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EFFECTS OF INHIBITORS OF PLATELETS AGGREGATION ON OXIDATIVE PHOSPHORYLATION IN RAT LIVER MITOCHONDRIA *IN VITRO*

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Abstract—The effects of inhibitors of platelets aggregation used in clinical practice (nicergoline, sulphinpyrazone, dipyridamole and aspirin) on respiration and phosphorylation of rat liver mitochondria *in vitro* are investigated. All the drugs studied, except aspirin, act as "inhibitors–uncouplers" in a same concentration range, 50–300 nmol/mg protein: they stimulate state 4 respiration, inhibit simultaneously state 3 oxidation (inhibition not reversed by 2-4 dinitrophenol), reduce the ADP/O ratio and respiratory control index (RCI) and stimulate the latent ATPase activity. The latest stimulation is inhibited by oligomycin 6 μg/mg protein. In presence of 200–800 nmol of aspirin/mg protein, the state 3 oxidation is only inhibited with succinate as substrate; aspirin would be a DNP-like uncoupler. It is observed that Triton X 100 acts in the same qualitative way and some similarities are suggested between inhibitors of platelets aggregation and detergents. Correlation between anti-aggregating properties and drug effects on mitochondrial functions are discussed.

In a previous paper [1], we have shown that ticlopidine, a new inhibitor of platelets aggregation [2–4] inhibits the energy transduction of rat liver mitochondria *in vitro*. In this work, we study the effects of some drugs now used as inhibitors of platelets aggregation in clinical practice—nicergoline, sulphinpyrazone, aspirin and dipyridamole—to investigate possible and common characteristics of action.

MATERIALS AND METHODS

Mitochondrial isolation. Wistar male rats weighing 200–300 g were used. Liver mitochondria were prepared according to the method of Schneider and Hogeboom [5] with modifications [6]. The mitochondrial pellet, after discarding the fluffy layer, was washed three times and suspended in the isolation medium at 25–35 mg/ml concentration.

Protein evaluation. Mitochondrial protein was determined through the biuret method [7] by using deoxycholate for solubilisation.

Oxydative phosphorylation. Oxygen uptake was tested at 25° using a Clark oxygen electrode. The reaction system consisted of 90 mM KCl, 12.5 mM K_2HPO_4, 5 mM $MgCl_2$, 1 mM EDTA, 25 mM Hepes–NaOH pH 7.3 and mitochondria equivalent to 3–3.5 mg protein for 1.4 ml final volume. Substrates (5 mM in final concentration) were succinate, glutamate–malate, ascorbate (with 250 μM TMPD: *N,N,N',N'* tetramethylphenylenediamine, as electron donor). When succinate was the substrate, the reaction system also contained 3 μM rotenone. ADP was added in the amount of 227 nmol to initiate state 3 conditions. The ADP/O ratios were calculated according to Chance and William [8]. All aqueous solutions were prepared in demineralised then distilled water.

State 3 is the mitochondrial respiration occurring in the presence of substrate with ADP; state 4 is the rate of oxygen uptake measured after the ADP has been converted to ATP [8].

ATPase activity. ATPase activity was measured in the previously described medium (1.4 ml) without phosphate in the presence and absence of oligomycin. Mitochondria were added in a 0.1 ml volume to obtain 3 mg/ml final concentration. The reaction was started by the addition of 5 mM ATP, carried out at 25° for 5 min and stopped by adding 1 ml of 10 per cent (w/v) trichloracetic acid. After centrifugation, the supernatant fraction was analysed for inorganic phosphate by the method of Fiske and Subbarow [9].

Chemicals. Oligomycin, rotenone, DNP, ADP, ATP, Hepes, substrates and TMPD were purchased from Sigma. Inhibitors of platelets aggregation were a generous gift from: Spécia, nicergoline and aspirin; Geigy, sulphinpyrazone; and Boehringer, dipyridamole.

RESULTS

The comparative results are summarized in Figs. 1–4.

State 4 oxidation is activated (Fig. 1) with all inhibitors of platelets aggregation and all substrates. This activation is maximum when glutamate–malate is used as substrate: it is 275 per cent of control with aspirin, 140 per cent with nicergoline and about 110 and 60 per cent with sulphinpyrazone and dipyridamole respectively. When ascorbate is the substrate, this activation is minute.

Irrespective of the substrates used, state 3 oxygen consumption (Fig. 2) is inhibited by the four drugs studied, except aspirin which stimulates respiration with glutamate–malate and ascorbate. The inhibition observed is the most important, 40–60 per cent of control, using succinate as substrate; it does not exceed

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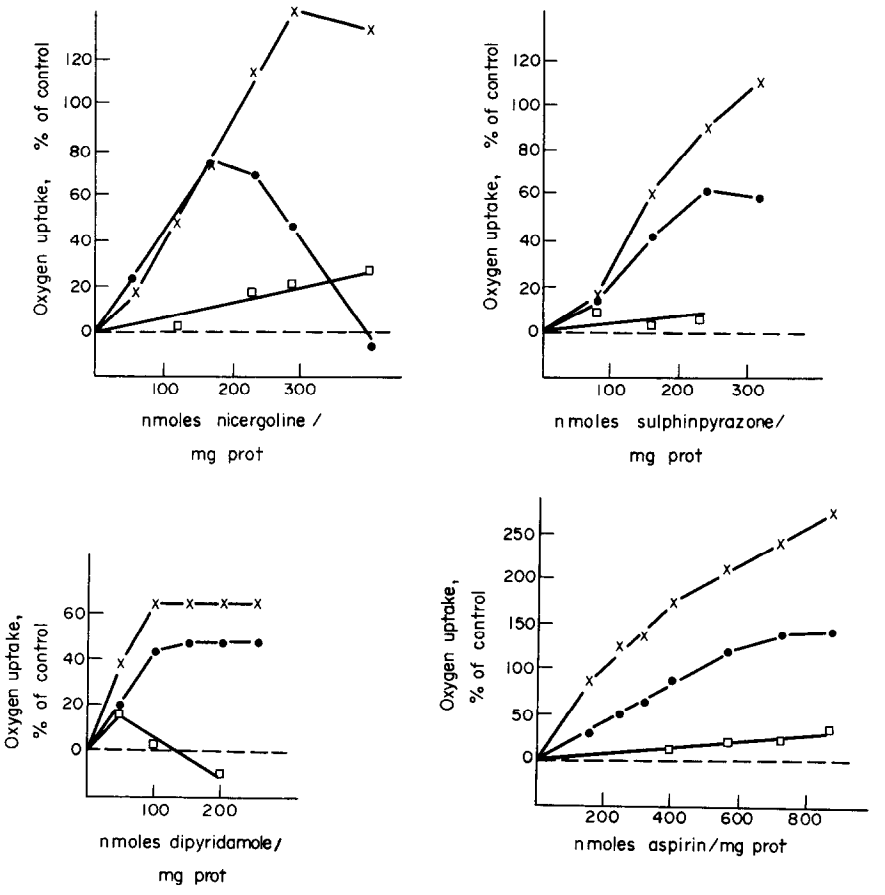

Fig. 1. Effect on state 4 respiration of nicergoline * sulphinpyrazone, dipyridamole and aspirin with ×——× glutamate–malate, ●——● succinate and □——□ ascorbate as substrates. The values are expressed as the average of four (*) or two independent determinations. Conditions are those described in Materials and Methods.

Control respiration are: 11.00 ± 0.22 natomes O/mn/mg prot with glutamate–malate, 21.57 ± 1.18 with succinate and 46.65 ± 6.57 with ascorbate.

20 per cent of control with ascorbate. 2, 4-Dinitrophenol (DNP) 50 μM does not relieve this inhibition.

The results in Fig. 3 show that the four compounds decrease the ADP/O ratio. The action on the respiratory control index, RCI, is similar (not shown) by stimulating state 4 respiration and inhibiting state 3 respiration.

Addition of inhibitors of platelets aggregation stimulates the latent ATPase activity (Fig. 4) from 224 per cent with nicergoline to 788 per cent with dipyridamole. This stimulation is inhibited by oligomycin (6 μg/mg protein).

These activations of state 4, inhibitions of state 3, decreases of the ADP/O and RCI ratios and stimulations of latent ATPase activity have been previously described with ticlopidine [1] and partly observed with detergents [10, 11]. In Fig. 5, we can ascertain that Triton X100 acts in a same qualitative way as inhibitors of platelets aggregation on respiration and phosphorylation: it increases state 4 oxidation and inhibits state 3 respiration, especially with succinate and glutamate–malate respectively; in the presence of ascorbate we observe a biphasic effect. The state 3 inhibition is not relieved by DNP (50 μM). A decrease of the ADP/O ratio (and RCI—not shown) and a stimulation of the latent ATPase activity (Fig. 6), inhibited by 6 μg oligomycin/mg protein is also observed.

DISCUSSION

The results presented in this paper indicate a remarkable agreement in activity between the different inhibitors of platelets aggregation studied which, with the exception of aspirin, act in a same concentration range, 50–300 nmol/mg protein, as "inhibitors–uncouplers" of oxidative phosphorylation [12]: state 3 respiration depressed and not reversed by DNP, state 4 respiration activated simultaneously, ADP/O ratios decreased and stimulation of the ATPase activity inhibited by oligomycin. In presence of aspirin, the inhibition of state 3, elicited at higher doses, 200–800 nmol/mg protein, is only observed with succinate. This comparatively weakest state 3 inhibition is in concordance with a DNP-like action reported by Brody [13].

The facts that inhibition of state 3 is not reversed by DNP and that effects on respiration and phosphorylation are qualitatively similar only with the NAD and the FAD dependent substrate—with ascorbate the de-

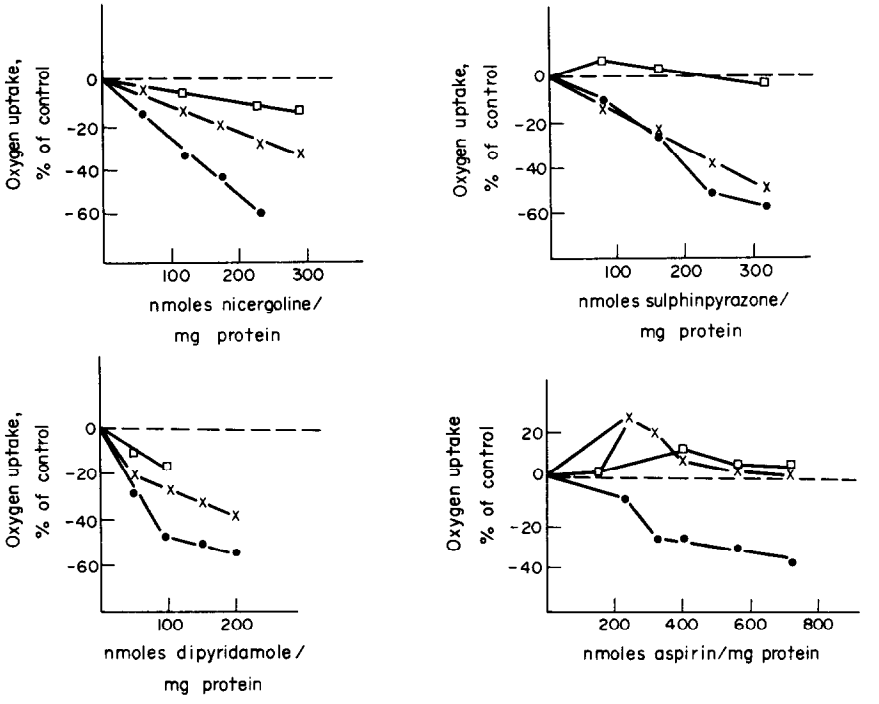

Fig. 2. Effect on state 3 respiration of nicergoline*, sulphinpyrazone, dipyridamole and aspirin with ×——× glutamate–malate, ●——● succinate and □——□ ascorbate as substrates. The values are the means of four (*) or two independent determinations. Conditions are those described in Materials and Methods. Control respiration are: 48.89 ± 1.91 natomes O/mn/mg prot with glutamate–malate, 86.91 ± 8.34 with succinate and 84.23 ± 10.44 with ascorbate.

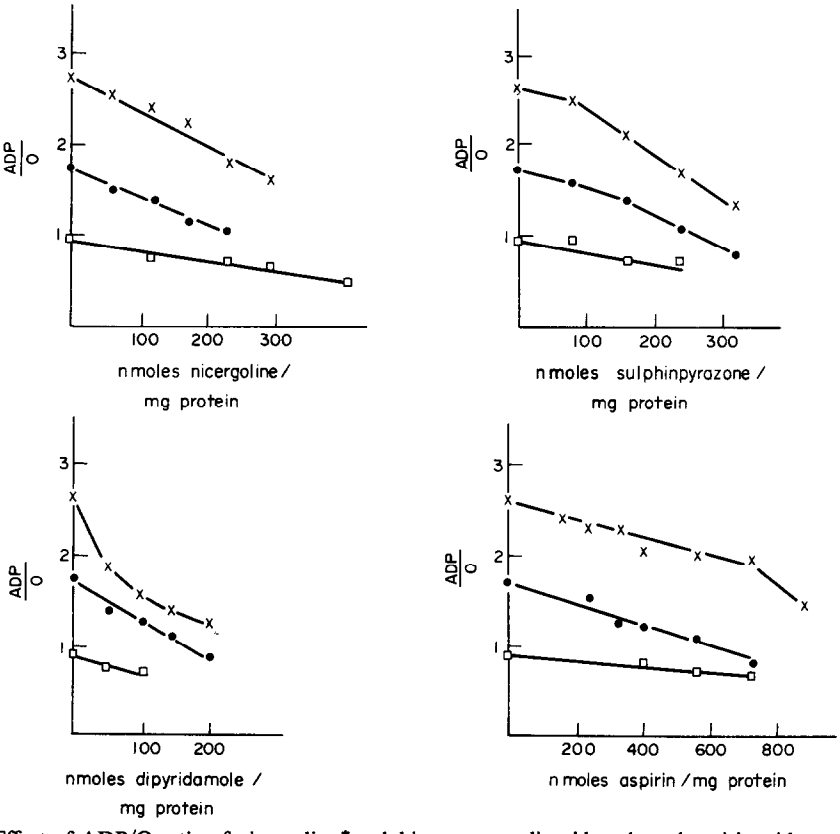

Fig. 3. Effect of ADP/O ratio of nicergoline*, sulphinpyrazone, dipyridamole and aspirin with ×——× glutamate–malate, ●——● succinate and □——□ ascorbate as substrates. The values are the means of four (*) or two independent determinations. Conditions are those described in Materials and Methods.

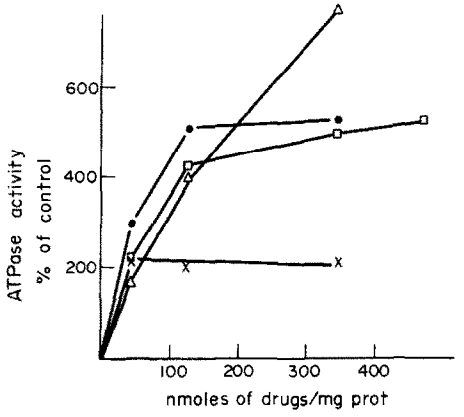

Fig. 4. Effect on the latent ATPase activity of x——x nicergoline, ●——● sulphinpyrazone, △——△ dipyridamole and □——□ aspirin. The values are the means of three independent determinations. For experimental details see Materials and Methods. ATPase activity of control are 157.6 ± 30.6 nmoles of Pi/mg prot/5 mn.

crease of the ADP/O ratios and the respiratory action are minute—suggest a drug action essentially on the two first sites of phosphorylation and on the respiratory chain between NADH dehydrogenase and cytochrome *c* (an action on substrate carriers and (or) dehydrogenases cannot been excluded).

Triton X100 acts in a same qualitative way as inhibitors of platelets aggregation, especially with succinate. Alterations of mitochondrial metabolism by inhibitors of platelets aggregation could have some similarities with that of detergents. Triton X100 is able to disrupt the inner membrane of liver mitochondria [14]; inhibitors of platelets aggregation could act in the same way; it appears that all procedures which impair the integrity of the inner membranes, freezing and thawing, sonication, aging [15], and incubation with phospholipases [16], also decrease respiratory control and increase the ATPase activity.

The data reports a similar qualitative action between the drugs studied and that previously observed with ticlopidine [1] and confirms the blocking effect of the inhibitors of platelets aggregation on liver mitochondrial energy. Platelet mitochondria have not been used regarding difficulties in isolation procedures; however, fundamental differences have not been reported on structure and function of respiratory chain and phosphorylation system for mitochondria from different sources.

Others drugs have the same "inhibitor–uncoupler" effect; 5-5′ diphenyl-2-thiohydantoin [17], anti-inflammatory steroids [18] or 2-aryl-1,3-indandiones [19] but nothing has been reported on their anti-aggregating properties. The mechanism of inhibition of platelets aggregation is complex and it appears difficult to correlate the drug effects on mitochondrial functions and on platelets aggregation. However, the great similarity of action between the drugs studied in this paper and other inhibitors of platelets aggregation such as indometha-

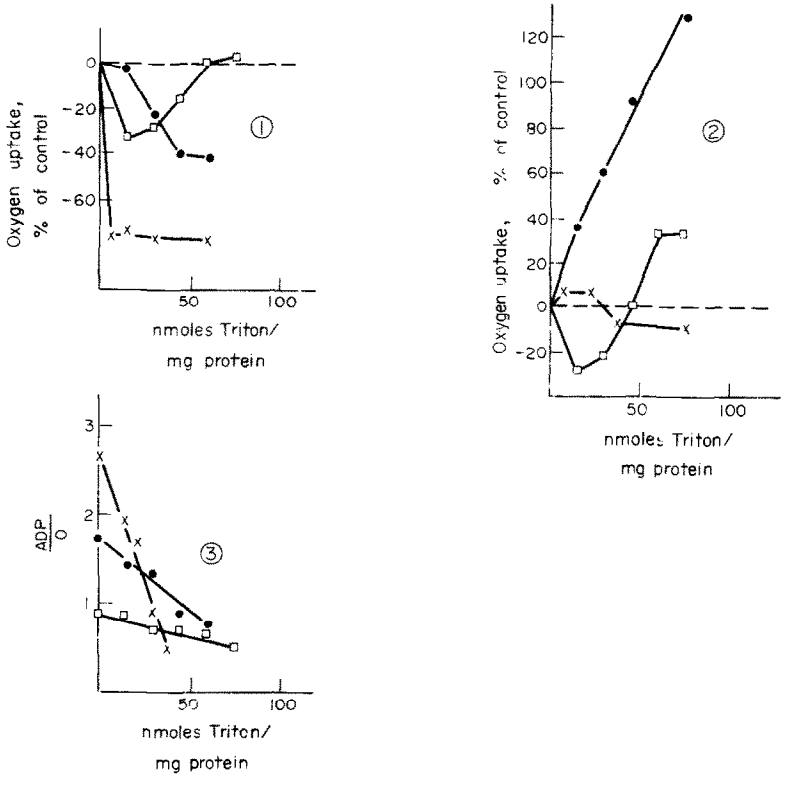

Fig. 5. Effect on state 4 oxidation ①, state 3 oxidation ② and ADP/O ratio ③ of Triton X100 with x——x glutamatemalate, ●——● succinate and □——□ ascorbate as substrates. The values are the means of two independent determinations. Conditions are those described in Materials and Methods.

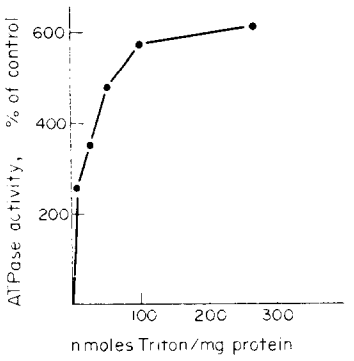

Fig. 6. Effect on the latent ATPase activity of Triton X100. The values are the means of three independent determinations. For experimental details see Materials and Methods. ATPase activity of controls are 157.6 ± 30.6 nmoles of Pi/mg prot/5 mn.

cin [20] and clofibrate [21] suggests that such mitochondrial action could be a reasonable assumption of anti-aggregating properties.

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