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SWELLING OF MITOCHONDRIA BY THE PLATELET ANTIAGGREGATING AGENT TICLOPIDINE

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Abstract—Our studies on the effects of ticlopidine on mitochondrial functions led us to an intriguing observation related to its interaction with mitochondrial membranes. Liver mitochondria were isolated from Sprague–Dawley rats and assayed for swelling by spectrophotometry. When ticlopidine was added to mitochondria preincubated in an isotonic test medium, an induced-swelling activity was observed. This activity was time and concentration dependent and occurred in different isosmotic solutions. Several analogues of ticlopidine, assayed under identical conditions, produced only a minor effect. Respiratory chain inhibitors, uncouplers, ATP, and phosphate protected the mitochondria against the ticlopidine-induced swelling, whereas oligomycin did not. Comparative studies with the drugs chloramphenicol, nitroso-chloramphenicol, and salicylate (known for their association with mitochondrial injury) showed the first two to have little effect while the third one caused swelling as expected. On the other hand, oxypolarographic tests of respiring mitochondria in the presence of ticlopidine showed that the drug is not an uncoupling agent. These results indicate that the antiaggregating agent ticlopidine interacts with mitochondrial membranes causing swelling which, in turn, may alter mitochondrial permeability; however, unlike some other swelling agents, it does not act as a classical uncoupler.

Presently, the interaction of ticlopidine (5-[2-chlorophenyl - methyl] - 4,5,6,7 - tetrahydrothieno - [3,2-c]pyridine hydrochloride), the new platelet antiaggregating agent [1], with subcellular organelles is not well understood. Because its clinical administration has occasionally been associated with severe granulocytopenia [2, 3], and because a direct toxic cellular effect of ticlopidine has been observed on granulopoiesis *in vitro* [4], we have recently studied the influence of this drug and several of its analogues on different subcellular functions localized at the mitochondrial level. Our results [5] show that, while ticlopidine has little or no effect on protein synthesis and DNA polymerase activity in isolated mitochondria, it causes a concentration-dependent inhibition of the oxidative phosphorylation activity. Since this latter activity is mainly a membrane function, we have here studied the interaction of ticlopidine with the membranes of intact mitochondria by measuring spectrophotometrically their swelling in isotonic solutions. The present paper describes the occurrence of a swelling-induced activity by ticlopidine and shows that the drug does not act as an uncoupler.

METHODS AND MATERIALS

Isolation of mitochondria. The livers of Sprague–Dawley male rats were used to isolate mitochondria as previously described [5, 6].

Measurement of mitochondrial swelling. The isolated mitochondria were added at room temperature to different isosmotic reaction media as stated in the figure legends. The reaction took place in a spectrophotometric cell mounted on an air-driven stirrer device and contained 2 mg mitochondrial protein/4 ml of the medium studied. Mitochondrial swelling was then initiated by addition of a swelling agent and measured by the increase in percentage transmittance (T) at 520 nm in a Baush & Lomb Spectronic 70 spectrophotometer. The increase in T was recorded immediately with a Linear-255 recorder connected to the spectrophotometer as in Ref. 7. Four to seven swelling assays from four different mitochondrial preparations were accomplished with reproducible results. The figures shown are representative traces of the swelling assays.

Mitochondrial respiration. The mitochondrial respiration was determined by measuring oxygen consumption by oxypolarography using a Clark-type oxygen electrode as previously described [8].

Protein determination. Mitochondrial protein was determined by the biuret method [9] using bovine serum albumin (fraction V) as a standard.

Materials. Ticlopidine (PCR 5332) and its analogues were provided by Clin-Midy/Sanofi, Montpellier, France. Other chemicals were from the Sigma Chemical Co., St. Louis, MO, U.S.A., or were of analytical grade.

RESULTS

Mitochondrial swelling by ticlopidine. Figure 1 shows the mitochondrial swelling as induced by different concentrations of ticlopidine. In an isotonic medium routinely used for mitochondrial studies,

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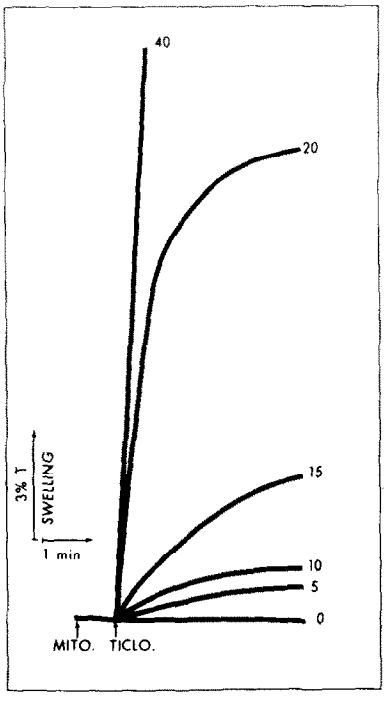

Fig. 1. Mitochondrial swelling induced by different concentrations of ticlopidine. Rat liver mitochondria (Mito.) (2 mg protein/4 ml) were preincubated for 1 min at room temperature in a reaction medium consisting of 87 mM sucrose, 24 mM glycylglycine, and 60 mM KCl (pH 7.4). The swelling, measured by the increase in time of the percent transmittance (%T) at 520 nm, was initiated by adding ticlopidine (Ticlo.). The numbers along the traces indicate ticlopidine concentrations in μg/ml.

the swelling was time and concentration dependent. High amplitudes of swelling were reached at 20 μg/ml of ticlopidine. This concentration was found previously to be over 90% inhibitory of mitochondrial oxidative phosphorylation [5]. Moreover, the swelling-induced activity was similarly observed in different isosmotic sucrose–buffer (such as sucrose–Tris, sucrose–Hepes*–KCl and sucrose–Tris–EDTA) and KCl–Hepes solutions (not shown), suggesting that the observed swelling was not directly related to the composition of the buffer used.

Compared to several of its PCR analogues (see Ref. 5 for PCR formulas), ticlopidine was the only agent that induced significant mitochondrial swelling. Thus, PCR 5325 (a close analogue) produced only 22% swelling relative to ticlopidine; likewise, analogues PCR 4099, PCR 2362, PCR 3787, PCR 4499, and PCR 0665 produced 14, 12, 12, 12, and 6% swellings respectively.

Protection against ticlopidine-induced swelling. The presence in the preincubation medium of respiratory chain inhibitors such as rotenone, antimycin A, or cyanide, and also of oxidative phosphorylation uncouplers such as 2,4-dinitrophenol or carbonyl cyanide *m*-chlorophenyl hydrazone resulted in 72–89% protection against the swelling effect of ticlopidine (Table 1). However, the addition of the ATP-synthase-ATPase inhibitor oligomycin did not affect the swelling (Table 1). Since the isolated mitochondria had a low endogenous respiratory rate which was rotenone- and cyanide-sensitive, and because the ticlopidine-induced swelling increased after addition of exogenous respiratory substrates such as glutamate (not shown), the protection observed in the presence of the respiratory chain inhibitors (Table 1) may indicate that the type of mitochondrial volume change caused by ticlopidine is of the "active" type as characterized by its requirement for endogenous respiration. The lack of protection in the presence of oligomycin suggests that the ATP-synthase is not involved in the swelling activity.

Table 1. Effects of inhibitors of oxidative phosphorylation on the mitochondrial swelling induced by ticlopidine

Additions*	Relative swelling†
None	100
Oligomycin	96
Rotenone	28
Potassium cyanide	22
Antimycin A	18
2,4-Dinitrophenol	15
C-CCP	11

Experimental conditions, except for the preincubation time, were as in Fig. 1.

* The inhibitors were incubated with mitochondria for 90 sec (prior to ticlopidine addition) at the following concentrations: oligomycin (2 μg/mg protein), rotenone (2 μg/mg protein), potassium cyanide (1 mM), antimycin A (2 μg/mg protein), 2,4-dinitrophenol (0.1 mM), and C-CCP (0.5 μM).

† The swelling at 4 min with 20 μg/ml of ticlopidine in the absence of inhibitors, as determined in Fig. 1, was taken as 100%.

Adding ATP to both ticlopidine-swollen mitochondria or to mitochondria prior to ticlopidine addition (Fig. 2A) caused, respectively, a partial reversal of the induced-swelling and a partial protection. The reversal by ATP, however, was observed only in a KCl buffer and not in a sucrose buffer (cf. Fig. 2A and 2B). Similar results were obtained previously with thyroxine-induced swelling of isolated mitochondria [10]. Figure 2 also shows that the swelling caused by addition of ATP was not inhibited by ticlopidine, nor did the drug block the respiratory chain driven swelling (not shown).

The presence of increasing concentrations of phosphate in the preincubation medium, prior to ticlopidine addition, also provided increasing protection against its swelling-induced effect. Complete protection by phosphate was reached at a concentration of 20 mM (Fig. 3).

Comparative effects with other drugs. Because ticlopidine has been associated occasionally with hematopoietic toxicity [1, 11], and has been reported to cause inhibition of oxidative phosphorylation in isolated mitochondria [5, 12], we compared its swell-

* Abbreviations: Hepes, *N*-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; PCR, Parcor; Mito., mitochondria; and Ticlo., ticlopidine.

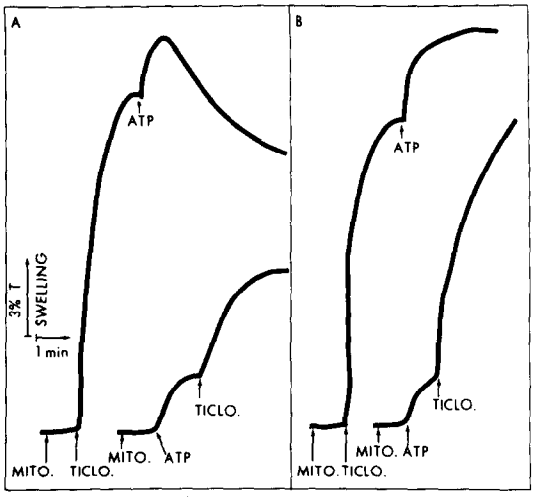

Fig. 2. Effect of ATP on ticlopidine-induced mitochondrial swelling. Mitochondria in panel A were added to a KCl medium consisting of 120 mM KCl and 20 mM Hepes (pH 7.4), and in panel B to a sucrose medium consisting of 250 mM sucrose, 10 mM Tris, and 1 mM EDTA (pH 7.4). ATP (5 mM) and ticlopidine (20 μg/ml) were added as indicated.

ing-induced effect with those of both chloramphenicol and nitroso-chloramphenicol (known for their hematological toxicity at the mitochondrial level [13, 14]), and also with that of salicylate which causes mitochondrial swelling [15]. Using the isotonic sucrose–glycylglycine–KCl test medium, Table 2 shows that, compared to ticlopidine, both chloramphenicol and its analogue nitroso-chloramphenicol caused very little swelling, whereas salicylate produced 23% swelling relative to ticlopidine. Since the effect of salicylate on mitochondrial swelling is reported to be considerably less in a KCl medium as compared to an NH_4Cl medium [15], we tested the latter in our assays. Figure 4 shows that the ticlopidine-induced effect which occurred in NH_4Cl was similar to that in a KCl medium; however, as previously reported [15], the swelling by salicylate in NH_4Cl was considerably higher.

Table 2. Comparative swelling effects of ticlopidine and chloramphenicol, nitroso-chloramphenicol, and salicylate on mitochondria

Additions	Relative swelling*
Ticlopidine	100
Chloramphenicol	8
Nitroso-chloramphenicol	17
Salicylate	23

Conditions were as in Fig. 1. Mitochondrial swelling was induced by the addition of ticlopidine (20 μg/ml), chloramphenicol (323 μg/ml), nitroso-chloramphenicol (154 μg/ml), or salicylate (3.2 mg/ml).

* The swelling at 4 min with ticlopidine, as determined in Fig. 1, was taken as 100%.

Ticlopidine—not a classical uncoupler. When ticlopidine was added to respiring mitchondria oxidizing an NAD-linked substrate such as glutamate, or an

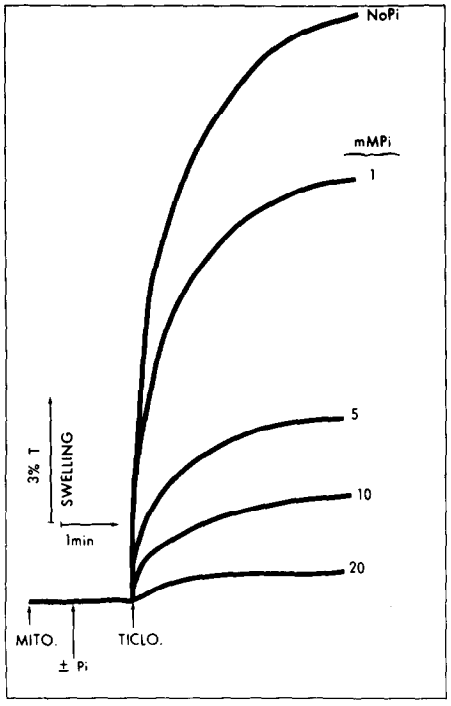

Fig. 3. Protection by phosphate against ticlopidine-induced mitochondrial swelling. When present, P_i was added at various concentrations, as shown, to a medium consisting of 250 mM sucrose, 10 mM Tris, and 1 mM EDTA (pH 7.4). Ticlopidine was 20 μg/ml.

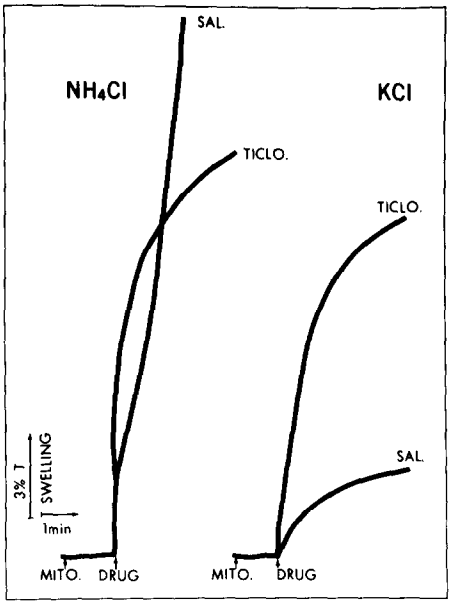

Fig. 4. Mitochondrial swelling induced by ticlopidine (Ticlo.) and salicylate (Sal.) in isotonic medium of either ammonium or potassium chloride. Mitochondria were added to either 130 mM NH_4Cl, 20 mM Tris (pH 7.4), or to 120 mM KCl, 20 mM Hepes (pH 7.4). When present, ticlopidine was 20 μg/ml, and salicylate 3.2 mg/ml.

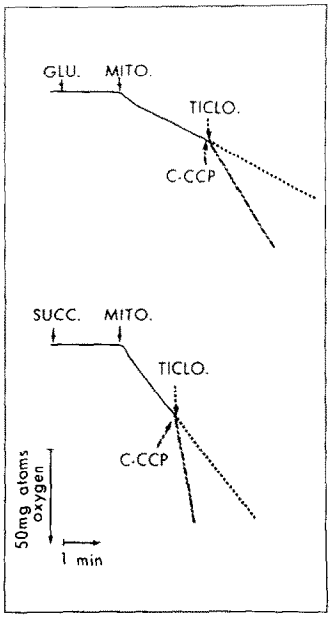

Fig. 5. Effects of ticlopidine (Ticlo.) and carbonyl cyanide *m*-chlorophenyl hydrazone (C-CCP), an uncoupler of oxidative phosphorylation, on mitochondrial glutamate (Glu.) and succinate (Succ.) supported respiration. Mitochondria (Mito.) (1 mg protein/2 ml) were incubated at 30° in a reaction medium consisting of 87 mM sucrose, 24 mM glycylglycine, 60 mM KCl, and 5 mM P_i (pH 7.4). The addition of either Ticlo. (20 μg/ml) or C-CCP (1 μM) was made as indicated. Oxygen consumption was measured as described under Methods and Materials.

FAD-linked substrate such as succinate as shown in Fig. 5, there was less than 20% activation of oxygen consumption with glutamate and no activation of oxygen consumption with succinate. However, as expected, the addition of an uncoupler such as carbonyl cyanide *m*-chlorophenyl hydrazone (C-CCP) resulted in 300–400% activation. Accordingly, ticlopidine appears to act not as an uncoupler, but, rather, to interact with mitochondria to inhibit uncoupling-induced activity of 2,4-dinitrophenol as previously shown [5].

DISCUSSION

The interaction of the antiaggregating agent ticlopidine with several mitochondrial functions was investigated recently, and the drug was found to inhibit oxidative phosphorylation [5, 12]. Although there is no significant experimental evidence to suggest that mitochondria may be a physiological target for the ticlopidine antiaggregating effect, this study and the one described in the accompanying paper [16] clearly demonstrate that the drug produces significant mitochondrial changes.

It is well established that changes in the morphology of mitochondria may have profound effects on mitochondrial functions. A number of agents, such as thyroxine, Ca^{2+}, reduced glutathione, L-ascorbate, phosphate, fatty acids, heavy metals (reviewed in Ref. 17), some uncoupling agents [18] and salicylate [15] are known to cause various degrees of mitochondrial swelling. Our investigations show that ticlopidine is also a mitochondrial swelling agent. The induction of swelling was observed in different isotonic solutions. Unlike the passive type of swelling that occurs in response to variations in the concentration of solutes in the medium [17], the swelling by ticlopidine appears to be of the "active" type in that it was inhibited by both respiratory chain blockers and by uncouplers. Whereas ticlopidine and some of its analogues were capable of inhibiting the energy-conserving mechanism in mitochondria, ticlopidine was the only compound among all the tested analogues to cause significant swelling.

The partial reversal by ATP of ticlopidine-induced swelling resembles that observed with thyroxine [10] when mitochondria were in a KCl medium. Such reversal did not occur in a sucrose medium. Also, in a KCl medium, ATP partially protected mitochondrial swelling by ticlopidine (Fig. 2A), whereas there was no protection in the sucrose medium (Fig. 2B). Protection of the ticlopidine-induced swelling in a sucrose medium (in the absence of respiratory inhibitors), however, was observed in the presence of increasing concentrations of phosphate (Fig. 3). Whereas the physiological significance of phosphate protection is not readily apparent, it suggests that the ticlopidine-induced effect may be subject to control by physiological factors.

Despite the increase of the mitochondrial ATPase activity caused by ticlopidine [16], our oxypolarographic data on the effect of the drug on respiring mitochondria (see Results) provide good evidence that ticlopidine does not behave as a classical uncoupler. Although many swelling agents are known to cause uncoupling in isolated mitochondria [17], some others do not. For example, a well documented case is that of butacaine [19] which inhibits ATP formation and, like the uncoupler C-CCP, stimulates passive swelling in sodium nitrite, but, unlike C-CCP, does not uncouple respiring mitochondria.

Our results appear to be consistent with recent studies on the interaction of ticlopidine with erythrocyte membranes [20, 21] which showed that the drug causes an increase in the fluidity of the membrane phospholipidic core and acts not by direct interaction with hemoglobin but via binding to the cell membrane. The molecular mechanism of ticlopidine–membrane interaction in mitochondrial swelling, which may have occurred through an alteration in membrane permeability or structure, or both, remains to be elucidated.

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