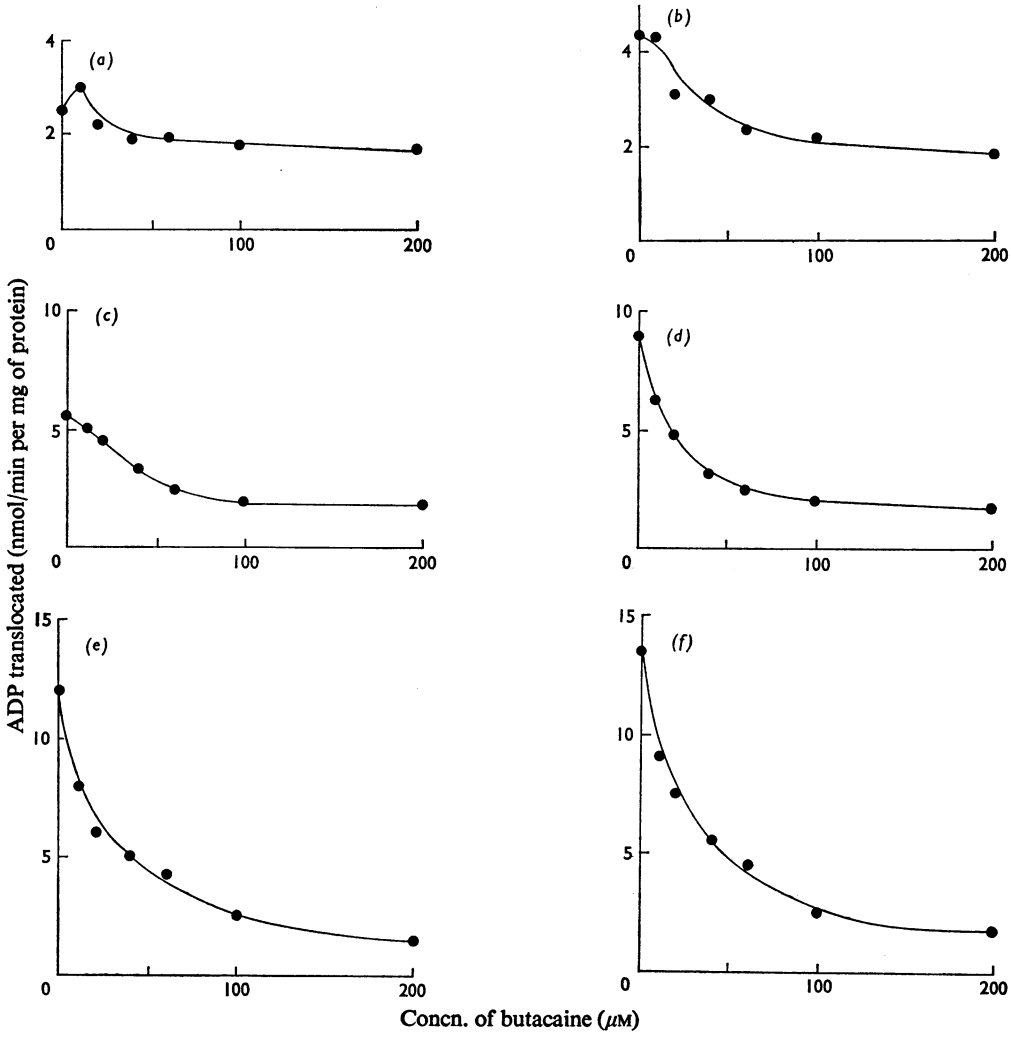

Fig. 5. *Effect of butacaine concentration on the translocation of different concentrations of ADP*

Each concentration of ADP (as indicated) was independently examined as a function of butacaine concentration. Figs. 5(*a*)–5(*f*) are data obtained with 10, 20, 50, 100, 200 and 400μM-ADP respectively.

inhibition (K_i) decreases from 280μM at 10μM-ATP to 28μM at 400μM-ATP.

Effect of butacaine concentration on translocation of various concentrations of ADP

Data presented in Fig. 5 show the effect of butacaine concentration on the translocation of increasing concentrations of ADP. Again, stimulation is observed at low ADP concentrations, but is not as great as that observed for ATP (see Fig. 4) and is not seen above 10μM-ADP. Maximal inhibition increases from 40% at 10μM-ADP to 86% at 400μM-ADP. Basal translocation rates varied from 1.5 to 1.8nmol/min per mg of protein over the range of ADP concentrations tested. As with ATP, the K_i for butacaine decreases, with increasing ADP concentrations, from 60μM to 18μM over the range of ADP concentrations used (see Table 2).

Effect of butacaine on the affinity of the translocase for ADP and ATP

The effect of a single concentration (50μM) of butacaine on the translocation of ATP, measured

as a function of ATP concentration, is shown in Fig. 6(*a*). As predicted from the data presented in Fig. 3, butacaine stimulates translocation at low ATP concentrations, but at high concentrations translocation of ATP is inhibited. A similar type of experiment carried out with ADP is shown in Fig. 6(*b*). In this case butacaine is inhibitory at all ADP concentrations tested.

A more direct comparison between the effects of butacaine on ATP and ADP translocation is seen when these data are replotted in a double-reciprocal form so as to compare the K_m and $V_{max.}$ values. The Lineweaver–Burk plot of the data obtained exhibits non-linearity, indicating two binding sites in the translocation reaction (Pffaff *et al.*, 1969; T. L. Spencer & F. L. Bygrave, unpublished work). Thus two apparent K_m values may be obtained. Data in Table 3 show the values obtained for the kinetic constants from these two plots as well as those obtained in the presence of several fixed concentrations of butacaine. As the concentration of the anaesthetic is increased from 0 to 50μM, both the high and low K_m values for ATP decrease, the former becoming non-existent. At the same time the 'low' K_m value falls from 15μM to 5μM, i.e. the affinity for ATP increases some threefold.

With ADP the 'high' K_m value increases from 50μM to 100μM and the 'low' K_m value decreases from 8μM to 6μM as the butacaine concentration is increased to 50μM. ATP translocation seems to be more sensitive to inhibition by butacaine than does that of ADP, as is clear from the greater percentage inhibition for ATP.

One of the most interesting aspects of this experiment is that in the presence of 50μM-butacaine the affinity of the translocase for both ATP and ADP is approximately the same.

Table 2. *Effect of ADP concentration on the kinetic parameters for butacaine inhibition/stimulation of ADP translocation*

Translocation of ADP at the concentrations shown was determined as a function of butacaine concentration (see Fig. 5). Maximal inhibition and K_I values were determined from the data contained in Fig. 5 by the use of double-reciprocal plots.

Concn. of ADP (μM)	Maximal inhibition (%)	K_I (μM)
10	40	60
20	65	32
50	72	28
100	83	20
200	85	18
400	86	18

Influence of local anaesthetics on the translocation of a low concentration of ATP

Data in Fig. 7 show the effect of increasing concentrations of nupercaine, tetracaine and procaine on the translocation of 10μM-ATP. Previously (see Fig. 4) it had been shown that the translocation rate is stimulated by low butacaine concentrations under these conditions, whereas inhibition occurs with high concentrations. This stimulation–inhibition phenomenon was mimicked by both nupercaine and

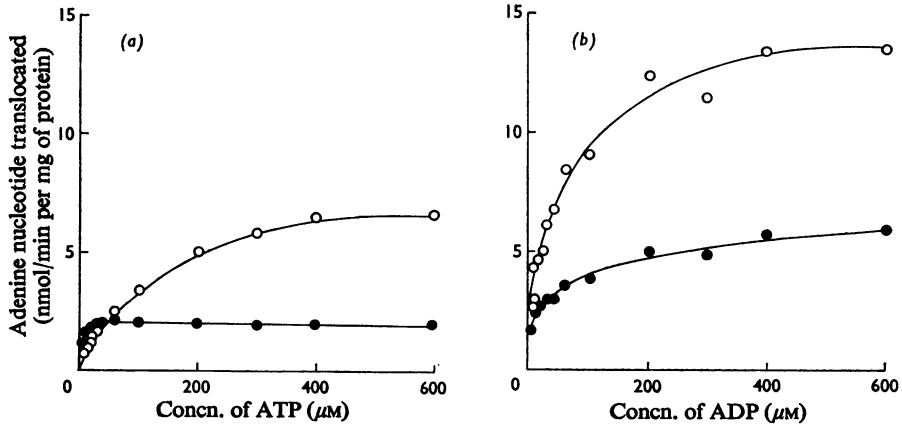

Fig. 6. *Effect of a fixed concentration of butacaine on the translocation of ATP (a) and ADP (b)*

Adenine nucleotide translocase activity was measured as a function of ADP and ATP concentration in the absence (○) and presence (●) of 50μM-butacaine.

Table 3. *Effect of several butacaine concentrations on the kinetic parameters for ATP and ADP translocation*

Experiments of the type shown in Fig. 6 were carried out at three different concentrations of butacaine as indicated. $V_{max.}$ and apparent K_m values were determined from these data by using double-reciprocal plots.

Concn. of butacaine (μM)	ATP			ADP		
	$V_{max.}$ (nmol/min per mg)	Low K_m (μM)	High K_m (μM)	$V_{max.}$ (nmol/min per mg)	Low K_m (μM)	High K_m (μM)
0	7.7	14.5	125	14.7	8	50
10	5.6	14	83	11	8	70
20	3.7	13	45	9	7	85
50	2.2	5	—	7	6	100

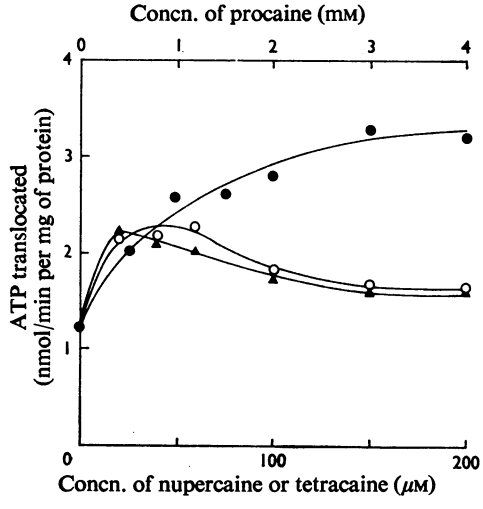

Fig. 7. *Effect of nupercaine, tetracaine and procaine concentration on the translocation of a low ATP concentration*

Mitochondria were incubated as described in the Experimental section with 10μM-ATP and increasing concentrations of nupercaine (▲), tetracaine (○) or procaine (●).

tetracaine. There is one difference, however, in that the inhibition does not result in a return to a rate equal to or less than that in the basal state, i.e. in the absence of anaesthetic.

Procaine also stimulates the translocation of ATP, with a plateau being observed at 5mM, the highest concentration tested in this experiment. Presumably at higher concentrations than this, inhibition would occur. Kinetic parameters calculated from these plots showed that the maximal stimulation was approximately the same for butacaine, nupercaine and tetracaine (77–85%), but with procaine this value was increased to 200%. The apparent K_a values for the stimulation were 10, 12, 15 and 90μM for nupercaine, tetracaine, butacaine and procaine respectively.

Influence of K^+ on butacaine inhibition of ADP and ATP translocation

Both local anaesthetics and K^+ ions are thought to interact with mitochondrial membranes and compete with Ca^{2+} for Ca^{2+}-binding sites (see Azzi & Scarpa, 1967; Johnson & Swartz, 1969). Thus it could be expected that K^+ and Ca^{2+} would interfere with each other in their interactions with these membranes. Data in Table 4 show the effect of 20mM-K^+ on the kinetic parameters obtained for the inhibition of adenine nucleotide translocation by butacaine. In all cases only a slight decrease in the maximal stimulation is obtained over that in the control state. The most striking effect, however, is the large increase in the K_i values obtained in the presence of K^+. This effect is even more pronounced when Ca^{2+} was also present in the incubation medium. Similar results were obtained with ADP, although the effect was not as pronounced.

Influence of pH on the inhibition by butacaine of ATP translocation

In order to characterize further the effects of butacaine, the inhibition was studied in incubation media of different pH values. Data in Fig. 8 show the effect of pH, varied in the range 5.4–8.4, on the kinetic values obtained on this inhibition. The inhibition is least at the lower pH values, being approx. 55% at pH5.4, but as the pH increases, inhibition also increases to reach 80% at pH7.4. No significant change in the K_i of butacaine occurs as a function of pH. Attempts were made to correlate these changes as a function of pH with changes in the proportion of the ionic species of butacaine present in the incubation system. Results from a pH-titration curve for butacaine (data not shown) indicated that this proportion does not change significantly over the pH range tested.

As a comparison with the effect of pH on the inhibitory properties of butacaine, the effect of pH on the stimulation of the translocation of 10μM-ATP by butacaine was also investigated. The degree of stimulation decreased only slightly under acidic

Table 4. *Effect of K^+ on the inhibition of adenine nucleotide translocation by butacaine*

Mitochondria were incubated as described in the legend to Fig. 2 with increasing amounts of butacaine, 200μM-ATP or -ADP, and 200μM-Ca^{2+} when present. KCl when added was at 20mM. Maximal percentage inhibition and K_i values for butacaine were determined by using double-reciprocal plots.

Substrate translocated	Added Ca^{2+}	Maximal inhibition (%)		K_i (μM)	
		K^+ absent	K^+ present	K^+ absent	K^+ present
ATP	Absent	73	67	43	90
	Present	77	75	36	200
ADP	Absent	84	74	22	90
	Present	81	78	27	190

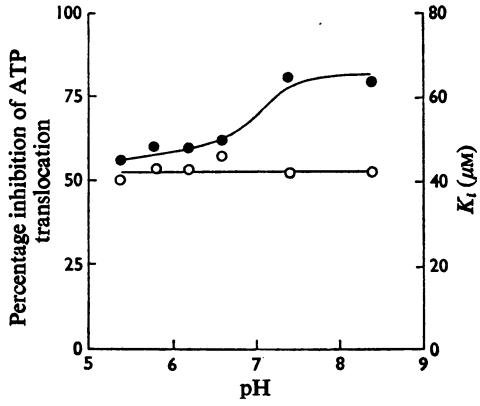

Fig. 8. *Effect of pH on the apparent K_i and percentage maximal inhibition of ATP translocation by butacaine*

Mitochondria were incubated with 200μM-ATP as described in the Experimental section at the pH values indicated and with various concentrations of butacaine. The kinetic constants were determined by using double-reciprocal plots. Percentage in inhibition of translocation (●); apparent K_i (○).

conditions, from 39% at pH7.4 to 32% at pH5.4; the K_m for butacaine remained the same at 10μM-ATP at all pH values tested.

Discussion

The main conclusions to be gathered from the data presented in this paper are first, that several local anaesthetics inhibit the basic translocation of adenine nucleotides by rat liver mitochondria, and secondly, that at low concentrations anaesthetics can preferentially inhibit the Ca^{2+}-stimulated portion of adenine nucleotide translocation, particularly that of ATP. Thus this study has shown that these compounds might be useful in elucidating aspects of the mechanism of adenine nucleotide translocation in mitochondria.

Of the four anaesthetics tested, butacaine was by far the most potent. Both the degree of inhibition and the efficiency of butacaine in inducing this inhibition (as measured by the K_i value) were dependent not only on the particular adenine nucleotide examined, but also on the concentration of ATP or ADP present. Inhibition of the translocase occurred at high adenine nucleotide concentrations, whereas stimulation occurred at low adenine nucleotide concentrations.

The greater potency exhibited by butacaine is most likely attributable to the more hydrophobic nature of its structure (Fig. 1), which would allow it to penetrate the interior of the membrane more readily. The possibility that local anaesthetics are capable of inducing configurational or conformational changes in membranes of mitochondria, has been considered already by Johnson *et al.* (1973).

Inhibition of Ca^{2+}-stimulated translocation

Each of the local anaesthetics tested inhibited the stimulation by Ca^{2+} of ATP translocation. In general, the relative potency of these compounds correlated with their potency of blocking nerve conduction (Seeman, 1972). The exception to this was butacaine. The concentrations necessary for 50% inhibition of Ca^{2+}-stimulated ADP translocation were higher than those required for an equivalent inhibition of Ca^{2+}-stimulated ATP translocation.

Low concentrations of anaesthetics potentiated the stimulatory effect of Ca^{2+} on translocation of ADP but not of ATP. The biphasic nature of this stimulation at low anaesthetic concentrations is of particular interest since it is known that many other surface-active agents stabilize membranes at low concentrations (Seeman, 1966). Similar adenine nucleotide specificity has been observed previously from our laboratory with respect to La^{3+}, a known Ca^{2+} antagonist, which inhibits the stimulation of ATP translocation, but as shown here, potentiates the stimulation of ADP translocation by Ca^{2+} (Spencer & Bygrave, 1972*a*). Thus there appears to

be some form of co-operative interaction between both La^{3+} and Ca^{2+} on the one hand and local anaesthetics and Ca^{2+} on the other, resulting in the stabilization of the mitochondrial membrane which highlights the stimulatory effect of this ion on ADP translocation. In relation to this, Mela (1969) has observed that low concentrations of butacaine stimulate the membrane-alkalinization changes associated with Ca^{2+} accumulation by mitochondria.

Membrane charge seems to be an important factor involved in the ability of butacaine to inhibit translocation. In this regard it is the gross membrane charge elicited by 20mM-K^+ and not the localized positive charge brought about by 50μM-La^{3+} that is the more important. We have shown previously that K^+- and Ca^{2+}-mediated stimulation of ATP translocation are partially additive indicating different binding sites on the mitochondrial membrane for these effectors (Spencer & Bygrave, 1972*a*). Further, the Ca^{2+} but not the K^+ effect is sensitive to low concentrations of La^{3+} (Spencer & Bygrave, 1972*b*).

There is considerable evidence that the physiological sites of action of local anaesthetics are the membranes of various cells and subcellular organelles (Seeman, 1972). Thus it is quite likely that one of the functions of the mitochondria that is influenced by anaesthetic action *in vivo* is adenine nucleotide translocation. This is especially so for butacaine which, as we have shown, has such a high affinity for inhibition of this process.

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