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EFFECT OF HIGHLY SPECIFIC INHIBITORS OF OXIDATIVE PHOSPHORYLATION, DICYCLOHEXYLCARBODIIMIDE, ANTIMYCIN A AND PIERICIDIN A, ON CYCLIC AMP ACCUMULATION INDUCED BY NORADRENALINE AND THEOPHYLLINE IN RAT ADIPOSE TISSUE

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Abstract—The effect of three inhibitors of oxidative phosphorylation (acting at different levels), dicyclohexylcarbodiimide, antimycin A and piericidin A, was tested on cyclic AMP accumulation and lipolysis in rat epididymal fat.

Dicyclohexylcarbodiimide, antimycin and piericidin, showed a marked inhibitory effect on cyclic AMP accumulation induced by noradrenaline plus theophylline. The same drugs inhibited also the lipolytic response of adipose tissue to noradrenaline, theophylline and dibutyryl cyclic AMP. The effect on cyclic AMP was quantitatively similar to that on lipolysis in the case of antimycin and dicyclohexylcarbodiimide. In contrast, piericidin was less effective on the cyclic AMP level than on lipolysis.

These data suggest that the synthesis of cyclic AMP in adipose tissue, is dependent on oxidative phosphorylation, but to a lesser degree than the whole final lipolytic process.

THIS paper reports further investigations on the effect of oxidative phosphorylation inhibitors on the hormonal control of lipolysis. These studies aim to establish in which way, and to what extent, the lipolytic effect of catecholamines on rat white adipose tissue is linked to energy availability in the adipocyte. Changes in ATP level could affect cyclic AMP synthesis and/or the activation of lipolysis by cyclic AMP.

Previous studies have indicated inhibition of hormone-induced lipolysis by several inhibitors and uncouplers of oxidative phosphorylation (as reviewed by Fain[1]). Some of these drugs (rotenone, dinitrophenol, oligomycin and ionophorous antibiotics) were also tested on cyclic AMP accumulation induced by noradrenaline and/or theophylline.[2–5] The effects on cyclic AMP level were, sometimes, quantitatively discordant, or even in contrast, with the inhibitory effect shown by the same drugs on lipolysis. Oligomycin and dinitrophenol induced changes in the rate of lipolysis and on cyclic AMP levels, were quantitatively similar.[2] Rotenone was, however, less effective on cyclic AMP level than on lipolysis. The fact could be correlated with the different site and mechanism of action of rotenone on oxidative phosphorylation. This hypothesis led us to investigate the effect on cyclic AMP levels of three other specific inhibitors of oxidative phosphorylation acting at different levels on the process: (a) piericidin A, which inhibits electron transport in the respiratory chain at the same site as rotenone,[6,7] (b) antimycin A, one of the most potent inhibitors of the mitochondrial respiration whose action is localized between cytochromes *b*

and c_1,[7] (c) dicyclohexylcarbodiimide (DCCD) which produces the same effect on oxidative phosphorylation as oligomycin,[7,8] but whose action is much more specific.[9]

The effect of these three inhibitors was tested on cyclic AMP level and was compared with their action on lipolysis. All the drugs strongly inhibited cyclic AMP accumulation induced by noradrenaline and theophylline. In our experimental conditions, the effect on cyclic AMP was quantitatively similar to that on hormone-stimulated lipolysis in the case of antimycin and of DCCD. In contrast, piericidin affected cyclic AMP accumulation to a lesser extent than lipolysis, similar to the effect of rotenone.

MATERIALS AND METHODS

Epididymal fat was obtained from fed Sprague–Dawley rats weighing 200–230 g. Preparation of fat pads for determination of lipolysis and cyclic AMP accumulation, was as previously described.[10] Samples of adipose tissue were weighed (100 ± 5 mg for lipolysis, 200 ± 10 mg for cyclic AMP determination) and added to 1·90 ml of Krebs–Ringer bicarbonate, pH 7·2, containing 2·5% bovine albumin and, where indicated, the metabolic inhibitors. After a preliminary incubation in a metabolic shaker at 37° for 30 min, noradrenaline and theophylline were added (each dissolved in 50 μl of 0·9 NaCl) and the incubation continued. Glycerol and free fatty acids were measured after 150 min, while cyclic AMP was measured after 10 min.

At the end of the incubation, in samples for lipolysis determination the reaction was stopped by addition of 2·5 N H_2SO_4 (0·1 ml/assay) and, after centrifugation, the free fatty acids determined in the incubation medium according to Dole[11] and glycerol according to Korn.[12] In the samples for cyclic AMP determination tissue and medium were, at the end of incubation, immediately separated by filtration and washed (1 ml of 0·9 NaCl) under vacuum. The fat was rapidly added to 1 ml of ice-cold 3% TCA and homogenized in a Potter homogenizer with teflon pestle. Ice-cold TCA (50%) (0·15 ml) was added to the incubation medium.

Isolation and assay of cyclic AMP. The tissue homogenates and the incubation medium were neutralized with 1 M Tris and centrifuged as previously described in detail.[2] Cyclic AMP was purified from the neutralized supernatants by the $BaSO_4$ method of Krishna *et al.*[13] with subsequent ion exchange chromatography (AG 50W-X8 columns) according to Kuo and Greengard,[14] as reported in detail in a previous paper.[2]

The nucleotide levels were measured by the method of Kuo and Greengard[14] based upon the ability of a protein kinase from bovine heart to catalyse the transfer of ^{32}P from γ^{32} P-ATP to histone in a cyclic AMP-dependent reaction. The enzyme preparation was that obtained after the DEAE–cellulose step of purification as described by Kuo *et al.*[15] Histone-bound ^{32}P was measured in a Beckman liquid scintillation counter. The scintillation solvent used was 0·5% PPO in (50%, v/v) toluene and methyl cellosolve. Details of the standard curve, the K_m-value for cyclic AMP and the overall recovery of the synthetic nucleotide added to the control samples, were reported previously.[2]

Materials. DEAE–cellulose (medium mesh, 0·85 m-equiv./g), histone (Type II), cyclic AMP, ATP and bovine serum albumin (Fraction V) were purchased from Sigma. AG 50W-X8 resin (200–400 mesh) in the hydrogen form, analytical grade, from BioRad Laboratories, was repeatedly washed in distilled water to remove the

fine particles. ^{32}P-ATP was obtained from the Radiochemical Centre, Amersham. Noradrenaline bitartrate monohydrate was from Recordati, DCCD (A grade) from Calbiochem, antimycin A (Type III) from Sigma, piericidin A, originally from Dr. N. Takahashi (University of Tokyo) through the courtesy of Dr. S. Luciani. The three inhibitors were dissolved in ethanol. An equal amount (50 μl) of ethanol was added to the control samples. Other chemicals were reagent grade from standard suppliers.

$^{6}NC_2$'-dibutyryl cyclic 3',5'-adenosine-monophosphate was a generous gift from Dr. M. Carissimi (Maggioni, Milano, Italy).

RESULTS

Effect of piericidin, antimycin and dicyclohexylcarbodiimide (DCCD), on the basal level and on the increase of cyclic AMP induced by noradrenaline and theophylline. Piericidin (Table 1) and DCCD (Table 3) did not significantly alter the basal level of cyclic AMP in epididymal fat or in the medium, while antimycin (Table 2) slightly increased the cyclic nucleotide concentration in adipose tissue.

Only the synergistic effect of noradrenaline and theophylline on the cyclic nucleotide synthesis and accumulation, was suitable for investigating the effect of inhibitors. When present alone, noradrenaline or theophylline induced only a small and somewhat variable increase in cyclic AMP level, in accordance with previous data.[2,14,16,17]

Piericidin (Table 1), antimycin (Table 2) and DCCD (Table 3), were all found to inhibit the effect of noradrenaline plus theophylline in elevating the level of cyclic AMP both inside adipose tissue as well as in the medium. The total amount of cyclic AMP which accumulated in tissue plus medium is reported in the last column of all Tables. The total inhibitory effect of piericidin at a concentration as low as 2 nmoles/mg tissue protein, of antimycin at 10 nmoles/mg tissue protein, and of DCCD at 126 nmoles/mg tissue protein, was 70, 90 and 50 per cent, respectively.

It should be noted that these quantitative data are only indicative, because of the presence of albumin in the incubation medium (12·5 mg bovine serum albumin/mg tissue protein). It has been reported that antimycin inhibition of the respiratory chain may be reversed by addition of serum albumin.[18] We found, however, that DCCD and piericidin were much less effective against cyclic AMP accumulation, when adipose tissue was incubated in a medium deprived of albumin. The quantitative data are interesting mostly in view of the comparison between the effects of these drugs on cyclic AMP and on lipolysis.

Effect of piericidin, antimycin and DCCD, on lipolysis induced by noradrenaline, theophylline and dibutyryl cyclic AMP. The three drugs had no effect on basal lipolysis. The free fatty acids (FFA) and glycerol release induced by noradrenaline from adipose tissue, were both inhibited by piericidin (Table 4) and by DCCD (Table 5). Previous data for the effect of piericidin on isolated adipose cells[19] agree with our findings. The inhibitory effect of piericidin and DCCD was also seen with theophylline and dibutyryl cyclic 3',5'-AMP stimulated lipolysis (Tables 4 and 5).

Antimycin has been previously shown to inhibit lipolysis stimulated by noradrenaline, by theophylline, and by dibutyryl cyclic AMP[20] in analogous experimental conditions. It was possible therefore to compare the effect of the three inhibitors tested, on the rate of hormone-stimulated lipolysis and on the level of cyclic AMP increased by noradrenaline plus theophylline (Table 6). The changes induced by DCCD on the rate of lipolysis, as well as on cyclic AMP levels, are similar. Antimycin

Table 1. Inhibitory effect of piericidin A on the basal level and on the increase of cyclic AMP induced by noradrenaline and theophylline in rat adipose tissue

Drugs in the medium (M)	Cyclic AMP pmoles/g fresh tissue			
	Tissue	Medium	Tissue + medium	
	143·82 ± 21·36	76·25 ± 10·90	220·07	
Piericidin $3·8 \times 10^{-6}$	91·70 ± 7·79 ($P > 0·05$)	156·49 ± 20·90 ($P > 0·02$)	248·19	
Noradrenaline 10^{-5} + theophylline 0·003	25413·79 ± 1377·50	652·45 ± 110·00	26066·24	
Noradrenaline 10^{-5} + theophylline 0·003 + piericidin $3·8 \times 10^{-6}$	7616·41 ± 159·27 (−70%) ($P < 0·001$)	287·76 ± 18·47 (−56%) ($P < 0·02$)	7904·17	(−70%)

Rat epididymal fat (200 ± 5 mg) was added to 2 ml of Krebs–Ringer bicarbonate containing bovine albumin and, where indicated, piericidin. After pre-incubation for 30 min at 37°, theophylline and noradrenaline were added and the samples incubated for another 10 min. At the end of the incubation, tissue and medium were treated as indicated in the Methods. Cyclic AMP was then extracted, purified and titrated by the use of bovine heart protein kinase (8 μg). The data are the means (± S.E.) of three to four assays. Piericidin $3·8 \times 10^{-6}$ M corresponds to 0·14 nmole/mg total protein (tissue + medium albumin) and to 2 nmoles/mg tissue protein.

TABLE 2. INHIBITORY EFFECT OF ANTIMYCIN A ON THE BASAL LEVEL AND ON THE INCREASE OF CYCLIC AMP INDUCED BY NORADRENALINE AND THEOPHYLLINE IN RAT ADIPOSE TISSUE

Drugs in the medium (M)	Cyclic AMP pmoles/g fresh tissue		
	Tissue	Medium	Tissue + medium
—	64·62 ± 5·50	0	64·62
Antimycin 2×10^{-5}	145·26 ± 9·47*	0	145·26
Noradrenaline 10^{-5} + theophylline 0·003	21868·00 ± 805·25	344·01 ± 42·00	22170·01
Noradrenaline 10^{-5} + theophylline 0·003 + antimycin 2×10^{-5}	2412·15 ± 339·31* (−89%)	0 (−100%)	2412·15 (−90%)

Experimental conditions are described under Table 1. The data are the means (±S.E.) of three to five assays. Antimycin 2×10^{-5} M corresponds to 0·8 nmole/mg total protein (tissue + medium albumin) and to 10 nmoles/mg tissue protein.
* $P < 0{\cdot}01$.

has a somewhat lower effect on lipolysis than on cyclic AMP accumulation induced by noradrenaline and theophylline. Piericidin was less effective on the cyclic AMP level than on lipolysis inhibiting cyclic AMP accumulation by 70 per cent, at a concentration ten times higher ($3{\cdot}8 \times 10^{-6}$ M) than that ($3{\cdot}8 \times 10^{-7}$ M) inhibiting lipolysis to the same extent. This is quite in accordance with the behaviour of rotenone[2] and, therefore, with the previously mentioned hypothesis.

TABLE 3. INHIBITORY EFFECT OF DICYCLOHEXYLCARBODIIMIDE (DCCD) ON THE BASAL LEVEL AND ON THE INCREASE OF CYCLIC AMP INDUCED BY NORADRENALINE AND THEOPHYLLINE IN RAT ADIPOSE TISSUE

Drugs in the medium (M)	Cyclic AMP pmoles/g fresh tissue		
	Tissue	Medium	Tissue + medium
	47·61 ± 26·44	219·37 ± 22·93	266·98
DCCD $2{\cdot}5 \times 10^{-4}$	141·40 ± 36·40 (NS)	136·15 ± 34·19 (NS)	277·55
Noradrenaline 10^{-5} + theophylline 0·003	31039·60 ± 930·50	1585·49 ± 96·46	32625·09
Noradrenaline 10^{-5} + theophylline 0·003 + DCCD $2{\cdot}5 \times 10^{-4}$	16087·00 ± 1138·17* (−49%)	403·98 ± 44·38* (−75%)	16490·98 (−50%)

Experimental conditions are described under Table 1. Each value represents the means (± S.E.) of three to four assays. DCCD $2{\cdot}5 \times 10^{-4}$ M corresponds to 10 nmoles/mg total protein (tissue + medium albumin) and to 126 nmoles/mg tissue protein.
* $P < 0{\cdot}001$. NS, not significant.

TABLE 4. INHIBITORY EFFECT OF PIERICIDIN A ON LIPOLYSIS STIMULATED BY NORADRENALINE, THEOPHYLLINE AND DIBUTYRYL CYCLIC AMP (cAMP-DB) IN RAT ADIPOSE TISSUE

Drugs in the medium (M)	Δ FFA* (μEq/g fresh tissue)	Δ Glycerol* (μmoles/g fresh tissue)
Noradrenaline 10^{-5}	31·00 ± 1·67	18·03 ± 1·35
Noradrenaline + piericidin $3{\cdot}8 \times 10^{-8}$	15·17 ± 0·96 (−51%)	11·03 ± 0·63 (−39%)
Noradrenaline + piericidin $3{\cdot}8 \times 10^{-7}$	7·19 ± 1·87 (−77%)	4·14 ± 0·38 (−77%)
Noradrenaline + piericidin $3{\cdot}8 \times 10^{-6}$	0·16 ± 0·12 (−100%)	0·64 ± 0·35 (−100%)
Theophylline 0·003	31·17 ± 1·83	17·17 + 1·12
Theophylline + piericidin $3{\cdot}8 \times 10^{-8}$	15·67 ± 0·91 (−50%)	12·41 ± 0·67 (−28%)
Theophylline + piericidin $3{\cdot}8 \times 10^{-7}$	9·48 ± 0·77 (−70%)	5·38 ± 0·31 (−69%)
Theophylline + piericidin $3{\cdot}8 \times 10^{-6}$	2·31 ± 0·69 (−93%)	1·76 ± 0·28 (−90%)
cAMP-DB 0·005	24·62 ± 0·72	14·48 ± 0·99
cAMP-DB 0·005 + piericidin $3{\cdot}8 \times 10^{-8}$	13·69 ± 0·52 (−44%)	9·65 ± 0·37 (−33%)
cAMP-DB 0·005 + piericidin $3{\cdot}8 \times 10^{-7}$	7·86 ± 0·66 (−68%)	4·13 ± 0·71 (−71%)
cAMP-DB 0·005 + piericidin $3{\cdot}8 \times 10^{-6}$	1·85 ± 0·88 (−92%)	0·48 ± 0·19 (−97%)

Rat epididymal fat (100 ± 5 mg) was added to 2 ml of Krebs–Ringer bicarbonate containing 2·5% bovine albumin and, where indicated, piericidin. After a pre-incubation for 30 min at 37°, noradrenaline, theophylline or cAMP-DB was added and the samples incubated for another 120 min. Each value represents the mean (±S.E.) of four to six assays. Piericidin $3{\cdot}8 \times 10^{-6}$ M corresponds to 0·14 nmole/mg total protein (tissue + medium albumin) and to 4 nmoles/mg tissue protein.

* Free fatty acid (FFA) and glycerol absolute increase from control (fat incubated without noradrenaline, theophylline or dibutyryl cyclic AMP).

TABLE 5. INHIBITORY EFFECT OF DICYCLOHEXYLCARBODIIMIDE (DCCD) ON LIPOLYSIS STIMULATED BY NORADRENALINE, THEOPHYLLINE AND DIBUTYRYL CYCLIC AMP (cAMP-DB) IN RAT ADIPOSE TISSUE

Drugs in the medium (M)	Δ FFA* (μEq/g fresh tissue)	Δ Glycerol* (μmoles/g fresh tissue)
Noradrenaline 10^{-5}	29·55 ± 1·25	16·52 ± 1·30
Noradrenaline 10^{-5} + DCCD $2{\cdot}5 \times 10^{-4}$	11·72 ± 1·27 (−60%)	4·79 ± 0·94 (−70%)
Noradrenaline 10^{-5} + DCCD 0·003	4·43 ± 0·30 (−85%)	2·66 ± 0·06 (−84%)
Theophylline 0·003	31·63 ± 0·73	17·89 ± 0·66
Theophylline 0·003 + DCCD $2{\cdot}5 \times 10^{-4}$	16·21 ± 1·10 (−49%)	9·48 ± 0·05 (−47%)
Theophylline 0·003 + DCCD 0·003	11·64 ± 0·10 (−63%)	6·05 ± 0·59 (−66%)
cAMP-DB 0·005	27·82 ± 1·25	13·43 ± 2·02
cAMP-DB 0·005 + DCCD $2{\cdot}5 \times 10^{-4}$	15·46 ± 2·06 (−44%)	5·78 ± 0·77 (−57%)
cAMP-DB 0·005 + DCCD 0·003	9·05 ± 0·12 (−68%)	4·96 ± 1·11 (−63%)

Experimental conditions are described under Table 4. Each value represents the mean (±S.E.) of three to six assays from three experiments. DCCD $2{\cdot}5 \times 10^{-4}$ M corresponds to 10 nmoles/mg total protein (tissue + medium albumin) and to 126 nmoles/mg tissue protein.

* Free fatty acid (FFA) and glycerol absolute increase from control (fat incubated without noradrenaline, theophylline or dibutyryl cyclic AMP).

TABLE 6. COMPARISON BETWEEN THE EFFECT OF PIERIDICIN, ANTIMYCIN AND DCCD ON HORMONE-STIMULATED LIPOLYSIS AND CYCLIC AMP SYNTHESIS

Drugs		Lipolysis* (% inhibition)		Cyclic AMP† level (% inhibition)
		Noradrenaline	CAMP-DB	
Piericidin	$3{\cdot}8 \times 10^{-6}$ M	100	100	70 ± 1
Piericidin	$3{\cdot}8 \times 10^{-7}$ M	77 ± 2	70 ± 2	
Antimycin	2×10^{-5} M	60 ± 2	59 ± 1	89 ± 3
DCCD	$2{\cdot}5 \times 10^{-4}$ M	65 ± 3	44 ± 4	50 ± 4

* Lipolysis induced by noradrenaline 10^{-5} M or by dibutyryl cyclic AMP 0·005 M in adipose tissue incubated in Krebs–Ringer bicarbonate containing bovine albumin, at 37° for 150 min in a metabolic shaker.

† Cyclic AMP accumulation induced by noradrenaline 10^{-5} M plus theophylline 0·003 M, in adipose tissue incubated in Krebs–Ringer bicarbonate containing bovine albumin, at 37° for 10 min in a metabolic shaker.

DISCUSSION

Antimycin A piericidin A and dicyclohexylcarbodiimide (DCCD), showed a marked inhibitory effect on cyclic AMP accumulation induced by noradrenaline plus theophylline in adipose tissue. The same drugs also inhibited the lipolytic response of adipose tissue to noradrenaline, theophylline and dibutyryl cyclic AMP. The result clearly confirms and emphasizes the relationship between hormone-induced lipolysis and oxidative phosphorylation. In this regard, the action of DCCD, and that of piericidin, are the more interesting. DCCD produces the same effect on oxidative phosphorylation as oligomycin[7,8] but its action is much more specific.[9] In fact, while oligomycin in a comparable range of concentrations is able to influence several mammalian ATPases associated with membranes,[21,22] DCCD inhibits other ATPase systems from mammalian cells at concentrations 1000-times higher than mitochondrial ATPase.[9] DCCD is therefore much more suitable to evaluate the contribution of aerobically generated ATP to energy-requiring processes in systems containing intact cells, as in the case of hormone-induced lipolysis in adipose tissue, where a relationship between hormonal action on lipolysis and (Na^+, K^+)-ATPase has been indicated.[1,23] A highly active bivalent cation-dependent ATPase purified from rat epididymal fat cell ghosts[24] was also significantly inhibited by oligomycin, but DCCD is not active on the same purified enzyme. The inhibitory effect of oligomycin on cyclic AMP accumulation[2] could thus have been dependent on its activity at plasma membrane level and to related changes in the ionic composition of cytoplasm.

The parallel inhibitory effect shown by DCCD on lipolysis and on cyclic AMP accumulation, excludes the fact that the effect of oligomycin[2] on cyclic AMP synthesis could have been dependent on its activity at plasma membrane level.

Piericidin was tested in view of its site and action mechanism on electron transport. Similar to rotenone,[6,7] it blocks the respiratory chain at the level of the NADH-cytochrome *b* reductase segment. The small inhibitory effect of rotenone on cyclic AMP accumulation if compared with the effect of the same drug on lipolysis,[2] was thought to be dependent on the possibility that a certain amount of ATP furnished

by oxidation of succinate could be available for cyclic AMP formation. The hypothesis is supported by the results obtained with piericidin. This drug, like rotenone, was less effective on cyclic AMP accumulation than on lipolysis. Therefore, the present data seem to suggest that the synthesis of cyclic AMP in adipose tissue, is dependent on oxidative phosphorylation, but to a lesser degree than the whole final lipolytic process.

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