The Action of Local Anaesthetics on Lipolysis and on Adenosine 3':5'-Cyclic Monophosphate Content in Isolated Rat Fat-Cells

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1. Local anaesthetics inhibited hormone-stimulated lipolysis in isolated rat fat-cells. The most potent anaesthetic was dibucaine, which inhibited adrenaline-stimulated lipolysis by 50% at a concentration of 0.16 mM. 2. The amount of inhibition produced by a given concentration of anaesthetic was very similar with adrenaline, theophylline and dibutyryl cyclic AMP, at submaximal and maximal concentrations. 3. The inhibitory effect of dibucaine on lipolysis was apparent within 5 min and was constant over 1 h. 4. Dibucaine inhibited basal, adrenaline-stimulated and insulin-stimulated glucose uptake at concentrations 6-10-fold higher than those inhibiting lipolysis. 5. The effects of dibucaine on lipolysis and glucose uptake were reversed after removal of anaesthetic and washing of cells. 6. Dibucaine further elevated the concentration of cyclic AMP in the presence of adrenaline or adrenaline plus theophylline. 7. Dibucaine had no effect on ATP content at concentrations causing 80% inhibition of lipolysis, but lowered ATP content at higher concentrations. 8. The relative potency of different local anaesthetics as inhibitors of hormone-stimulated lipolysis paralleled their potency as inhibitors of ion movements in other systems. 9. The possibility is discussed that Ca^{2+} ions are involved in the regulation of lipolysis, and that local anaesthetics inhibit lipolysis by interfering with Ca^{2+} translocation.

The involvement of cyclic AMP in the lipolytic action of hormones such as adrenaline has been well documented (Robison *et al.*, 1971). The possibility that cations are of regulatory importance in adipose tissue has also been considered. It has been shown that changes in the ionic composition of incubation media can modify hormone action and the activity of hormonally sensitive processes in intact cells *in vitro* and in broken-cell preparations (Bleicher *et al.*, 1966; Mosinger & Vaughan, 1967; Hales *et al.*, 1968; Gorski & Krishna, 1972). Further, agents which inhibit ion translocation can mimic some hormonal effects (Mosinger & Kujalova, 1966; Ho *et al.*, 1967; Martin *et al.*, 1972) and both lipolytic and antilipolytic hormones can modify ion fluxes and membrane potential (Perry & Hales, 1970; Krishna *et al.*, 1970) in adipose tissue. The action of ions at specific sites between initial hormone binding and final effect has not been defined in detail. Recently, interest has focused particularly on the possible role of Ca^{2+} ions in the hormonal control of lipolysis (Efendic *et al.*, 1970; Werner *et al.*, 1972; Exton *et al.*, 1972; Schimmel, 1973), and the interactions between Ca^{2+} ions and cyclic AMP in various tissues have been discussed (Rasmussen, 1970).

One approach to the study of the role of ions in intracellular regulation is the use of compounds that

interfere with ion translocation. Local anaesthetics such as procaine and dibucaine exert their action on nervous tissue by inhibition of cation movement across the cell membrane (Ritchie *et al.*, 1970). Local anaesthetics have been shown to affect Ca^{2+} -dependent processes in various tissues (Jaanus *et al.*, 1967; Feinstein & Paimre, 1969; Johnson & Inesi, 1969; Friedmann & Rasmussen, 1970) and in artificial membranesystems (Papahadjopoulos, 1972).

We have studied the effect of local anaesthetics on lipolysis, glucose uptake and the content of ATP and cyclic AMP in isolated rat fat-cells. The anaesthetics inhibited hormone-stimulated lipolysis while further increasing the concentration of cyclic AMP. Preliminary reports of parts of this work have been presented (Hales, 1970; Siddle & Hales, 1972).

Experimental

Materials

Collagenase was obtained from Worthington Biochemical Corp., Freehold, N.J., U.S.A.; bovine serum albumin (fraction V) was from Armour Pharmaceutical Co., Ltd., Eastbourne, Sussex, U.K.; adrenaline was from Hopkin and Williams Ltd., Chadwell Heath, Essex, U.K.; dibutyryl cyclic AMP

(*N*-6, *O*-2'-dibutyryladenosine cyclic 3':5'-mono-phosphate) was from Boehringer Corp. (London) Ltd., London W.5, U.K.; crystalline ox insulin (24 i.u./mg) was a gift of Dr. B. A. L. Hurn, Wellcome Research Laboratories, Beckenham, Kent, U.K.; porcine glucagon was a gift from Ely Lilly and Co., Indianapolis, Ind., U.S.A.; human growth hormone was kindly given by Dr. A. Stockell-Hartree and porcine adrenocorticotrophic hormone by Dr. H. B. F. Dixon, both of the Department of Biochemistry, University of Cambridge, U.K.; dibucaine hydrochloride was from CIBA Ltd., Horsham, Sussex, U.K.; other local anaesthetics, as their hydrochlorides, were obtained from the hospital pharmacy.

Methods

Isolated rat fat-cells (Rodbell, 1964) were prepared from epididymal fat-pads of male albino Wistar rats weighing 120–140 g and given free access to food and water. Incubations were carried out in Krebs–Ringer bicarbonate buffer (Cohen, 1957) containing 4% (w/v) bovine serum albumin and 2 mM-glucose. Portions (1 ml) of cell suspension were incubated in capped polythene vessels (75 mm × 25 mm), with a gas phase of O₂+CO₂ (95:5) in shaking incubators at 37°C. Each experiment was performed with cells prepared in a single batch, with three or four portions of cells for each measurement. The dry weight of cells in 1 ml of suspension was used as the basis for expression of results. Incubations were terminated by addition of 1 ml of 10% (w/v) trichloroacetic acid. The acid was removed from the deproteinized extract by shaking with 4 × 5 ml of diethyl ether.

Glucose uptake was determined by measuring the glucose content of extracts of cell suspension before and after incubation, by the method of Huggett & Nixon (1957). Glycerol formation, measured by the method of Garland & Randle (1962), was taken as an index of the rate of lipolysis.

Cyclic AMP concentration was determined by radioimmunoassay (Siddle *et al.*, 1973) after the extracts of cell suspension had been concentrated 3-fold by freeze-drying and redissolving in 100 mM-potassium phosphate buffer, pH 7.0. When cyclic AMP was to be measured separately in cells and incubation medium, a portion of cell suspension was centrifuged for 15 s and the infranant removed. Trichloroacetic acid (10%, w/v) was added to the separated cells and medium, and extracts were prepared as for the whole cell suspension.

ATP was assayed by the method of Addanki *et al.* (1966) in extracts of cell suspension freeze-dried and redissolved in 0.1 M-NaOH. When ATP was to be measured, samples were kept on ice during the cell extraction procedure, and standard ATP solutions were extracted in the same way from incubation medium.

Results

Relative potency of local anaesthetics

Various structurally related anaesthetics (Ritchie *et al.*, 1970) were tested as inhibitors of lipolysis in isolated fat-cells by measuring their effect on glycerol formation during a 1 h incubation with adrenaline. The estimated concentrations causing 50% inhibition of lipolysis were: procaine, 7.2 mM; lignocaine, 3.8 mM; metycaine, 1.3 mM; tetracaine, 0.35 mM; dibucaine, 0.16 mM (Fig. 1). Dibucaine, the most potent anaesthetic tested, was used in further experiments.

The relative potencies of these compounds as inhibitors of lipolysis and in systems studied by other workers are shown in Table 1. The correspondence between the potencies in the different systems suggests that there may be a common basis for the various effects of the anaesthetics.

Effect of local anaesthetics with various lipolytic stimuli

The effect of dibucaine on lipolysis was tested in the presence of adrenaline, theophylline and dibutyryl cyclic AMP, representing three different types of lipolytic stimulus (Table 2). The amount of inhibition produced by 0.25 mM-dibucaine was very similar

Fig. 1. Potency of local anaesthetics as inhibitors of adrenaline-stimulated lipolysis

Glycerol formation was measured in cells incubated with anaesthetic and 55 μ M-adrenaline for 1 h. Incubations were terminated by addition of 10% trichloroacetic acid, and extracts were prepared as described in the text. Cell concentration was 20 mg dry wt./ml. Glycerol formation in the absence of anaesthetic was 50 μ mol/h per g. Each point is the mean of three observations. Abbreviations: P, procaine; L, lignocaine; M, metycaine; T, tetracaine; D, dibucaine.

Table 1. *Relative potencies of local anaesthetics*

Potency is expressed relative to that of procaine in each system. The potency for inhibition of lipolysis is the reciprocal of the concentration giving 50% inhibition in the presence of 55 μ M-adrenaline.

	Procaine	Lignocaine	Metycaine	Tetracaine	Dibucaine
Inhibition of lipolysis in fat-cells	1	1.9	5.5	21	46
Anaesthetic potency (Blaustein & Goldman, 1966)	1	3.8	3.5	36	54
Inhibition of Ca ²⁺ binding to phospholipid (Blaustein & Goldman, 1966)	1	3.5	4.7	8.7	10
Inhibition of Ca ²⁺ -induced Na ⁺ permeability in phospholipid vesicles (Papahadjopoulos, 1972)	1	2.5	—	45	135
Inhibition of Ca ²⁺ -mediated catecholamine secretion in adrenals (Jaanus <i>et al.</i> , 1967)	1	—	—	33	122

Table 2. *Inhibition of lipolysis by dibucaine with various lipolytic stimuli*

Cell suspension (1 ml; 11 mg dry wt./ml) was incubated with adrenaline, dibutyryl cyclic AMP or theophylline, with or without 0.25 mM-dibucaine, for 1 h. Basal glycerol formation was $2.3 \pm 0.2 \mu\text{mol/h}$ per g dry wt. Each value is the mean $\pm$ S.E.M. of three observations.

Stimulus	Glycerol formation ($\mu\text{mol/h}$ per g dry wt.)		Inhibition by dibucaine (%)
	No dibucaine	0.25 mM-Dibucaine	
Adrenaline			
0.55 μM	7.5 ± 0.3	3.6 ± 0.3	52 ± 4
5.5 μM	80.4 ± 7.6	24.0 ± 2.7	70 ± 4
55 μM	83.8 ± 6.7	31.2 ± 3.2	63 ± 5
Dibutyryl cyclic AMP			
0.5 mM	8.9 ± 0.8	4.4 ± 1.4	51 ± 16
2 mM	130.0 ± 15.1	44.5 ± 4.3	66 ± 5
8 mM	130.1 ± 8.0	51.2 ± 2.5	61 ± 3
Theophylline			
0.25 mM	21.4 ± 1.1	8.1 ± 0.3	62 ± 2
1 mM	136.5 ± 7.0	42.4 ± 7.2	69 ± 5
4 mM	109.3 ± 7.0	40.6 ± 9.2	63 ± 9

with all three stimuli at maximal and submaximal concentrations. In other experiments using both dibucaine and metycaine, the sensitivity of lipolysis to inhibition by anaesthetic was also similar when the stimulus was glucagon (1 $\mu\text{g/ml}$), adrenocorticotrophic hormone (10 $\mu\text{g/ml}$) or growth hormone (10 $\mu\text{g/ml}$) plus cortisol (5 $\mu\text{g/ml}$). In some experiments it appeared that hormone-stimulated lipolysis was more sensitive to inhibition by dibucaine than was lipolysis stimulated by dibutyryl cyclic AMP (Fig. 3), but this was not a consistent finding (Table 2).

Time-course of inhibition of lipolysis

Glycerol formation in fat-cells stimulated with adrenaline was measured at several times over a 1 h period in the presence and absence of dibucaine, adrenaline and dibucaine being added to the cells simultaneously at the start of the incubation (Fig. 2).

The inhibitory effect of dibucaine on lipolysis was apparent after 5 min, the earliest time of measurement, and remained constant over 1 h.

Effect of dibucaine on glucose uptake

Dibucaine inhibited basal and stimulated glucose uptake in fat-cells. The concentration-dependence of this effect was compared with that for inhibition of lipolysis in the presence of adrenaline and dibutyryl cyclic AMP (Fig. 3). In this experiment dibucaine appeared to inhibit adrenaline-stimulated lipolysis at slightly lower concentrations than were effective with dibutyryl cyclic AMP-stimulated lipolysis. With both stimuli, in several similar experiments, lipolysis was 6–10-fold more sensitive than glucose uptake to inhibition by dibucaine. Basal glucose uptake and glucose uptake stimulated by 0.7 nM-insulin were very similar to adrenaline-stimulated glucose uptake in their sensitivity to dibucaine.

Reversibility of effects of dibucaine

The inhibitory effect of dibucaine on lipolysis and glucose uptake was fully reversible (Table 3). When dibucaine (4 mM) was present throughout incubation with adrenaline, stimulation of lipolysis was completely inhibited, and basal and stimulated glucose uptakes were decreased almost to zero. When adrenaline was added immediately after washing and resuspension of cells that had been preincubated with dibucaine, lipolysis was stimulated to 50% of maxi-

Fig. 2. Time-course of lipolysis with and without dibucaine

Cells were incubated with $5.5 \mu\text{M}$ -adrenaline with or without dibucaine, all additions being made simultaneously at the start of the incubation. Cell concentration was 20 mg dry wt./ml. Each point is the mean $\pm$ S.E.M. of three observations. $\bullet$, No dibucaine; $\circ$, 0.25 mM-dibucaine; $\blacksquare$, 1 mM-dibucaine.

mum and glucose uptake to almost 100%. After a wash period of 2 h between resuspension of dibucaine-preincubated cells and addition of adrenaline the

Fig. 3. Effect of dibucaine on glucose uptake and lipolysis

Glycerol formation and glucose uptake were measured in cells incubated with dibucaine and either 4 mM-dibutyryl cyclic AMP or $5.5 \mu\text{M}$ -adrenaline for 1 h. Results are expressed as percentage of values in the absence of dibucaine for each set of values. Absolute values of responses without dibucaine were: glycerol formation: adrenaline, $80 \mu\text{mol/h per g}$ ($\bullet$); dibutyryl cyclic AMP, $105 \mu\text{mol/h per g}$ ($\circ$); glucose uptake: adrenaline, $75 \mu\text{mol/h per g}$ ($\blacksquare$); dibutyryl cyclic AMP, $40 \mu\text{mol/h per g}$ ($\square$). Cell concentration was 15 mg dry wt./ml. Each point is the mean of three observations.

Table 3. Reversibility of effect of dibucaine on glucose uptake and lipolysis

Cells were preincubated without (A) or with (B) 4 mM-dibucaine for 15 min and then washed with $3 \times 10 \text{ ml}$ of medium without dibucaine. Glycerol formation and glucose uptake were measured over a 1 h period with and without $55 \mu\text{M}$ -adrenaline either immediately or after a wash period of 2 h followed by resuspension in fresh medium. Other cells (C) were preincubated with 4 mM-dibucaine and then incubated for 1 h with and without $55 \mu\text{M}$ -adrenaline in the continued presence of dibucaine. Cell concentration was 15–25 mg dry wt./ml. Each result is the mean $\pm$ S.E.M. of four observations.

	Wash period (h)	Glycerol formation ($\mu\text{mol/h per g dry wt.}$)		Glucose uptake ($\mu\text{mol/h per g dry wt.}$)	
		Basal	+Adrenaline ($55 \mu\text{M}$)	Basal	+Adrenaline ($55 \mu\text{M}$)
(A) Preincubated without dibucaine for 15 min	2	1.3 ± 0.3	51.7 ± 1.3	25.0 ± 0.6	55.4 ± 1.7
(B) Preincubated with 4 mM-dibucaine for 15 min	0	1.1 ± 0.1	28.7 ± 1.3	26.2 ± 2.1	57.0 ± 0.8
	2	2.0 ± 0.2	57.8 ± 1.7	24.7 ± 2.9	57.6 ± 0.9
(C) Incubated with 4 mM-dibucaine throughout	—	0.3 ± 0.1	0.3 ± 0.1	1.8 ± 1.8	0.2 ± 0.1

stimulation of lipolysis was the same as in cells pre-incubated without anaesthetic. The effect of dibucaine was therefore fully reversible but not instantaneously so after removal of extracellular anaesthetic. The delay in recovery of normal stimulation of lipolysis, compared with recovery of glucose uptake,

Table 4. *Effect of dibucaine on cyclic AMP concentration*

Total cyclic AMP content in the cell suspension was measured after 5 min incubation with dibucaine, with and without 55 μ M-adrenaline. Cell concentration was 13 mg dry wt./ml. Each result is the mean $\pm$ S.E.M. of four observations.

Dibucaine (mM)	Cyclic AMP content (nmol/g dry wt.)	
	No adrenaline	+Adrenaline (55 μ M)
0	0.30 $\pm$ 0.01	1.07 $\pm$ 0.11
0.015	0.29 $\pm$ 0.02	0.93 $\pm$ 0.10
0.06	0.24 $\pm$ 0.03	1.05 $\pm$ 0.09
0.25	0.29 $\pm$ 0.03	1.57 $\pm$ 0.09
1	0.32 $\pm$ 0.02	3.53 $\pm$ 0.29
4	0.42 $\pm$ 0.02	12.90 $\pm$ 1.54

Fig. 4. *Effect of dibucaine on cyclic AMP content of cells and incubation medium*

Cells were incubated with 55 μ M-adrenaline plus 1 mM-theophylline, with or without dibucaine. Cyclic AMP concentration was measured separately in (a) cells and (b) incubation medium. Cell concentration was 20 mg dry wt./ml. Each point is the mean $\pm$ S.E.M. of four observations. ●, No dibucaine (glycerol formation 58.2 μ mol/h per g); ○, 1 mM-dibucaine (glycerol formation 13.6 μ mol/h per g).

was probably due to the relative sensitivities of the two processes (Fig. 3) to inhibition by traces of anaesthetic remaining after washing the cells or initially retained within the cells.

Effect of dibucaine on cyclic AMP content

The effect of dibucaine on total cyclic AMP content of the cell suspension was investigated after 5 min incubation in the presence and absence of adrenaline (Table 4); 4 mM-dibucaine increased basal cyclic AMP content by only 40%, whereas in the presence of adrenaline cyclic AMP content was increased 12-fold. The magnitude of the dibucaine effect varied from one cell preparation to another and in some experiments the increases in cyclic AMP content were only about half of those shown in Table 4.

Fig. 5. *Effect of dibucaine on ATP content*

Cells were incubated with dibucaine and 5.5 μ M-adrenaline for 5 min and 30 min. Glycerol formation (a) measured after 30 min was expressed as a percentage of the value in the absence of anaesthetic. ATP content (b) measured at 5 min (○) and 30 min (●) was expressed as a percentage of the value at the start of the incubation. Mean glycerol formation was 18.5 μ mol/30 min per g dry wt. and mean initial ATP content was 266 nmol/g dry wt. Each point is the mean $\pm$ S.E.M. from three separate experiments using different batches of cells, with cell concentrations in the range 19–37 mg dry wt./ml.

The effect of 1 mM-dibucaine was also tested on cells incubated with adrenaline plus theophylline. Cyclic AMP was measured separately in cells and incubation medium at various times up to 1 h (Fig. 4). Although the concentration of cyclic AMP produced by adrenaline plus theophylline was very high relative to concentrations produced by either compound alone, dibucaine caused a further increase in cyclic AMP in both cells and incubation medium. The time-course of cyclic AMP content in the cells, which showed an early peak followed by a decline, was not affected by the local anaesthetic.

Effect of dibucaine on ATP content

ATP content was measured in fat-cells after 5 min and 30 min of incubation with adrenaline (Fig. 5). ATP content was expressed as a percentage of the initial value before addition of adrenaline and dibucaine. Incubation with adrenaline caused a small fall in ATP content, which was greater after 30 min than at 5 min. Dibucaine at concentrations up to 1 mM, which was sufficient to inhibit lipolysis by more than 80%, had no significant effect on ATP content; 4 mM-dibucaine caused a considerable, though rather variable, fall in ATP content, the decrease relative to values in the absence of anaesthetic being the same at 30 min as at 5 min. The same pattern of effects of dibucaine on ATP content was seen in the absence of adrenaline.

Discussion

Local anaesthetics inhibited hormone-stimulated lipolysis and glucose uptake, elevated cyclic AMP content and lowered ATP content in isolated rat fat-cells. When the most potent anaesthetic, dibucaine, was used, effects on lipolysis were observed at concentrations 6–10-fold lower than those required to cause changes in the other parameters studied. Local anaesthetics share with a variety of compounds, including tolbutamide (Fain *et al.*, 1972; Brown *et al.*, 1972), naphthoquinones (Kuo, 1969; Fain, 1971), tricyclic anti-depressants (Lovrien *et al.*, 1972) and phenformin (Brown *et al.*, 1973), the property that they inhibit hormone-stimulated lipolysis while further increasing elevated concentrations of cyclic AMP. Insufficient is known about the mechanism of action of any of these compounds to indicate whether they share any common features in their actions on lipolysis and cyclic AMP concentration. The fact that a given concentration of dibucaine produced a similar inhibition of lipolysis with a variety of stimuli (Table 2), and did not prevent a rise in cyclic AMP content with adrenaline (Table 4, Fig. 4), implies that the anaesthetic acts at one of the later reactions in the sequence between initial hormone binding and final activation of triglyceride lipase. This sequence prob-

ably involves phosphorylation of the lipase by a cyclic AMP-dependent protein kinase (Robison *et al.*, 1971; Huttunen & Steinberg, 1971; Soderling *et al.*, 1973). It is possible that local anaesthetics affect this process by inhibiting the activity of protein kinase or triglyceride lipase. These possibilities have not been studied, and investigation of the direct effect of local anaesthetics on lipase activity in cell-free systems is of doubtful value considering the low lipase activity obtained after disruption of cells exposed to lipolytic hormones (Hales *et al.*, 1968; Huttunen & Steinberg, 1971).

The action of local anaesthetics did not appear to be dependent on an effect on the concentration of ATP within the cell, as a concentration of dibucaine causing more than 80% inhibition of adrenalin-stimulated lipolysis did not significantly alter fat-cell ATP content (Fig. 5). Higher concentrations of dibucaine caused a lowering of ATP content (Fig. 5), but this did not prevent a considerable elevation of cyclic AMP concentration (Table 4). The action of dibucaine did not appear to be dependent on a progressive effect on cell metabolism or structure, as it was very rapid in onset and constant over 1 h (Fig. 2), was readily reversible (Table 3) and was not accompanied by any changes in ultrastructure visible under the electron microscope (L. Herman & C. N. Hales unpublished work).

A further possibility to be considered is that the action of local anaesthetics on lipolysis is related to an effect on ion translocation, as suggested by the relative potencies of anaesthetics in a variety of systems (Table 1). The effect of tetracaine on perfused liver is of particular interest in that glucagon-stimulated glycogenolysis, but not basal glucose output, is inhibited, and the normal stimulation of Ca^{2+} release by glucagon is prevented, but there is no effect on elevation of hepatic cyclic AMP content (Friedmann & Rasmussen, 1970). In skeletal and cardiac muscle it has been shown that Ca^{2+} concentration is important in addition to cyclic AMP in the control of glycogenolysis (Namm *et al.*, 1968; Heilmeyer *et al.*, 1970; Ozawa, 1972). It has been suggested that the rate of lipolysis in adipose tissue may similarly be dependent on intracellular Ca^{2+} concentration (Efendic *et al.*, 1970; Werner *et al.*, 1972; Exton *et al.*, 1972). Using antimonate precipitation and analytical electron microscopy, Hales *et al.* (1974) demonstrated the existence of Ca^{2+} ions in the smooth endoplasmic reticulum of fat-cells, and suggested that this system may be functionally analogous to the sarcoplasmic reticulum of skeletal muscle. The inhibitory effect of local anaesthetics on hormone-stimulated lipolysis in adipose tissue lends further support to the idea that, as in other tissues (Rasmussen, 1970), changes in Ca^{2+} concentration, in addition to the content of cyclic AMP, may be important in mediating hormone action, in this case the activation of triglyceride lipase.

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