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MECHANISM OF INTERACTION OF TICLOPIDINE AND ITS ANALOGUES WITH THE ENERGY-CONSERVING MECHANISM IN MITOCHONDRIA

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Abstract—It was shown recently that the antiaggregating agent ticlopidine and some of its analogues inhibit the energy-conserving mechanism in mitochondria [Abou-Khalil *et al.*, *Biochem. Pharmac.* **33**, 3893 (1984)]. In the present investigation, the mechanism of inhibition by these drugs was investigated by studying their effects on key reactions of oxidative phosphorylation. Liver mitochondria were isolated from Sprague–Dawley male rats, and the interactions of ticlopidine and six of its analogues with those key reactions were tested. We found: (A) The transport of phosphate, glutamate and succinate into mitochondria was not affected significantly by ticlopidine or any of its analogues; however, it was inhibited by both mersalyl and *N*-ethylmaleimide as expected. (B) There was no inhibitory effect of the tested drugs on the mitochondrial [^{3}H]ADP translocation activity; rather, ticlopidine produced a concentration-dependent increase of that activity, reaching 54% with 20 μg/ml. (C) Ticlopidine and its analogue, PCR 5325, increased the latent ATPase activity by about 400% and the DNP-dependent ATPase by about 50%. Also, PCR 4099 caused a 115% increase in the latent activity, whereas the effects of the remaining analogues varied from slight activation to slight inhibition. (D) Under non-phosphorylation conditions, the mitochondrial H^+ extrusion resulting from succinate oxidation was inhibited by ticlopidine in a concentration-dependent manner reaching a quasi total inhibition with 40 μg/ml. While PCR 5325 gave results similar to ticlopidine, PCR 4099 was less inhibitory and the other analogues were ineffective. These data indicate that the inhibitory action caused by ticlopidine and some of its analogues on oxidative phosphorylation does not reside at one particular site in the mitochondrial membrane; rather, the inhibition seems to be the outcome of profound alterations in mitochondrial ADP translocase, latent ATPase, and proton translocation in the respiratory chain.

Ticlopidine, 5-[2-chlorophenyl-methyl]-4,5,6,7-tetrahydrothieno[3,2-c] pyridine hydrochloride, is a drug which inhibits platelet aggregation, prolongs bleeding time, and has a potent antithrombotic activity (see Ref. 1 for review). The inhibition of platelet aggregation by the drug is more prominent when the aggregating agent is ADP. The mechanism of this inhibition, however, has not yet been elucidated. Some of the recent studies directed to this problem have yielded the following results: (*a*) ticlopidine does not reduce the formation of prostacyclin (PGI_2) which inhibits the aggregation [2, 3]; (*b*) the drug inhibits the binding of ADP to its low affinity sites but does not modify its binding to the high affinity sites [4]; (*c*) it enhances the stimulation by prostaglandin E_1 of adenylate cyclase activity [5] (see, however, Ref. 6); and (*d*) ticlopidine has no apparent effect on the level of cyclic AMP in platelets [5, 7].

Since mitochondria, site of the major energy source in the cell, were found recently to be inhibited by ticlopidine and some of its analogues [8], and since platelet aggregation appears to be an energy-requiring process [9, 10], we carried out the present studies on the mode of interaction of these drugs with the energy-conserving mechanism in mitochondria.

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METHODS AND MATERIALS

Isolation of mitochondria. The isolation of mitochondria was accomplished as in the accompanying paper [11].

Transport of substrates. The transport of phosphate, glutamate, and succinate into mitochondria was measured by mitochondrial swelling in the corresponding isotonic ammonium salts according to Ref. 12. Briefly, mitochondria were preincubated as indicated in the legend of Table 1. Swelling was induced by adding 0.5-ml aliquots of preincubated mitochondria (1 mg protein) to 3.5 ml of the ammonium salt (final concentration 114 mM) with 1 mM ethylene glycol bis-(amino-ethyl) tetraacetic acid and 0.5 μg rotenone/ml. The induced swelling, quantified by the increase in percentage transmittance at 520 nm, was recorded immediately and followed in time until a plateau was reached. The amplitude of swelling after 3–4 min was used to calculate percent inhibition.

Transport of [^{3}H]*ADP*. The Millipore filtration procedure as described [13] in combination with the inhibitor-stop technique using carboxyatractyloside [14] were employed to determine transport of the labeled ADP. Briefly, mitochondria (0.5 mg protein/ml) were preincubated for 30 sec at 2° in a medium

consisting of 24 mM glycylglycine, 87 mM sucrose, 60 mM KCl, and 1 mM ethylenediamine-tetraacetic acid (pH 7.4) in the absence (control) or the presence of drugs. The transport reaction was started by addition of 100 μM [^{3}H]ADP (sp. act. 13 μCi/mmole) and stopped after 30 sec by addition of 15 μM carboxyatractyloside followed by immediate filtration on a Millipore filter (pore size 0.64 μm).

ATPase activity. This activity was measured as follows. Mitochondria (1 mg protein/2 ml) were preincubated at 30° for 2 min with ticlopidine or with one of its analogues in a reaction medium consisting of 87 mM sucrose, 24 mM glycylglycine, 60 mM KCl, and 5 mM $MgCl_2$ (pH 7.4). The reaction was started by the addition of 2.5 mM ATP without (latent ATPase) or with (DNP-dependent ATPase) 0.25 mM 2,4-dinitrophenol (DNP). When present, oligomycin (4 μg/mg protein) was added to the medium. The reaction was stopped after 5 min by adding ice-cold trichloroacetic acid (12% final concentration). The inorganic phosphate released was measured as described [15].

Proton movement due to mitochondrial respiration. The protons released by respiring mitochondria were measured with a Beckman Futura pH glass electrode as described [16] with a minor modification. Mitochondria (6 mg protein/6 ml) were added to a medium consisting of 3 mM glycylglycine, 40 mM KCl, 9 mM $MgCl_2$, 150 mM sucrose (pH 7.4) with 250 nmoles *N*-ethylmaleimide, 3 μg oligomycin, 1.2 μg valinomycin, and 6 μg rotenone, with or without the drug studied. After 2 min of incubation, the addition of 1 mM succinate started the reaction.

Protein determination. Mitochondrial protein was determined by the biuret method [17] 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

The inhibitory mechanism of ticlopidine and some of its analogues (see Ref. 8 for formulas of analogues) on oxidative phosphorylation was investigated by determining the interactions of these drugs with major mitochondrial functions known to be essential to the energy-conserving mechanism. They are:

Transport of phosphate and oxidative substrates. Since it is well established that mitochondrial membranes possess separate specific transporters for phosphate and certain oxidative substrates [18], we determined the effects of ticlopidine and its analogues on the transport of such substrates into mitochondria. Table 1 depicts the percent inhibition caused by these drugs on the transport of phosphate, and on that of glutamate or succinate which are NAD- and FAD-linked substrates respectively. Neither ticlopidine nor any of its analogues caused significant inhibition of the transport systems studied. However, mersalyl or *N*-ethylmaleimide, two thiol reagents known as inhibitors of these systems [19–21], gave over 84% inhibition, as expected (Table 1).

It should be noted that, in the transport assays, mitochondria were preincubated with ticlopidine at a concentration of 20 μg/ml prior to the addition of the substrate to the medium in which transport occurred. As described under Methods and Materials, such addition resulted in ticlopidine dilution in the transport medium. This was corrected by adding 20 μg/ml of ticlopidine to that medium in order to maintain its concentration at the same level. Under such conditions, the results were similar to those of Table 1 (not shown).

Translocation of [^{3}H]ADP. To make ATP, mitochondria must be able to transport ADP from the surrounding medium into their matrix. The transport occurs through an ADP translocase which is a well characterized carrier located in the inner mitochondrial membrane [22, 23]. Table 2 shows that, when the activity of that carrier was assayed in the

Table 1. Effects of ticlopidine and its analogues on phosphate, glutamate, and succinate transport in mitochondria

Additions	Percent inhibition*		
	NH_4-Phosphate	NH_4-Glutamate	NH_4-Succinate
Ticlopidine (PCR 5332)	7	7	9
PCR 5325	10	10	10
PCR 4099	7	6	11
PCR 3787	0	10	0
PCR 2362	0	4	3
PCR 4499	0	4	5
PCR 0665	0	0	5
Mersalyl	95		
NEM		87	84

Transport was measured by mitochondrial swelling at 520 nm in the respective isotonic ammonium salts. Prior to swelling, mitochondria were preincubated for 2 min in a reaction medium consisting of 24 mM glycylglycine, 60 mM KCl, and 87 mM sucrose (pH 7.4), in the absence (control assays) or in the presence of 20 μg/ml of each PCR. When present, mersalyl and NEM (*N*-ethylmaleimide) were 100 and 200 μM respectively.

* Percent inhibition from three mitochondrial preparations was calculated after 3–4 min of swelling as compared to the controls (42, 24 and 13% transmittance for phosphate, glutamate and succinate respectively).

Table 2. Effects of ticlopidine and its analogues on [^{3}H]ADP transport in mitochondria

Additions	Concn (μg/ml)	[^{3}H]ADP transport (cpm/mg protein/min)	% Control
None (Control)		6,710 ± 440	100
Ticlopidine (PCR 5332)	5	7,450 ± 470	110
	10	8,060 ± 490	121
	20	10,420 ± 390	154
PCR 5325	20	9,480 ± 550	140
PCR 4099	20	8,260 ± 390	125
PCR 3787	20	7,660 ± 270	112
PCR 2362	20	6,770 ± 280	101
PCR 4499	20	7,250 ± 200	109
PCR 0665	20	6,960 ± 280	104
Carboxyatractyloside		140 ± 20	2

Conditions are described under Methods and Materials. Concentrations of ticlopidine and analogues varied from 5 to 20 μg/ml as indicated. Carboxyatractyloside was 15 μM. Values are means of seven to eleven assays ± S.E. Counting efficiency for labeled ADP was identical in the different samples.

presence of ticlopidine (from 5 to 20 μg/ml), there was no inhibition of ADP transport. Rather, the transport was observed to increase with increasing concentrations of the drug, reaching a 54% activation at 20 μg/ml. This concentration was found previously to block oxidative phosphorylation by over 90% [8]. Moreover, when ticlopidine analogues were used under these conditions, their effects on ADP transport ranged from 40% activation to no activation in the following order: PCR 5325 > PCR 4099 > PCR 3787 > PCR 4499 > PCR 0665 > PCR 2362 (Table 2). On the other hand, the addition of the translocase inhibitor carboxyatractyloside [22] inhibited transport totally as anticipated (Table 2).

Latent and DNP-dependent ATPase activities. Normally, mitochondrial ATPase activity (which occurs through the reverse reaction of membranous ATP-synthase) is a latent enzymatic reaction that can be activated by uncouplers such as 2,4-dinitrophenol (DNP). Thus, latent ATPase and DNP-dependent ATPase refer to the enzymatic activities in the absence or the presence of DNP respectively. Table 3 shows the effects of ticlopidine and its analogues on these activities as determined by the release of inorganic phosphate from ATP. Unexpectedly, ticlopidine, which does not act as a classical uncoupler in respiring mitochondria [8, 11], increased consistently both the latent and the DNP-dependent activities by 403 and 49% respectively. The ticlopidine-induced increase of the ATPase activities was inhibited by oligomycin (Table 3). The use of PCR 5325, which is structurally the analogue closest to ticlopidine, gave similar results, whereas PCR 4099 induced a 105% increase of the latent activity with no effect on the DNP-dependent one. All the other analogues produced only minor effects on both ATPase activities, as shown in Table 3.

Proton release from respiring mitochondria. According to the chemiosmotic theory of oxidative phosphorylation [24], the respiratory chain of respiring mitochondria ejects protons out of the organelles, generating thus an electrochemical proton gradient. This gradient is then utilized for energy conservation by synthesizing ATP through the membranous ATP-synthase which operates as a reverse proton pump. Since ticlopidine and some of its analogues inhibit oxidative phosphorylation, and since measurement of protons was experimentally possible, we deter-

Table 3. Effects of ticlopidine and its analogues on the latent and DNP-dependent ATPase activities in mitochondria

Additions	Latent ATPase (nmoles P_i released/ mg protein/min)	Latent ATPase % A or I*	DNP-dependent ATPase (nmoles P_i released/ mg protein/min)	DNP-dependent ATPase % A or I*
None	40 ± 5		162 ± 5	
Ticlopidine (PCR 5332)	201 ± 5	+403	241 ± 8	+49
PCR 5325	187 ± 14	+369	258 ± 13	+59
PCR 4099	86 ± 8	+115	162 ± 6	0
PCR 3787	50 ± 5	+25	137 ± 5	−16
PCR 2362	48 ± 4	+21	138 ± 11	−15
PCR 4499	47 ± 5	+19	140 ± 8	−14
PCR 0665	47 ± 4	+19	128 ± 10	−21
Ticlopidine + oligomycin	14 ± 5	−93	11 ± 5	−93

Experimental details are given under Methods and Materials. Latent ATPase represents ATPase activity in the absence of 2,4-dinitrophenol (DNP), and DNP-dependent ATPase represents such activity in the presence of DNP. Additions were as follows: 20 μg/ml of each PCR and, when present, 4 μg/mg protein of oligomycin.

* Percent activation (A+), or inhibition (I−) was calculated with relation to the respective controls. Values are means of eight to twenty-two assays ± S.E.

Table 4. Effects of ticlopidine and its analogues on the proton release from respiring mitochondria

Additions	Concn (μg/ml)	H^+ (nmoles released/min)	% Inhibition
None (Control)		130 ± 6	
Ticlopidine (PCR 5332)	20	69 ± 9	48
	40	18 ± 4	86
PCR 5325	40	4	97
PCR 4099	40	48	63
PCR 3787	40	145	NI*
PCR 2362	40	117	10
PCR 4499	40	141	NI
PCR 0665	40	131	NI
Antimycin A	2	0	100

Proton measurement in the presence of 6 mg mitochondrial protein is given under Methods and Materials. Values for control and ticlopidine determinations are means of five to seven assays ± S.E.; other values are means of two assays.

* No inhibitory effect.

mined the effects of ticlopidine and six analogues on the extrusion of protons by respiring mitochondria. Non-phosphorylation conditions (e.g. absence of P_i and presence of oligomycin) were chosen to ensure net translocation of protons. Table 4 shows that ticlopidine inhibited proton translocation of mitochondria. The inhibition was concentration dependent, reaching 86% inhibition at 40 μg/ml. When ticlopidine concentration was expressed as μg drug/mg mitochondrial protein, the 40 μg/ml of ticlopidine shown in Table 4 was the equivalent of 40 μg/mg mitochondrial protein (which is the same drug concentration used to inhibit oxidative phosphorylation [8]). Also, paralleling our previous oxypolarographic data [8], PCR 5325 and PCR 4099 produced significant inhibition of proton ejection by mitochondria, whereas the other tested PCRs had little or no effect (Table 4). On the other hand, the use of antimycin A in the system produced the predicted total inhibition of proton translocation (Table 4).

DISCUSSION

The present data provide some insights about the inhibitory actions of the antiaggregating agent ticlopidine and some of its analogues on mitochondrial oxidative phosphorylation activity. A cascade of enzymatic reactions is known to occur at the mitochondrial level as part of the energy transduction mechanism associated with oxidative phosphorylation. Our study was designed to address the question of whether one (or more) of these reactions was affected by ticlopidine and its analogues.

Since the mitochondrial inner membrane possesses a considerable permeability barrier, all substrates needed for ATP biosynthesis must be transported into mitochondria by specific carriers before they are metabolized. Thus, the carriers of the two oxidative substrates glutamate and succinate were tested in the presence of ticlopidine and each of its analogues. The results showed only 10% inhibition or less. Likewise, when the phosphate carrier was assayed, it was not affected significantly by these drugs. These studies indicate that neither the transport of oxidative substrates nor that of phosphate is involved in the action of ticlopidine. Likewise, the well-defined carrier translocase (which is specific for ADP and ATP and carries out a one-for-one exchange of these adenine nucleotides [22, 23]) was not blocked by ticlopidine. However, ticlopidine caused a concentration-dependent increase of [^{3}H]ADP accumulation in mitochondria (see Table 2). This increase appears to be the result of mitochondrial membrane alteration, leading to a net uptake of the compound by a mechanism other than the one-for-one exchange. The possibility of ticlopidine-ADP binding to the membrane, however, is not excluded. Whatever the mechanism is, it is tempting to speculate that the increased mitochondrial uptake of ADP induced by ticlopidine may somehow be related to the inhibition by the drug of ADP-induced platelet aggregation.

Even more intriguing was the effect of ticlopidine on mitochondrial ATPase and proton extrusion from respiring mitochondria. While we have shown previously that ticlopidine (i) does not act as a classical uncoupler, and (ii) inhibits the uncoupling-induced activity by 2,4-dinitrophenol [8, 11], at 20 μg/ml the drug was found to promote the latent ATPase activity by about 5-fold, exceeding the levels produced by 2,4-dinitrophenol. The drug was also found to inhibit proton release from respiring mitochondria when the incubation was also under no-phosphorylation conditions. It appears therefore that, while ticlopidine effectively reversed the enzymatic reactions of ATP synthesis by activation of latent ATPase, it also blocked the formation of the electrochemical proton gradient which is necessary for the recuperation of the liberated energy and the formation of ATP.

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