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EFFECT OF IONOPHOROUS ANTIBIOTICS (NIGERICIN, GRAMICIDIN AND VALINOMYCIN) ON CYCLIC AMP SYNTHESIS IN RAT ADIPOSE TISSUE

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Abstract—The antibiotic nigericin increases the permeability of natural and artificial membranes to hydrogen ions and promotes potassium–hydrogen exchange. It has been shown to inhibit the catecholamine-induced lipolysis in rat epididymal fat. The effect of nigericin was also tested, together with two other ionophorous antibiotics, gramicidin and valinomycin, on cyclic AMP synthesis stimulated in adipose tissue by noradrenaline and theophylline. The last two agents have previously been found to inhibit hormone-induced lipolysis in a manner similar to that of nigericin. In contrast to their common antilipolytic activity, the three drugs had different effects on cyclic AMP synthesis. Valinomycin did not depress the increase of the cyclic nucleotide, while gramicidin and nigericin caused its inhibition. The comparison between the three drugs is interesting as they differ in cation (mainly K^+ and Na^+) and proton specificity, whilst having a similar final metabolic effect, i.e., the discharge or reduction of mitochondrial energy. The similar effect on lipolysis, in contrast to the different action on cyclic AMP synthesis, suggests that the three ionophorous antibiotics depress or inhibit the activation of the lipolytic process by lowering the availability of ATP after the synthesis of the cyclic nucleotide.

IONOPHOROUS antibiotics such as gramicidin[1,5] valinomycin[1–5] and nonactin[3] have been shown to inhibit lipolysis induced by catecholamines and by dibutyryl cyclic AMP in rat adipose tissue and cells. Very little is known about the site of action of these agents on the metabolic sequence[6] representing the activation of lipolysis by catecholamines. According to Kuo and Dill[3] the effect of valinomycin and nonactin is at least in part due to an inhibition of adenyl cyclase, since the two antibiotics lowered the accumulation of cyclic AMP induced by catecholamines and methylxanthines. Phosphodiesterase was not affected. In contrast, Fain and Loken[4] found that, in the presence of valinomycin, cyclic AMP concentration in adipose cells was still above the amount needed to maximally activate lipolysis. The antilipolytic effect of valinomycin would therefore follow the inhibition of cyclic AMP action. This is in accordance with the inhibitory effect shown by the antibiotic against dibutyryl cyclic AMP stimulated lipolysis.[1,5]

The mechanism of the inhibitory effect shown by ionophorous antibiotics on lipolysis, is still not clear. These agents are known to increase the permeability of natural and artificial membranes to some monovalent cations[7–13] and also to influence ion transport. The cell membrane interaction occurs parallel to the metabolic effects, the most studied of which is the uncoupling of oxidative phosphorylation.[7–12,14]

The inhibition of hormone-stimulated lipolysis could be explained by one of the following hypotheses; (1) an altered energy balance in adipose cell, or, more specifically, a lack of ATP required for the lipolytic process;[15–22] (2) a direct antibiotic–

membrane interaction influencing cell membrane permeability; (3) a variation of electrolyte balance inside the cytoplasm or in some cell compartment.

The present results on the antilipolytic effect of nigericin, and on the effect of nigericin, gramicidin and valinomycin on cyclic AMP concentration in rat adipose tissue, seem to indicate the first of the aforementioned hypothesis.

MATERIALS AND METHODS

Materials. DEAE–cellulose (medium mesh, 0·85 m-equiv./g) histone (Type II) cyclic AMP and bovine serum albumin (Fraction V) were purchased from Sigma. γ^{32}P-ATP was obtained from the Radiochemical Centre, Amersham. AG 50W-X8 resin (200–400 mesh), purchased as analytical grade from BioRad Laboratories in the hydrogen form, was washed repeatedly in distilled water to remove fines and kept at 4° as a 50% (v/v) suspension in distilled water. Noradrenaline bitartrate monohydrate was from Recordati, theophylline from C. Erba and gramicidin D from Sigma. Valinomycin and nigericin were generous gifts from Professor V. V. Zakusov, Moscow and Dr. B. C. Pressman, Philadelphia.

Preparation and incubation of rat epididymal fat pads. Treatment of adipose tissue and determination of lipolysis, were carried out as previously described.[23] Free fatty acids were titrated in the incubation medium according to Dole[24] and glycerol according to Korn.[25] Fat pads from albino Wistar rats, were pooled, weighed (200 ± 5 mg) and placed into 1·90 ml of Krebs–Ringer bicarbonate, pH 7·2, containing 2·5% bovine albumin, and where indicated, the antibiotic to be tested. Nigericin, gramicidin and valinomycin were dissolved in absolute ethanol (50 μl), the same volume being added to the control samples. 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 samples incubated for a further 10 min at 37°. At the end of the incubation period, tissue and medium were immediately separated by filtration and washed (1 ml of 0·9% NaCl) under vacuum.

Isolation of cyclic AMP from tissue and medium. The tissue was rapidly added to 1 ml of ice-cold 3% TCA and homogenized in a Potter homogenizer with a Teflon pestle. 100 pmoles of cyclic AMP were added to a control sample to determine its recovery. The samples were then neutralized by the addition of 0·2 ml of 1 M Tris and centrifuged for 20 min at 10,000 *g*. The supernatant contained the basic material for purification of cyclic AMP. Fifty per cent ice-cold TCA (0·15 ml) was added to the incubation medium, the precipitate removed by centrifugation and the supernatant neutralized by the addition of 0·4 ml of 1 M Tris. Before centrifugation, 100 pmoles of cyclic AMP were added to a control sample to determine recovery.

Cyclic AMP was purified from the neutralized supernatants, of both tissue and medium, by the $BaSO_4$ method of Krishna *et al.*[26] 0·5 ml of both 5% $ZnSO_4$ and 2·6% $Ba(OH)_2$ were added to the tissue extracts and 1·5 ml of the same solutions, were added to the medium extracts. After the removal of the $BaSO_4$ precipitate by centrifugation, cyclic AMP was further purified by ion exchange chromatography according to Kuo and Greengard.[27] One ml aliquots of the supernatant were added to AG 50W-X8 columns (0·5 × 5 cm) and eluted with water. Cyclic AMP appeared in the third to the sixth ml of the column eluate; 2 ml of the cyclic AMP fraction from the columns were lyophilized together with 0·5 ml of histone solution (0·343 mg/ml water). The lyophilized residue was dissolved in 300 μl of distilled water

and the cyclic AMP was assayed in different amounts of this solution. In those samples having a very high concentration of cyclic AMP, the nucleotide was assayed directly in the column eluate.

Assay for cyclic AMP. The nucleotide levels were measured by the method of Kuo and Greengard[27] based upon the ability of a protein kinase from bovine heart to catalyse the transfer of ^{32}P to histone from γ^{32} P-ATP in a reaction dependent on the presence of cyclic AMP. Protein kinase was prepared according to Kuo and Greengard[20] as modified by Kuo *et al.*[29] The enzyme preparation was that obtained after DEAE-cellulose purification, and was stored at −50° in small lyophilized portions. Histone-bound ^{32}P was measured in a Beckman liquid scintillation counter. The scintillation solution was 0·5% PPO (2,4-diphenylisoxazole) in toluene and methyl cellosolve (50%, v/v). 0·5–12 pmoles of synthetic cyclic AMP were used to obtain a standard curve. In our experimental conditions, the slope of the curve relating the activity of the protein kinase to the concentration of cyclic AMP was constant between 0·5 and 10 pmoles and the apparent K_m-value for cyclic AMP was $1{\cdot}2 \times 10^{-8}$ M. Overall recovery of synthetic cyclic AMP added to the control samples, was between 60 and 75 per cent. The experimental data have been corrected for the recovery.

RESULTS

Inhibitory effect of nigericin on noradrenaline stimulated lipolysis. Nigericin inhibited the free fatty acid (FFA) and glycerol release from adipose tissue stimulated by noradrenaline 10^{-5} M (Table 1) while the basal release was not significantly affected. A 24 per cent inhibition of free fatty acid release was shown by nigericin at 10^{-6} M (0·03 μg/mg of total protein, tissue plus medium; 0·75 μg/mg of tissue protein) and a 65 per cent inhibition at 10^{-5} M. A similar degree of inhibition was shown by nigericin on glycerol release. This indicates that the antagonistic action of the antibiotic is due to a real inhibition of lipolysis.

TABLE 1. EFFECT OF NIGERICIN ON THE FREE FATTY ACID AND GLYCEROL RELEASE BY NORADRENALINE FROM RAT ADIPOSE TISSUE

Drugs in the medium (M)	Δ FFA* (μEq/g fresh tissue)	Δ Glycerol* (μmoles/g fresh tissue)
Noradrenaline 10^{-5}	28·19 ± 1·10 —	15·13 ± 1·50 —
Noradrenaline 10^{-5} + nigericin 10^{-6}	21·52 ± 1·78 (−24%)	10·59 ± 0·60 (−30%)
Noradrenaline 10^{-5} + nigericin 10^{-5}	9·92 ± 0·33 (−65%)	5·84 ± 0·25 (−61%)

Rat epididymal fat (100 ± 5 mg) was preincubated in 2 ml of Krebs–Ringer bicarbonate, pH 7·2, containing 2·5% bovine albumin and nigericin where indicated, at 37° for 30 min in a metabolic shaker. Noradrenaline was then added and the incubation continued for 150 min. Nigericin 10^{-6} M corresponds to 0·03 μg/mg total protein (tissue plus medium) and to 0·73 μg/mg tissue protein.

* Free fatty acids (FFA) and glycerol increase from control (fat incubated without addition of noradrenaline) in the incubation medium. Each value represents the mean ± S.E. of three to six assays. All the differences are statistically significant.

Effect of nigericin on the level of cyclic AMP in untreated adipose tissue, or in the presence of noradrenaline and of noradrenaline plus theophylline. The concentration of cyclic AMP present in untreated adipose tissue and in its incubation medium, was not consistently modified by nigericin 10^{-6} and 10^{-5} M. Only a slight but significant

TABLE 2. EFFECT OF NIGERICIN ON CYCLIC AMP LEVELS IN RAT ADIPOSE TISSUE AND IN ITS INCUBATION MEDIUM

Drugs in the medium (M)	Cyclic AMP (pmoles/g fresh tissue)		
	Tissue	Medium	Tissue + medium
	69·08 ± 17·71	86·27 ± 36·43	155·35
Nigericin 10^{-6}	170·07 ± 23·02 ($P < 0·025$)	94·26 ± 13·97 (NS)	264·33
Nigericin 10^{-5}	180·55 ± 11·15 ($P < 0·005$)	18·93 ± 4·46 (NS)	199·48

Rat epididymal fat (200 ± 5 mg) was incubated in 2 ml of Krebs–Ringer bicarbonate containing 2·5% bovine albumin, for 40 min at 37° in a metabolic shaker. At the end of incubation, tissue and medium were treated as indicated in the method. Cyclic AMP was then extracted, purified and titrated according to Kuo and Greengard[27] by the use of bovine heart protein kinase (8 μg). Nigericin 10^{-6} M corresponds to 0·03 μg/mg total protein (tissue plus medium) and to 0·73 μg/mg tissue protein. The data are the mean ± S.E. of four assays.

NS, not significant.

increase was evident in the tissue (Table 2). The effect of noradrenaline and theophylline on cyclic AMP is shown in Table 3. The incubation time was different from that in experiments for determining lipolysis. The difference is related to the maximum effect induced by noradrenaline in our experimental conditions on lipolysis (150 min)

TABLE 3. EFFECT OF NORADRENALINE, THEOPHYLLINE AND NORADRENALINE PLUS THEOPHYLLINE, ON THE LEVELS OF CYCLIC AMP IN RAT ADIPOSE TISSUE AND IN ITS INCUBATION MEDIUM

Drugs in the medium (M)	Cyclic AMP (pmoles/g fresh tissue)		
	Tissue	Medium	Tissue + medium
	111·87 ± 16·97	356·63 ± 110·67	468·50
Noradrenaline 10^{-5}	810·67 ± 86·84 (×7) ($P < 0·001$)	968·74 ± 37·63 (×2·7) ($P < 0·005$)	1779·41 (×3)
Theophylline 0·003	605·55 ± 26·24 (×5) ($P < 0·001$)	170·42 ± 44·12 (NS)	775·97 (×1·6)
Noradrenaline 10^{-5} + theophylline 0·003	37280·79 ± 1148·10 (×333) ($P < 0·001$)	2760·97 ± 71·05 (×7) ($P < 0·001$)	40041·76 (×85)

Rat epididymal fat (200 ± 5 mg) was preincubated in 2 ml of Krebs–Ringer bicarbonate containing bovine albumin, for 30 min at 37°. At that time, theophylline and/or noradrenaline were added and the incubation continued for 10 min. The experimental conditions for separating tissue from medium and for extracting, purifying and titrating cyclic AMP, were as described under Table 2. The data are the means ± S.E. of four assays.

NS, not significant.

and on cyclic AMP (10 min). In contrast, fat was preincubated in the presence of inhibitors for 30 min in both cases. The effects of noradrenaline 10^{-5} M and of theophylline 0·003 M alone, were most evident inside the tissue (five- to seven-times the control values). The cumulative effect of the two drugs was much higher: after incubation with the two drugs for 10 min, cyclic AMP increased in respect to its initial level by approx. 300-times inside the tissue, and by seven-times in the medium.

Nigericin 10^{-5} M inhibited the stimulatory action of noradrenaline on cyclic AMP (Table 4). This inhibitory effect was, however, more clearly evident in the presence of both noradrenaline and theophylline (Table 4). The whole inhibitory effect (tissue plus medium) was 30 per cent in the presence of nigericin at 10^{-6} M and 77 per cent at 10^{-5} M (Table 4).

TABLE 4. EFFECT OF NIGERICIN ON THE INCREASE OF CYCLIC AMP LEVEL INDUCED BY NORADRENALINE AND BY NORADRENALINE PLUS THEOPHYLLINE IN RAT ADIPOSE TISSUE

Drugs in the medium (M)	Cyclic AMP (pmoles/g fresh tissue)		
	Tissue	Medium	Tissue + medium
	69·08 ± 17·71	86·27 ± 36·43	155·35
Noradrenaline 10^{-5}	356·17 ± 40·84	352·21 ± 14·31	708·38
Noradrenaline 10^{-5} + nigericin 10^{-5}	295·53 ± 15·81	48·15 ± 18·61 (−86%)	343·68 (−51%)
Noradrenaline 10^{-5} + theophylline 0·003	28512·43 ± 854·27	4570·54 ± 99·50	33082·97
Noradrenaline 10^{-5} + theophylline 0·003 + nigericin 10^{-6}	21015·04 ± 758·24* (−27%)	2201·40 ± 293·00* (−52%)	23216·44 (−30%)
Noradrenaline 10^{-5} + theophylline 0·003 + nigericin 10^{-5}	5610·00 ± 675·10* (−81%)	2022·78 ± 96·13* (−56%)	7632·78 (−77%)

Rat epididymal fat (200 ± 5 mg) was added to 2 ml of Krebs–Ringer bicarbonate containing 2·5% bovine albumin and, where indicated, nigericin. After preincubation for 30 min at 37° in a metabolic shaker, theophylline and noradrenaline were added and the incubation continued for 10 min. The other experimental conditions were as described under Table 2. The data are the means ± S.E. of four assays.

* $P < 0·001$.

The comparison between the effect of nigericin on the rate of noradrenaline stimulated lipolysis (Table 1) and on the level of cyclic AMP (tissue + medium) increased by noradrenaline plus theophylline (Table 4) are in accord with each other.

Effect of valinomycin and gramicidin on the basal level and on the increase of cyclic AMP induced by noradrenaline and theophylline. The synergistic effect of noradrenaline and theophylline on the cyclic nucleotide synthesis and accumulation, was used for investigating the effect of valinomycin. It was found that valinomycin; (a) inhibits lipolysis stimulated not only by noradrenaline,[1–3,5] but also by dibutyryl cyclic AMP[1,5] and by theophylline;[2,3] (b) the antibiotic does not significantly influence the basal synthesis of cyclic AMP (Table 5); (c) the possibility that the antilipolytic action of valinomycin is due to an activation of phosphodiesterase, was excluded.[3] The

concentration of valinomycin to be tested inhibited lipolysis by about 50 per cent.[1,5]

In these experimental conditions valinomycin did not significantly affect the increase of cyclic AMP induced by noradrenaline and theophylline (Table 5).

Gramicidin was tested on cyclic AMP synthesis in the same experimental conditions as valinomycin. The drug concentration used was that inhibiting noradrenaline and dibutyryl cyclic AMP stimulated lipolysis by about 60 per cent.[1,5] Gramicidin did not induce a significant variation on the basal level of cyclic AMP either in the tissue or

TABLE 5. EFFECT OF VALINOMYCIN ON THE INCREASE OF CYCLIC AMP LEVEL INDUCED BY NORADRENALINE AND THEOPHYLLINE IN RAT ADIPOSE TISSUE

Drugs in the medium (M)	Cyclic AMP (pmoles/g fresh tissue)		
	Tissue	Medium	Tissue + medium
	111·87 ± 16·97	356·63 ± 110·67	468·50
Valinomycin 5×10^{-6}	37·14 ± 12·11 ($P < 0·02$)	553·54 ± 104·98 (NS)	590·68
Gramicidin 10^{-7}	94·23 ± 14·31 (NS)	201·98 ± 95·37 (NS)	296·21
Noradrenaline 10^{-5} + theophylline 0·003	37280·79 ± 1148·10	2760·97 ± 71·05	37557·76
Noradrenaline 10^{-5} + theophylline 0·003 + valinomycin 5×10^{-6}	33065·00 ± 1871·03 (NS)	2612·75 ± 248·08 (NS)	35677·75
Noradrenaline 10^{-5} + theophylline 0·003 + gramicidin 10^{-5}	14721·44 ± 677·02* (−61%)	2264·00 ± 78·58† (−18%)	16985·75 (−55%)

Rat epididymal fat (200 ± 5 mg) was added to 2 ml of Krebs–Ringer bicarbonate containing 2·5% bovine albumin and, where indicated, valinomycin. After preincubation for 30 min at 37° in a metabolic shaker, theophylline and noradrenaline were added and the incubation continued for 10 min. The other experimental conditions were as described under Table 2. Valinomycin 5×10^{-6} M corresponds to 0·14 μg/mg total protein and to 1·85 μg/mg tissue protein. Gramicidin 10^{-5} corresponds to 0·77 μg/mg total protein and to 10 μg/mg tissue protein. The data are the means ± S.E. of four assays.

* $P < 0·001$. † $P < 0·005$.

NS, not significant.

in the medium (Table 5). In contrast with valinomycin, the presence of gramicidin induced, like nigericin (Table 4), a clearcut inhibition (−55 per cent) on the stimulatory effect of noradrenaline plus theophylline. The inhibition was more evident in the tissue (Table 5). Finally, as in the case of nigericin, the variation induced by gramicidin on the level of cyclic AMP increased by noradrenaline and theophylline, paralleled the variation in hormone-stimulated lipolysis,[1,5] although different experimental conditions were used.

DISCUSSION

Nigericin, an antibiotic that renders the natural and artificial membranes freely permeable to protons and promotes potassium–hydrogen exchange,[9,12,30,31] was shown to inhibit the noradrenaline-stimulated lipolysis in rat adipose tissue. Two

other ionophorous antibiotics, gramicidin[1,5] and valinomycin,[1–3,5] have previously been found to possess the same antilipolytic activity. In contrast, the three drugs induced different effects when tested on cyclic AMP synthesis stimulated by noradrenaline and theophylline, i.e. valinomycin did not modify the increase of cyclic AMP, while gramicidin and nigericin strongly inhibited it.

The three antibiotics can be considered as representative of three groups of transport-inducing agents[12,13] differing in their cation and proton specificity.[8,9,30–37] Thus, the common antilipolytic activity shown by these drugs indicated that this effect was only indirectly related to particular changes in ion environment or in membrane permeability, but, preferentially, consequent to variations of energy balance in adipocytes. The three antibiotics have a final common metabolic effect represented by the discharge or reduction of mitochondrial energy.[7,12,14,39–43] This effect, even if linked to ion movements[11,38] is supported by different mechanisms. Valinomycin induces K^+-uptake by mitochondria and the cyclic transport of this cation can completely shortcircuit mitochondrial energy production. It is interesting to observe that, under particular conditions, even a stimulation of respiration and phosphorylation can be obtained with valinomycin.[7,39,40] Gramicidin also increases the cation transport[7,41] thus discharging mitochondrial energy. In contrast to valinomycin, gramicidin exhibits an inherent uncoupling activity.[42] Finally, nigericin induces a discharge of mitochondrial K^+ linked to hydrogen uptake without energy consumption,[30] but it uncouples oxidative phosphorylation.[12,14,43] The common final impairment of energy balance induced by all three antibiotics, could depress or inhibit the activation of the lipolytic process, by lowering the ATP available to it. This is in agreement with previous data concerning the effect of some specific inhibitors of oxidative phosphorylation both on lipolysis[5] and on cyclic AMP synthesis induced by noradrenaline and theophylline,[44] as well as with the demand for ATP at more than one step in hormone-activation of lipolysis, before[15–18] and after[18–22] cyclic AMP synthesis.

The ineffectiveness of valinomycin on cyclic AMP synthesis stimulated by noradrenaline and theophylline, indicates that the action of this antibiotic on the metabolic sequence representing the hormone-stimulated lipolysis, is located after the cyclic AMP formation. Finally, the ineffectiveness of valinomycin on cyclic AMP level could be related to its lack of conventional uncoupling effect, or, to its ability to stimulate mitochondrial phosphorylation.[7,39,40] The small amount of ATP required for cyclic AMP synthesis may be always available to the lipolytic process, even if the bulk of energy is dissipated into ion transport.

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