Biochemical Pharmacology, Vol. 27, pp. 2099–2102.

0006-2952/78/0901-2099$02.00/0

EFFECT OF AN INHIBITOR OF PLATELETS AGGREGATION, TICLOPIDINE ON ENERGY TRANSDUCTION OF RAT LIVER MITOCHONDRIA *IN VITRO*

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(*Received* 18 *October* 1977; *accepted* 18 *January* 1978)

Abstract—The new platelet aggregation inhibitor, ticlopidine (5-(2-chloro-benzyl)-4-5-6-7-tetrahydro-thieno (3-2-c) pyridine is an inhibitor of energy transduction in isolated rat liver mitochondria. Results show that the compound below concentrations of 100 nmoles/mg of mitochondrial proteins stimulates state 4 oxidation, inhibits state 3 oxidation (except with ascorbate as substrate), decreases ADP–O ratio and respiratory control, and stimulates the latent ATPase activity. This potent stimulation, ten and a half times greater than control, is inhibited by oligomycin (6 μg/mg protein) and is dependent on exogenous Mg^{2+}. The inhibition of state 3 oxidation, with glutamate–malate and succinate as substrates, is not reversed by 2–4 DNP and ticlopidine inhibits in a same extent the ADP and DNP-stimulated respiration. In the presence of succinate uncoupled respiration is more susceptible to inhibition by ticlopidine with sonic submitochondrial particles (I_{50} = 370 nmoles/mg protein) than with intact mitochondria (I_{50} = 75 nmoles/mg protein). Ticlopidine could act as an uncoupler and as an inhibitor of the electron transport chain, between NADH-dehydrogenase and cytochrome *c*, and as an inhibitor of the succinate translocation. It is suggested that the blocking effect on mitochondrial energy might play some part in the ticlopidine anti-aggregating properties.

Aspirin [1, 2], dipyridamole [3–5] have inhibiting properties on platelet aggregation and are used in human disease, particularly in thrombosis [6–8]. Studies on mitochondrial metabolism have proved that these two compounds block energy transduction; dipyridamole inhibits and uncouples oxidative phosphorylation [9], aspirin uncouples alone [10, 11].

A source of metabolic energy is essential for aggregation [12–14], and we know that glycolysis and oxydative phosphorylation are both required [14, 15]. If these two systems are inhibited, ADP induced platelet aggregation does not occur [16].

As regards inhibiting platelets aggregation, the properties of numerous compounds are often dependent on many associated mechanisms, among which modifications of mitochondrial metabolism can be important. In this light, we have studied the action of a new potent inhibitor of platelet aggregation in animal [17, 18] and man [19, 20], ticlopidine (5-(2-chlorobenzyl)-4,5,6,7-tetrahydrothieno (3-2-c) pyridine) on mitochondrial oxydative phosphorylation *in vitro*.

Owing to experimental difficulties regarding platelets mitochondrial isolation, rat liver mitochondria were used.

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 [21] with modifications [22]. The mitochondrial pellet, after discarding the fluffy layer, was washed three times and suspended in the isolation medium at 25–35 mg/ml concentration.

Submitochondrial sonic particles were prepared as described by Schwartz [22] with the following modifications; a 4 ml aliquot of mitochondrial suspension was exposed to sonic oscillations on ice for 4 min with a Branson Sonicator B30 set in position no. 4, and the particles were suspended in the above isolation medium at 10–15 mg/ml concentration.

Protein evaluation. Mitochondrial protein were determined through the biuret method [23] by using deoxycholate for solubilisation.

Oxydative phosphorylation. Oxygen uptake was tested at 25° using 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 2.5–3.5 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) and NADH (2 mM in final concentration) with membrane particles. When succinate was the substrate, the reaction system also contained 3 μM rotenone. ADP was added in the amount of 227 nmoles to initiate state 3 conditions. The ADP–O ratios were calculated according to Chance and Williams [24]. 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 4b is the rate of oxygen uptake measured after the ADP has been converted to ATP [24].

ATPase activity. ATPase activity was measured in the previously described medium (1.4 ml) without phosphate in the presence and absence of 5 mM

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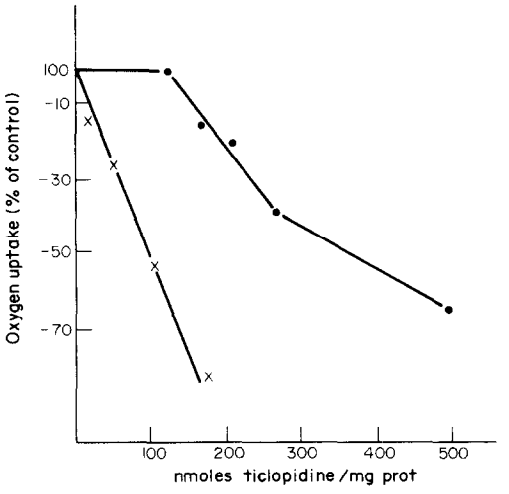

Fig. 2. Effect of ticlopidine on respiration of submitochondrial vesicles with NADH ×——× and succinate ●——● as substrates. Each point represents the average of five independent determinations. Conditions are those described in Methods. Control values for respiration are: 179.8 natomes O/mn/mg protein with NADH – 73.7 natomes O/mn/mg protein with succinate.

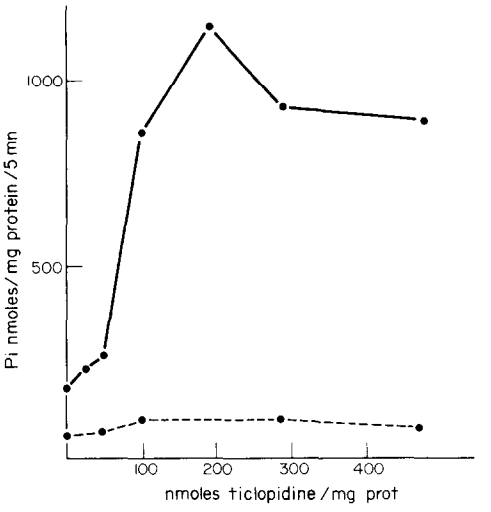

Fig. 3. Effect of ticlopidine on the latent ATPase activity with ●——● and without ●---● $MgCl_2$. Each point represents the average of five independent determinations. Conditions are those described in Methods.

stimulates the latent ATPase activity. Maximum stimulation is reached with a concentration of 188 nmoles of ticlopidine/mg protein and is ten and a half times greater than control. Further increase of concentration does not inhibit ATPase activity (Fig. 3). The stimulation of ATPase is Mg^{2+} dependent and is inhibited by oligomycin (6 μg/mg of protein).

DISCUSSION

The results presented in this paper indicate that ticlopidine behaves both as an inhibitor of respiration (unlike oligomycine, ticlopidine inhibits mitochondrial state 3 respiration, even in the presence of DNP, a classic uncoupler) and as an uncoupler (stimulation of state 4 oxidation, abolition of respiratory control, stimulation of latent ATPase activity, oligomycin sensitivity of stimulated ATPase). However, the fact that on the one hand the same doses of ticlopidine uncouple oxidative phosphorylation and inhibit simultaneously respiration, and that on the other hand the stimulation of ATPase activity is Mg^{2+} dependent, prevents assimilation of this ticlopidine uncoupling effect to that of DNP [27, 28]. The state 3 inhibition of respiration is not reversed by DNP which could suggest that ticlopidine is acting on the respiratory chain and above cytochrome *c* (any inhibition with ascorbate). Now, if we compare the ticlopidine inhibition with intact mitochondria to the inhibition with submitochondrial vesicles we can assume at least two possible sites of ticlopidine action: (1) On the electron transport chain, between NADH dehydrogenase and cytochrome *c*, probably on the complex I and III: the inhibition is observed with NADH, glutamate–malate and succinate in presence of mitochondrial vesicles. (2) On the succinate translocation; the inhibition of succinate oxidation observed with sonic fragments require an amount of ticlopidine five times greater than with intact mitochondria. These different effects on succinate oxidation in intact and sonicated mitochondria suggest that accessibility of substrate to its site of oxidation within mitochondria would be particularly affected (the succino dehydrogenase, like NADH dehydrogenase is located on the matrix side of the inner membrane [29]), ticlopidine straightly acting on dicarboxylate carriers or on their environment. When intact mitochondria respiration occurs with succinate, the effects observed would proceed from action both on this carrier and the complex III.

M. Hamberg and B. Samuelsson have imputed the anti-aggregating properties of aspirin and other non-steroidal anti-inflammatory drugs to their blocking effect on endoperoxide formation [30], i.e. to the inhibition of cyclo oxygenase [31–33]. It is difficult to think that part of the variations of oxygen uptake induced by ticlopidine is associated with a non mitochondrial function such as the cyclo oxygenase one; nothing has been reported on a possible localisation of this oxidase in mitochondrial membranes.

We have already noticed that dipyridamole and aspirin alter the synthesis of mitochondrial energy, ticlopidine acts in a similar manner by inhibiting and uncoupling the mitochondrial metabolism and stimulating the ATPase. We suggest that this blocking effect on the major source of cellular energy and the reducing one on cellular ATP energy stored might play some part in the anti-aggregating properties of ticlopidine.

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