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DIGITOXIN AND PROSTAGLANDIN E_1 AS INHIBITORS OF CATECHOLAMINE-STIMULATED LIPOLYSIS AND THEIR INTERACTION WITH CA^{2+} IN THE PROCESS

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Abstract—The antagonistic action of digitoxin and prostaglandin E_1 on the norepinephrine-stimulated lipolysis has been studied qualitatively and quantitatively. The actions of the two drugs are somewhat similar: (a) their antagonism appears, from the log dose–effect curves, to be of a mixed type; (b) Ca^{2+} concentration in the medium clearly influences their antagonistic effect.

A relation between the action of these drugs on lipolysis and active ion transport or oxidative phosphorylation cannot be entirely excluded. Calcium movements appear more directly involved. The importance of Ca^{2+} in the process studied is pointed out.

PROSTAGLANDIN E_1 antagonistic effect against norepinephrine-stimulated lipolysis *in vitro* was markedly influenced by the concentration of calcium ions in the incubation medium of adipose tissue.[1] Attention was therefore given to digitoxin, whose activity on cardiac tissue is also related to calcium ions.[2, 3] Digitoxin antagonized the norepinephrine-stimulated lipolysis. Moreover, the antilipolytic effect of prostaglandin was affected to some extent also by potassium concentration in the medium.[1]

These results led to consider the possibility that prostaglandin E_1 and digitoxin could influence lipolysis by affecting active ion transport. Thus, as regards the antilipolytic activity of these drugs, a possible common biochemical basis related to ionic transport, was looked for by studying the effects of prostaglandin E_1 on active transport in isolated frog skin, on the sodium and potassium-stimulated adenosine triphosphatase activity of human erythrocyte membranes, on the dinitrophenol-stimulated adenosine triphosphatase and on oxidative phosphorylation in isolated rat liver mitochondria.

MATERIALS AND METHODS

Sprague–Dawley male rats (200–230 g) were used. Samples of epididymal fat (100 ± 10 mg) from fed animals were incubated in 2 ml of Krebs–Ringer bicarbonate buffer pH 7·2 containing 3 per cent bovine albumin (fraction V, Sigma). After incubation in a metabolic shaker at 37°C, free fatty acids (FFA) released in the medium were determined according to Dole's[4] method. Norepinephrine and inhibitors were added to the incubation vessels before introduction of the fat. Prostaglandin E_1 was dissolved in water just before each experiment. Digitoxin was dissolved in absolute ethanol. The same volume (0·05 ml) of ethanol was introduced in the control test.

The action of prostaglandin E_1 was tested on the ATP-ase activity of human erythrocyte membranes prepared according to Dunham and Glynn.[5] The essay composition was the following: 10 mM Tris buffer pH 7·5; 100 mM NaCl; 25 mM KCl; 1 mM $MgSO_4$; 2 mM ATP; 0·1 mM prostaglandin E_1; membranes (about 9–10 mg of protein). The tubes were shaken for 60 min at 37°. The reaction was stopped with trichloroacetic acid (final concentration 0·86 M) and inorganic phosphate was measured by the Fiske and Subbarow method.[6]

Active sodium transport in isolated frog skin was determined as follows: the abdominal skin of *Rana esculenta* was dissected and washed in Ringer's solution. The clean skin (surface, 7·06 cm^2) was mounted between two lucite chambers containing frog Ringer's solution. Potential difference and short-circuit current across the skin were measured according to the technique of Ussing and Zerahn.[7] Drugs were added directly to the Ringer, bathing the internal or the external side of the skin, without replacing control solution.

The action of prostaglandin E_1 and digitoxin on oxidative phosphorylation was tested by using rat liver mitochondria prepared according to Hogeboom.[8] The final pellet was suspended in 0·25 M sucrose to a concentration of about 10 mg of protein/ml. The oxygen uptake was followed for 20 min at 30° by the conventional Warburg technique. Phosphate uptake was measured by the Fiske and Subbarow method,[6] and protein determined by the biuret procedure.[9] The medium composition was the following: 15 mM KH_2PO_4 buffer pH 7·5; 30 mM Tris buffer pH 7·5; 1 mM EDTA pH 7·5; 5 mM $MgSO_4$; 10 mM substrate (l-glutamate or succinate); 30 mM glucose; 0·5 mg yeast hexokinase (Sigma, type III); 1·3 mM ATP; 90 mM sucrose; 4–5 mg of protein rat liver mitochondria; 0·1 mM prostaglandin E_1. Final volume 2 ml.

The action of prostaglandin E_1 and digitoxin on ATP-ase activity of rat liver mitochondria was tested in a medium of the following composition in a final volume of 2 ml: 80 mM KCl; 30 mM Tris buffer pH 7·5; 25 mM sucrose; 2 mM ATP; rat liver mitochondria (about 3 mg of protein); 0·1 mM prostaglandin E_1. The tubes were shaken for 20 min at 30°; the reaction was stopped with trichloroacetic acid (final concentration 0·43 M) and inorganic phosphate was measured by Fiske and Subbarow method.[6]

Prostaglandin E_1 was a generous gift of Prof. Sune Bergström (Karolinska Institutet, Stockholm). and of the Upjohn Co., Kalamazoo, Michigan, U.S.A. Digitoxin was from Hoffmann–La Roche (Basel, Switzerland) and norepinephrine bitartrate monohydrate from Recordati (Milano, Italy).

RESULTS

Antagonistic effect of prostaglandin E_1 *and digitoxin on the norepinephrine-stimulated* FFA *release from rat epididymal fat* in vitro

In Fig. 1 the dose–action curves of norepinephrine in the presence of increasing concentrations of prostaglandin E_1 are reported. The calcium concentration in the medium was reduced to one half of the normal. In these experimental conditions it appears that the antagonism is of a mixed type (according to Ariëns and Van Rossum[10] nomenclature). The clear-cut non-competitive component of prostaglandin action given by the depression of the maximum ($pD'_2 = 5·7 \pm 0·3$) is combined with a parallel shift of the dose–response curves. Whether this really indicates the existence of a

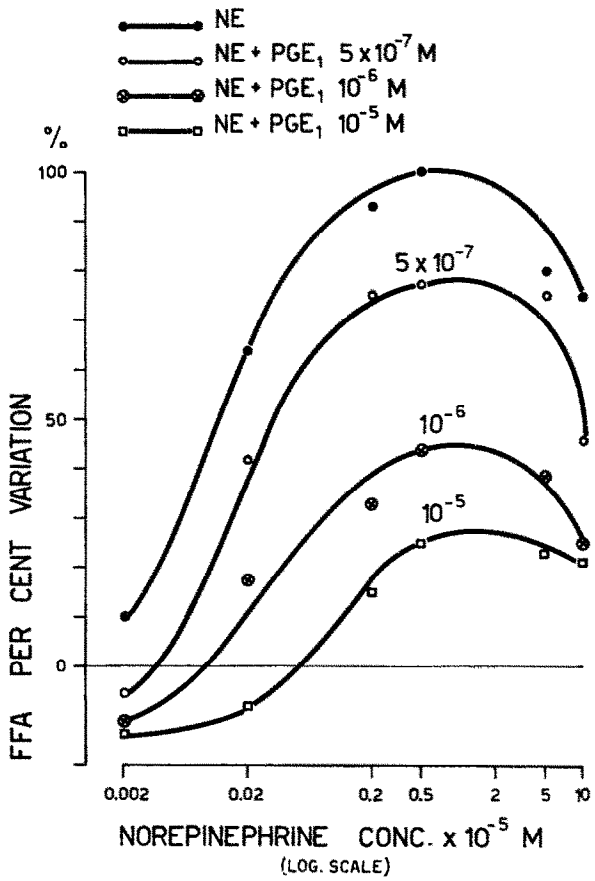

FIG. 1. Log dose-action curves for the antagonism between norepinephrine and prostaglandin E_1 on lipolysis *in vitro*.

Abscissa: molar concentration of norepinephrine (NE) in the medium. Ordinate: FFA per cent relative increase in the incubation medium. The FFA variation from control (fat incubated without drugs) induced by norepinephrine $0{\cdot}5 \times 10^{-5}$ M ($34{\cdot}75 \pm 2{\cdot}15$ μEquiv/g fresh tissue per 90 min) was taken as 100 per cent of the effect. Each point represents the mean of three experiments.

Rat epididymal fat (100 ± 10 mg) was incubated in 2 ml of modified Krebs–Ringer bicarbonate buffer pH 7·2 containing 3 per cent bovine albumin and ascorbic acid 200 μg/ml, at 37° for 90 min. The calcium concentration in the buffer was reduced to one half of the normal. Ca^{2+} was substituted with Na^{+}.

competitive component of prostaglandin action in antagonizing norepinephrine cannot be decided, of course, but the calculated pA_2 value is relatively high and relatively stable ($6{\cdot}0 \pm 0{\cdot}1$).

The competitive component becomes more manifest when calcium concentration in the medium is normal. In fact, when the medium is calcium deficient the higher doses of norepinephrine ($0{\cdot}5 \times 10^{-5}$ M) are inhibited to a greater extent (Table 1).

Digitoxin antagonizes the norepinephrine-stimulated lipolysis in a quite similar way to prostaglandin. In Fig. 2 the dose–action curves of norepinephrine in the presence of different concentrations of digitoxin are represented. Calcium in normal

concentration is present in the medium. Here too the antagonism appears to be of a mixed type: for the low concentrations of digitoxin a competitive interaction cannot be excluded; however, at the higher concentrations, digitoxin acts quite clearly in a non-competitive way. The parameters found are: $pA_2 = 5$ and $pD'_2 = 4{\cdot}1 \pm 0{\cdot}3$.

As in the case of prostaglandin, the omission of calcium in the medium emphasizes the non-competitive component of antagonism: when the medium is without calcium the higher doses of norepinephrine are proportionally antagonized to a greater extent (Table 2).

TABLE 1. INFLUENCE OF CALCIUM ON THE EFFECT OF PROSTAGLANDIN E_1 ON NOREPINEPHRINE-STIMULATED LIPOLYSIS

Drugs in the incubation medium M × 10^{-5}	FFA μEquiv/g/ per 90 min absolute increase from control	
	Ca^{2+} normal	1/2 Ca^{2+}
Norepinephrine 0·02	15·82	4·30
Norepinephrine 0·5	36·93	21·67
Norepinephrine 0·02 + PGE_1 0·05	2·66 (−83%)	3·52 (−18%)
Norepinephrine 0·5 + PGE_1 0·05	32·42 (−12%)	15·61 (−28%)
Norepinephrine 0·02 + PGE_1 1	0 (−100%)	0 (−100%)
Norepinephrine 0·5 + PGE_1 1	28·81 (−22%)	11·01 (−49%)

Epididymal fat (100 ± 10 mg) was incubated in 2 ml of Krebs–Ringer bicarbonate buffer (normal or modified as regards Ca^{2+} concentration) pH 7·2 containing 3 % bovine albumin and ascorbic acid 200 μg/ml, for 90 min. Ca^{2+} in the medium was substituted with Na^+. Norepinephrine and PGE_1 were added directly to the medium before introducing the fat. Each value represents the mean of three experiments (S.E. of the mean varies from 0·60 to 2·15).

Influence of the medium ionic composition on the prostaglandin E_1 and digitoxin inhibitory effect on norepinephrine-stimulated lipolysis

The antagonism by prostaglandin and digitoxin is affected by calcium and, in the case of prostaglandin, also by potassium concentration in the medium (Table 3). The antagonism by these drugs is the greatest in a medium without calcium, or, for prostaglandin, in a medium with calcium reduced to half the normal value and lacking potassium.

Action of prostaglandin on the adenosintriphosphatase activity of human erythrocyte membranes

Cardiac glycosides reduce or arrest the movement of potassium and sodium in a wide variety of animal cells.[11] There are close resemblances between the ATP-ase of erythrocyte membranes and the active ion transport system, especially in the way they are affected by cardiac glycosides.[12]

Prostaglandin 10^{-4} M was tested (as indicated in Methods) on the ATP-ase (Na^+ K^+ and Mg^{2+} stimulated) activity of human erythrocyte membranes and found to be without effect.

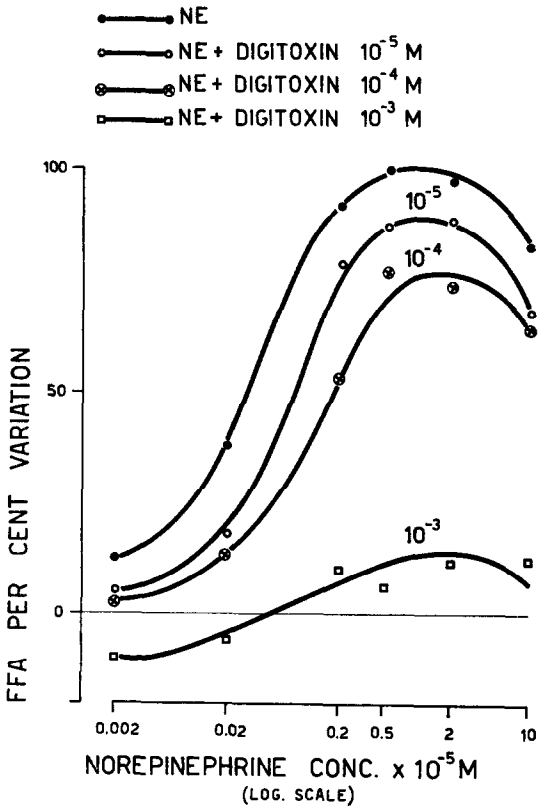

FIG. 2. Log dose-action curves for the antagonism between norepinephrine and digitoxin on lipolysis *in vitro*.

Abscissa: molar concentration of norepinephrine (NE) in the medium. Ordinate: FFA per cent relative increase in the incubation medium after 90 min of incubation. The FFA increase from control (fat incubated without drugs) induced by norepinephrine $0{\cdot}5 \times 10^{-5}$M was taken as 100 per cent of the effect. Each point represents the mean of five experiments.

Experimental conditions as in Fig. 1. Krebs–Ringer bicarbonate buffer containing a normal Ca^{2+} concentration.

TABLE 2. INFLUENCE OF CALCIUM ON THE EFFECT OF DIGITOXIN ON NOREPINEPHRINE-STIMULATED LIPOLYSIS

Drugs in the incubation medium M × 10^{-5}	FFA μEquiv/g per 150 min absolute increase from control		
	Ca^{2+} normal	1/2 Ca^{2+}	Without Ca^{2+}
Norepinephrine 0·02	12·04	5·64	1·79
Norepinephrine 0·5	32·58	25·55	20·81
Norepinephrine 5	31·84	23·82	24·02
Norepinephrine 0·02 + digitoxin 10	6·57 (−45%)	1·68 (70%)	0 (−100%)
Norepinephrine 0·5 + digitoxin 10	29·78 (−9%)	16·25 (36%)	2·18 (−90%)
Norepinephrine 5 + digitoxin 10	32·93 (−0%)	19·93 (16%)	9·58 (−60%)

Epididymal fat (100 ± 10 mg) was incubated as indicated in Table 1. Ascorbic acid 200 μg/ml was present in the incubation medium. Ca^{2+} in the medium was substituted with Na^{+}. Norepinephrine and digitoxin were added directly to the medium before introducing the fat. Each value represents the mean of six experiments (S.E.M. varies from 0·35 to 2·48).

TABLE 3. INFLUENCE OF THE MEDIUM IONIC COMPOSITION ON THE PROSTAGLANDIN E_1 AND DIGITOXIN INHIBITORY EFFECT ON NOREPINEPHRINE-STIMULATED LIPOLYSIS

Medium composition	Per cent inhibition	
	Prostaglandin E_1 10^{-5}M	Digitoxin 10^{-4}M
Normal	11 ± 4	12 ± 3
1/2 Ca^{2+}	20 ± 4	36 ± 4
Without Ca^{2+}	58 ± 7	43 ± 6
1/2 Ca^{2+}, without K^+	62 ± 5	32 ± 2

Epididymal fat incubated as in Table 1. The antagonistic activities of prostaglandin E_1 and digitoxin were tested toward norepinephrine 5×10^{-5} M. Ascorbic acid was present in the medium in concentration of 200 μg/ml. Calcium and potassium subtracted from the Krebs–Ringer bicarbonate buffer were substituted with sodium. Each value represents the mean ± S.E. of five experiments.

Effect of prostaglandin E_1 *on active ion transport in the short-circuited frog skin*

Prostaglandin 10^{-5} M significantly increased the short-circuit current when introduced in the solution bathing the internal side of the skin (Fig. 3). Cardiac glycosides, however, decrease the short-circuit current.[13]

Action of prostaglandin E_1 *at subcellular level*

The effect of prostaglandin 10^{-4} M and 5×10^{-6} M was tested in isolated rat liver mitochondria on oxidative phosphorylation with glutamate or succinate as substrates, and on 2,4-dinitrophenol stimulated ATP-ase activity. Prostaglandin E_1, like digitoxin,[14] did not affect the processes studied.

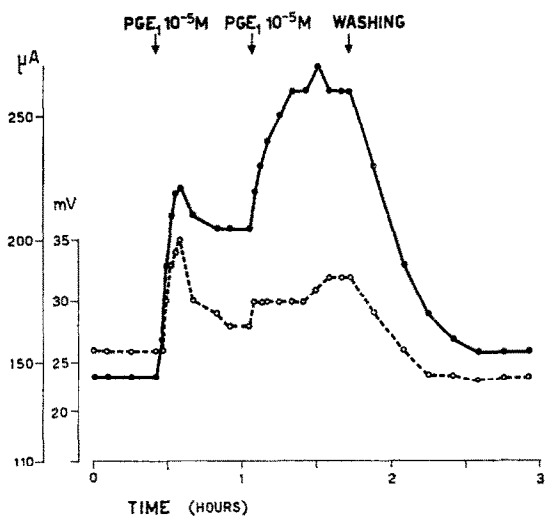

FIG. 3. The short-circuit current (●——●) and potential difference (○——○) for an isolated frog skin before and after the addition of prostaglandin E_1 (PGE_1) to the Ringer solution bathing the inside of the skin.

DISCUSSION

Prostaglandin E_1 and digitoxin show some similarities in their antilipolytic action *in vitro*. The antagonism of these drugs against norepinephrine appears to be of a mixed type, where not only a non-competitive interaction, but probably also a competitive component may be present, depending on the doses and on calcium concentration in the medium. The omission of Ca^{2+} emphasises the non-competitive component of antagonism.

The action of these drugs differs markedly from that of other inhibitors of norepinephrine-stimulated lipolysis. In fact, β-adrenergic blocking drugs such as propranolol and INPEA, behave as competitive antagonists[15] while inhibitors of oxidative phosphorylation (oligomycin, rotenone and 2,4-dinitrophenol) show in contrast typical behaviour of non-competitive antagonists (Fassina and Contessa[1] and Fassina *et al.*[16]). Further, rotenone action is not affected by calcium concentration in the medium.[1]

The experiments carried out with a view to establishing a biochemically common basis for the antilipolytic action of prostaglandin and digitoxin in their influence on ATP-ase activity, ion transport or oxidative phosphorylation, gave insufficient evidence. However calcium movements appear more directly involved in the antagonistic action of both these drugs. These results point out the possible influence of calcium ions in the norepinephrine-stimulated lipolysis.

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