Binding kinetics and antiplatelet activities of picotamide, a thromboxane A2 receptor antagonist.

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Binding kinetics and antiplatelet activities of picotamide, a thromboxane A_2 receptor antagonist

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1 Picotamide was shown to inhibit platelet binding of thromboxane A_2 (TxA_2)-mimetics and to cause a reduction of TxA_2 platelet receptors after *in vivo* administration. The present study aimed to investigate directly [^{3}H]-picotamide binding to human platelets and in particular the relationship between binding kinetics and antiaggregating properties.

2 [^{3}H]-picotamide time-dependently bound to a single class of platelet TxA_2 receptors with a K_D of 325 nmol l^{-1} at equilibrium. The binding was displaceable by TxA_2 analogues U46619 and ONO11120 (K_i 19 and 28 nmol l^{-1} respectively) but not by prostacyclin (PGI_2), prostaglandin E_2 (PGE_2) and TxB_2. Antiaggregating activity and TxA_2 formation inhibition paralleled with binding kinetics.

3 By prolonging the incubation time from 30 to 120 min, picotamide showed a progressively increasing non-displaceable binding, whereas specific displaceable binding decreased in comparison to the values reached at 30 min. Non displaceable binding was specific, temperature-dependent, saturable and followed a Michaelis-Menten kinetic (V_{maxapp} = 130 fmol per 10^8 platelets h^{-1}, K_{Mapp} = 330 nmol l^{-1}). Picotamide progressively underwent a specific stable interaction with its platelet receptor.

4 In conclusion, after an initial reversible binding, a progressive stabilization of picotamide binding takes place resulting in a progressively more stable interaction with platelets.

Keywords: Platelets; thromboxane; thromboxane receptor antagonist; thromboxane synthesis inhibitor; binding studies; aggregation; antiplatelet drug

Introduction

Picotamide (N,N'*bis*(3-picolyl)-4-methoxy-isophthalamide) is a new antiaggregating drug (Violi *et al.*, 1988; Berrettini *et al.*, 1990; Cattaneo *et al.*, 1991) which was found to be able to displace the platelet binding of both a labelled thromboxane A_2 (TxA_2)-mimetic ([^{3}H]-U46619) and a labelled TxA_2 antagonist ([^{125}I]-PTAOH) (Modesti *et al.*, 1989). These findings have caused picotamide to be considered as a TxA_2/PGH_2 competitive receptor inhibitor (Gresele *et al.*, 1989; Modesti *et al.*, 1989). However, no studies have been performed aimed at investigating directly the kinetics of picotamide binding to the TxA_2/PGH_2 receptors. Moreover TxA_2/PGH_2 binding sites have been found to be decreased after picotamide administration in man (Modesti *et al.*, 1991) and this observation apparently contrasts with the hypothesis of picotamide as a competitive receptor inhibitor. Indeed this pattern might suggest either that picotamide acts as a non competitive inhibitor with a stable interaction at the TxA_2/PGH_2 receptor site, or that picotamide follows a particularly low kinetic of dissociation from the TxA_2 receptor, with consequent functional inactivation of the TxA_2 receptor.

The present study aimed to investigate directly the kinetics of the binding of [^{3}H]-picotamide to human platelets and to assess the relationship between the kinetics of [^{3}H]-picotamide binding and antiplatelet activity.

Methods

Platelet aggregation studies

Blood was withdrawn by venipuncture from six overnight fasting healthy volunteers, aged 28 to 42 years, between 08 h 00 min–09 h 00 min and anticoagulated with 129 mmol l^{-1} trisodium citrate (9:1 v:v). No subject had taken any drugs for at least 15 days. Platelet aggregation studies were performed in platelet-rich plasma (PRP) with the optical method of Born, using an Elvi 840 dual channel aggregometer (Elvi Logos, Milan, Italy). PRP was prepared by centrifugation at 160 *g* at room temperature for 6 min. Platelet poor plasma (PPP) was obtained by centrifugation of the blood samples at 1,200 *g* at room temperature for 15 min. Platelet count in PRP was adjusted to 3×10^{11} platelets l^{-1} with autologous PPP in all experiments.

The stable endoperoxide analogue, U46619 (0.75 μmol l^{-1}) and collagen (2.5 μg ml^{-1}) were used as inducers. Platelet aggregation was recorded for 15 min and the extent of aggregation was evaluated by measuring the maximal height reached by the aggregation curves.

Percent inhibitions of U46619 and collagen induced platelet aggregation, by increasing concentrations of picotamide (0, 0.1, 1, 10, 100, 500 and 1,000 μmol l^{-1}) were calculated from the reduction of the maximal amplitude of the aggregation tracings in relation to the values obtained in the paired solvent experiments. The concentration of picotamide giving 50% inhibition of aggregation (IC_{50}) was calculated from the mean of at least four different experiments.

To assess the time-dependency of IC_{50}, the inhibitory effects of picotamide were evaluated after 2, 10 and 20 min of platelet incubation with picotamide.

After 15 min stirring in the aggregometer, PRP was centrifuged at 12,000 r.p.m. in an Eppendorf centrifuge and the supernatant stored at − 20°C for TxB_2 determination. TxB_2 was assayed by an enzymatic immunoassay (Cayman Chemical, Ann Arbor, MI, U.S.A.). Coefficients of variation of intraassay and inter-assay were 6.8% and 9.5%, respectively.

Binding studies

Blood sampling and platelet isolation Blood for the receptor binding studies was withdrawn from the same healthy volunteers into a syringe containing indomethacin (10 μmol l^{-1})

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and EDTA (5 mmol l^{-1}). PRP, obtained by centrifugation as described above, was centrifuged at 1,800 g for 30 min at 20°C. Platelets were then resuspended in 10 ml of phosphate buffer pH 7.2 (mmol l^{-1}: Na_2HPO_4 8, NaH_2PO_4 2, KCl 5, NaCl 135, EDTA 10) and again recentrifuged at 1,800 g for 30 min at 20°C. The supernatant was discarded and the platelets were resuspended in assay Tris-buffer pH 7.4 (NaCl 100 mmol l^{-1}, dextrose 5 mmol l^{-1}, indomethacin 10 μmol l^{-1} and Tris-HCl 5 mmol l^{-1}). If necessary assay Tris-buffer was added to obtain a platelet concentration of 5×10^{11} platelets l^{-1}.

Equilibrium analysis of [methylene^{3}H]-picotamide binding

Washed platelets (5×10^7 platelets) were incubated with 10 nmol l^{-1} (final concentrations are given) [methylene^{3}H]-picotamide and increasing concentrations of unlabelled picotamide (0 to 20 μmol l^{-1}) at 22°C in a final volume of 0.2 ml. The binding obtained in the presence of a large excess of picotamide (20 μmol l^{-1}) was considered as non-specific binding. Specific binding at each concentration was calculated as the difference between total and non-specific binding. After 30 min incubation four 4 ml aliquots of ice-cold buffer were added to each tube to stop the reaction and the content was rapidly filtered under reduced pressure through Whatman GF/C glass microfibre filters. The entire washing procedure was completed within about 15 s. Filters were dried under air flow and counted in a Beckman gamma counter with an overall efficiency of 50%. Binding data, dissociation constant (K_D) and maximum binding capacity (B_{max}), were calculated according to Scatchard (1949).

The competition for [methylene^{3}H]-picotamide binding by a thromboxane A_2 agonist (U46619), a thromboxane A_2 antagonist (ONO11120) (Narumiya *et al.*, 1989), PGI_2, PGE_2, TxB_2 and picotamide were evaluated by incubating 10 nmol l^{-1} [methylene^{3}H]-picotamide with platelets in the absence and in the presence of increasing concentrations (20 nmol l^{-1}–2 mmol l^{-1}) of competitors in a final volume of 0.2 ml. After 30 min the incubation was stopped by the addition of 5 ml of cold assay buffer and filtered. Inhibition constants of competitors were calculated according to Cheng & Prusoff (1973).

Kinetic analysis of [methylene^{3}H]-picotamide specific binding

The kinetics of association and dissociation of [methylene^{3}H]-picotamide to washed platelets were evaluated as previously described for other TxA_2 antagonists (Modesti *et al.*, 1989). To determine the rate of association of [methylene^{3}H]-picotamide, 5×10^7 washed platelets were suspended in assay buffer containing 10 nmol l^{-1} [methylene^{3}H]-picotamide (at 22°C in a final volume of 0.2 ml. After selected time intervals of incubation (20 s, 1, 2, 3, 5, 10, 15, 20, 30 min) samples were rapidly filtered. Specific binding was determined as the difference between the amount of [methylene^{3}H]-picotamide bound in the absence and in the presence of unlabelled picotamide (20 μmol l^{-1}).

To determine the rate of dissociation of [methylene^{3}H]-picotamide from its platelet binding sites, samples were prepared as described above. After 30 min incubation, picotamide (20 μmol l^{-1}) was added, and the amount of specific binding was measured after various time periods (20 s, 1, 2, 3, 5, 10, 15, 20, 30 min). Kinetic constants were calculated according to Weiland & Molinoff (1981).

Effect of prolonged incubation on [methylene^{3}H]-picotamide displaceable and non displaceable binding

The time-dependency of [methylene^{3}H]-picotamide displaceable and non displaceable binding was investigated in separate time course experiments.

The total, displaceable specific, non displaceable specific and non-specific binding at the different incubation times were investigated at 37°C by preparing three different sets of tubes.

The first set of tubes was aimed to evaluate the total binding. Tubes containing [methylene^{3}H]-picotamide at increasing concentrations (10^{-9}, 10^{-8}, 5×10^{-8}, 10^{-7}, 5×10^{-7}, 10^{-6}, 10^{-5} mol l^{-1}) plus platelets, were prepared as described above and filtered after different incubation times (t) (3, 10, 20, 30, 50, 60 and 120 min of incubation).

The second set of tubes was prepared to evaluate the non specific binding. Tubes contained the same incubation mixture (increasing concentrations of [methylene^{3}H]-picotamide and platelets) plus a high concentration (20 μmol l^{-1}) of unlabelled picotamide added before starting incubation (time 0). Samples were filtered after different incubation times (t) contemporary to the first set of tubes (3, 10, 20, 30, 50, 60 and 120 min of incubation).

In the third set of tubes, containing platelets and increasing concentrations of [methylene^{3}H]-picotamide, a large excess of unlabelled picotamide (20 μmol l^{-1}) was added at each time t (3, 10, 20, 30, 50, 60 and 120 min of incubation). Samples were filtered after 30 min and the residual binding was considered as non displaceable binding. The difference between total and non displaceable binding was considered as displaceable specific binding (displaceable receptor binding). The difference between non displaceable and non-specific (evaluated by the addition of 20 μmol l^{-1} picotamide at time 0) binding was considered as specific non displaceable binding (non displaceable receptor binding).

The characteristics (K_D, B_{max}) of displaceable specific receptor binding at 37°C after 30 min (at equilibrium) and after 60 and 120 min of incubation were calculated according to Scatchard (1949) as previously described.

The affinity constant (K_{Mapp}) and the maximal velocity (V_{maxapp}) of non-displaceable specific binding were calculated for each subject by the double reciprocal plot of Lineweaver-Burk (Cornish-Bowden & Eisenthal, 1978) and the lines of best fit were calculated by linear regression using the method of the least squares.

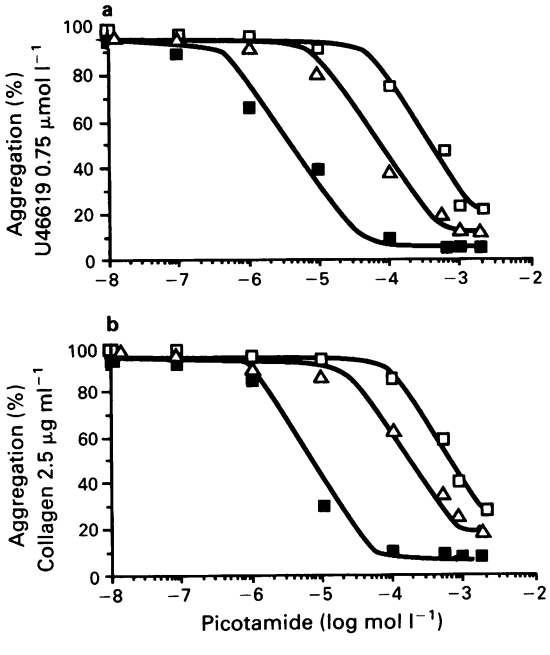

Figure 1 Platelet aggregation induced by U46619 (0.75 μmol l^{-1}) (a) and collagen (2.5 μg ml^{-1}) (b) with increasing concentrations of picotamide after different incubation times: (□) 2 min incubation; (△) 10 min incubation; (■) 20 min incubation.

The competition for [methylene^{3}H]-picotamide non-displaceable binding by picotamide, ONO11120, U46619, PGI_2, PGE_2 and TxB_2, was evaluated by incubating 10 nmol l^{-1} [methylene^{3}H]-picotamide with 5×10^7 platelets in the absence and in the presence of increasing concentrations (20 nmol l^{-1}–2 mmol l^{-1}) of competitors (added at time 0) at 37°C in a final volume of 0.2 ml. After 60 min the incubation was stopped by the addition of 5 ml of cold assay buffer and platelets were spun down by centrifugation. The pellet was then resuspended in assay buffer and filtered. Incorporated radioactivity was then counted as described.

Materials

ONO11120 (9,11-dimethylmethano-11,12-methane-16-phenyl-13, 14-dihydro-13-aza-15-tetranor-TxA_2) (Narumiya *et al.*, 1986) was a kind gift from Prof. Narumiya (Kyoto, Japan). Prostacyclin (PGI_2), PGE_2 and thromboxane B_2 (TxB_2) were obtained from Upjohn, Kalamazoo, MI, U.S.A. U46619 (9,11-dideoxy-11 α,9-α-epoxymethano-$PGF_{2\alpha}$) was obtained from SIGMA Chemicals, St. Louis, MO, U.S.A. Picotamide (N, N'*bis* (3-picolyl)-4-methoxy-isophthalamide, batch no. 870311) was kindly provided by the Samil Inc. (Sandoz group, Rome, Italy); [methylene^{3}H]-picotamide (29 Ci mmol l^{-1}, Amersham, Buckinghamshire, GB) was a kind gift of LPB (Milan, Italy).

All the other reagents were obtained from Merck (Darmstadt, Germany) and were of analytical grade.

Results

Aggregation studies

After 2 min of incubation, picotamide dose-dependently inhibited platelet aggregation induced both by collagen and U46619 with IC_{50}s of 4.5×10^{-4} mol l^{-1} and 6.1×10^{-4} mol l^{-1} respectively. Inhibition of platelet aggregation was also time-dependent and peaked after 20 min. Picotamide IC_{50} on collagen-induced platelet aggregation were 6.8×10^{-5} mol l^{-1} and 3.7×10^{-6} mol l^{-1} after 10 and 20 min of incubation respectively. Picotamide IC_{50} on U46619-induced aggregation was 5.9×10^{-5} mol l^{-1} and 4.7×10^{-6} mol l^{-1} after 10 and 20 min of incubation respectively (Figure 1).

Picotamide also exerted a time-dependent inhibitory activity on TxA_2 production. The EC_{50}s for TxA_2 production during collagen (7.5 μg ml^{-1}) induced aggregation were 5.4×10^{-5} mol l^{-1}, 2.2×10^{-6} mol l^{-1} and 4.3×10^{-7} mol l^{-1} after 2, 10 and 20 min of incubation respectively (Figure 2).

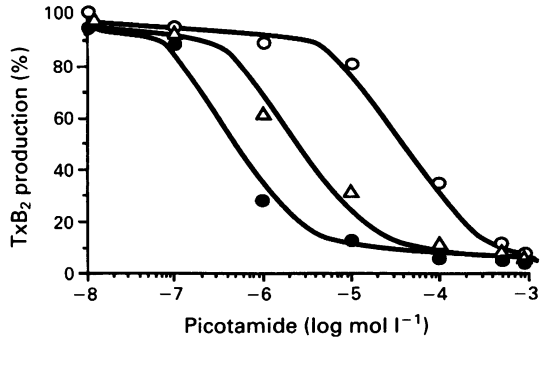

Figure 2 Effect of increasing concentrations and time of incubation of picotamide on thromboxane B_2 (TxB_2) synthesis by platelets during collagen induced (7.5 μg ml^{-1}) aggregation: (○) 2 min incubation; (△) 10 min incubation; (●) 20 min incubation.

[Methylene^{3}H]-picotamide binding

Equilibrium studies The binding of [methylene^{3}H]-picotamide at 22°C was saturable. Scatchard analysis of specific binding at equilibrium (i.e. after 30 min incubation) yielded a straight line, indicating a single class of binding sites for picotamide with K_D of 325 nmol l^{-1} and a B_{max} of 312 fmol per 10^8 platelets (Figure 3). The Hill coefficient (n_H) was 1.12 (Figure 4), suggesting that picotamide binds to a homogeneous individual class of binding sites without cooperativity.

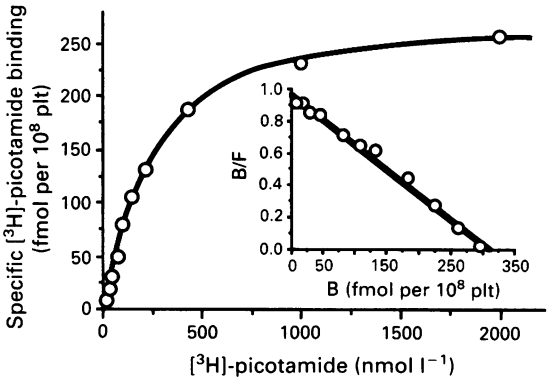

Figure 3 Saturation curve of the [methylene^{3}H]-picotamide binding to washed human platelets (fmol per 10^8 platelets) at 22°C. Scatchard analysis of the specific binding.

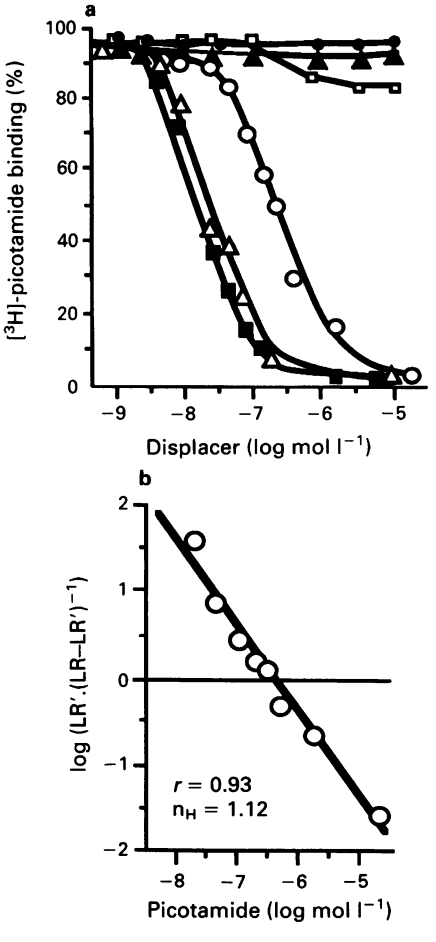

Figure 4 (a) Displacement of the specific [methylene^{3}H]-picotamide binding by increasing concentrations of different compounds: (■) U46619; (△) ONO11120; (○) picotamide; (□) TxB_2; (▲) PGE_2; (●) PGI_2. Data points represent means of triplicate determinations in at least four independent experiments. (b) Hill plot of the displacement curve by picotamide.

Binding of [methylene^{3}H]-picotamide was displaced by unlabelled picotamide, ONO11120 and by U46619 with a K_i of 361 nmol l^{-1}, 28 nmol l^{-1} and 19 nmol l^{-1} respectively. In contrast, PGI_2, PGE_2 and TxB_2 did not inhibit the binding (Figure 4).

Kinetic analysis The displaceable specific binding of [methylene^{3}H]-picotamide to platelets reached equilibrium after about 30 min. The observed rate constant of association (K_{obs}) was 0.110 min^{-1}. The dissociation curve performed after 30 min of incubation showed a displacement of about 65–70% of the total radioactivity bound. The analysis of the first order rate of dissociation showed a linear pattern with a K_{-1} of 0.107 min^{-1} ($n = 6$). The resulting association rate constant (k_1) was 0.00028 nmol l^{-1} min^{-1} with a calculated dissociation constant (K_D) of 382 nmol l^{-1} (Figure 5).

When the displaceable specific binding of [methylene^{3}H]-picotamide to platelets at 37°C was investigated after 30, 60 and 120 min, a progressive significant reduction of the binding capacity was observed. The B_{max} were 343, 243 and 153 fmol per 10^8 platelets after 30, 60 and 120 min respectively (2066, 1463, 921 binding sites/platelet respectively) with no significant changes in K_D (275, 251 and 222 nmol l^{-1} respectively) (Figure 6), thus indicating a progressive decrease of platelet receptors.

In contrast the non displaceable binding of [methylene^{3}H]-picotamide to platelets (i.e. the residual radioactivity after addition of 20 μmol l^{-1} picotamide to platelets at each time) at 37°C showed a slow regular increase (Figure 7). This time-dependent increase of non displaceable binding was almost completely inhibited when binding was performed at 4°C. The non displaceable binding of [methylene^{3}H]-picotamide did not increase when unlabelled picotamide was added at time 0 (non specific binding) (Figure 7).

The non displaceable binding at 37°C was concentration-dependent as it increased with increasing concentrations of [methylene^{3}H]-picotamide but was saturable. In fact, a progressive increase in binding velocity was found until a plateau level was reached with a Michaelis-Menten type kinetic of saturation (Figure 8). The Lineweaver-Burk plot was found to be linear (Figure 8) with a V_{maxapp} of 130 fmol per 10^8 platelets h^{-1} and a K_{Mapp} of 330 nmol l^{-1}. Therefore, about 35% of [methylene^{3}H]-picotamide bound to platelet receptors was irreversibly bound after 1 h.

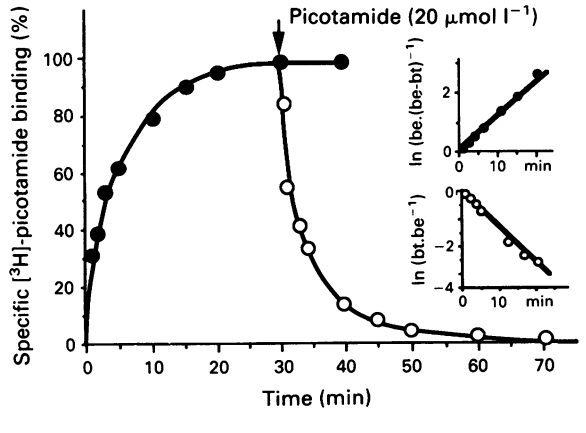

Figure 5 Time course of the association and dissociation phases of the specific [methylene^{3}H]-picotamide binding to washed human platelets at 22°C. Dissociation was obtained after 30 min incubation upon addition of 20 μmol l^{-1} (final concentration) picotamide to the incubation mixture. Data points represent means of triplicate determinations in at least four independent experiments. Upper inset: Specific binding association is plotted according to the pseudo-first order rate equation ($y = 0.331 + 0.110x$; $k_{obs} = 0.110$ min^{-1}). Lower inset: Dissociation is plotted as a first order reaction ($y = -0.461 - 0.107x$; $k_{-1} = 0.107$ min^{-1}; $k_1 = 0.00028$ nmol ml^{-1} min^{-1}; $K_D + 382$ nmol l^{-1}).

The non displaceable binding of [methylene^{3}H]-picotamide was inhibited by unlabelled picotamide, ONO11120 and U46619 when added at time 0 but not by PGI_2, PGE_2 and TxB_2 (data not shown).

Discussion

The present results indicate that [methylene^{3}H]-picotamide binds time-dependently to specific TxA_2/PGH_2 platelet receptors from which it is displaced by the TxA_2 agonist, U46619 and antagonist, ONO11120. Moreover, the time-dependent picotamide binding is associated with a progressive reduction

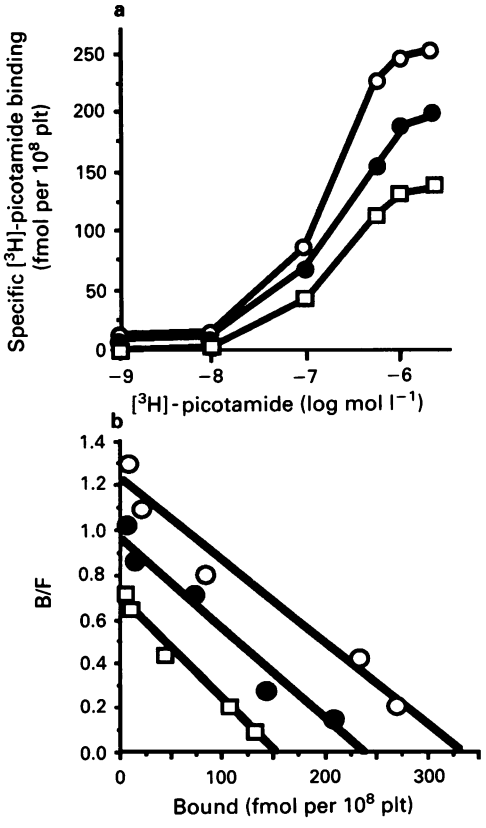

Figure 6 Saturation curve (a) and Scatchard analysis (b) of the specific [methylene^{3}H]-picotamide binding to washed human platelets (plt) at 22°C after 30 (○), 60 (●) and 120 min (□) of incubation.

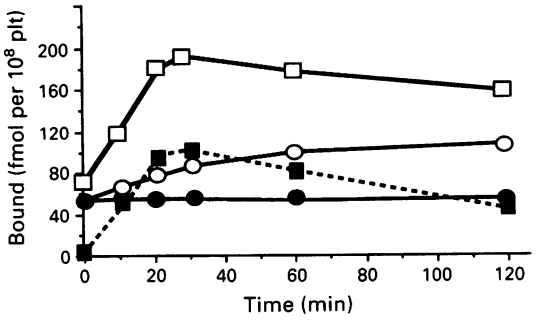

Figure 7 Time course of the binding of [methylene^{3}H]-picotamide 10 nmol l^{-1} to washed human platelets (plt) at 37°C. Total binding (□); specific displaceable binding at indicated time (t) (■) was considered as the radioactivity displaced by adding picotamide 20 μmol l^{-1} (final concentration) at time (t). Specific non-displaceable binding was defined as the difference between non displaceable binding (○) and non-specific binding (●).

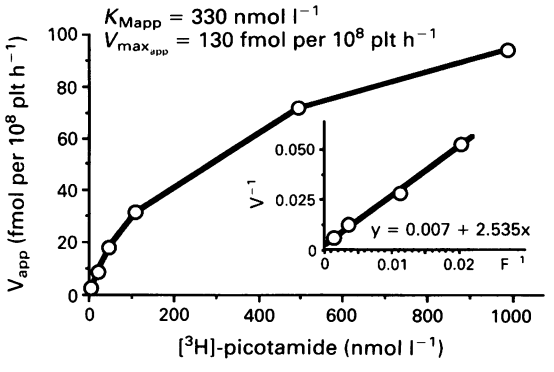

Figure 8 Analysis of the [methylene^{3}H]-picotamide uptake by human platelets (plt) in experiments performed at 37°C. Results shown are the means of experiments carried out in triplicate. Inset: Lineweaver-Burk's plot of the results.

in displaceable binding and a contemporary increase of specific non-displaceable binding.

These findings confirm previous indirect studies which had shown that picotamide shares the TxA_2/PGH_2 receptor (Modesti *et al.*, 1989). In addition they show that both the picotamide binding and the antiaggregating activity of picotamide occur slowly, reaching their maximum level after about 20 min incubation. The slow onset of picotamide binding accounts for the apparently low *in vitro* antiaggregating activity reported in previous studies (Gresele *et al.*, 1989; Berrettini *et al.*, 1990) in which the antiaggregating effect was assessed after only 2–10 min incubation, i.e. before that the binding equilibrium was reached.

The antiaggregating activity of picotamide is associated with reduced TxA_2 formation which may be due both to inhibition of TxA_2-synthase by picotamide (Gresele *et al.*, 1989) and to the reduced platelet aggregation resulting from TxA_2 receptor blockade. Indeed, TxA_2 formation during collagen-induced platelet aggregation was found to be reduced by the simple receptor antagonists not provided with TxA_2 synthase inhibitory properties (Hornby & Skidmore, 1984).

Previous studies on the antiaggregating effects of picotamide (Gresele *et al.*, 1989; Berrettini *et al.*, 1990) led to the suggestion that picotamide could act as a competitive TxA_2 inhibitor. However, the present kinetic analysis of the [methylene^{3}H]-picotamide binding showed a more complex pattern. Indeed [methylene^{3}H]-picotamide was readily and almost completely displaced from the TxA_2 receptor during the first 20 min of incubation whereas after the first 20 min the non displaceable amount progressively increased and paralleled the reduction of specific binding. This pattern conformed to that of non-competitive receptor inhibitors (Patscheke, 1990) and suggests a stable interaction of picotamide with the TxA_2 platelet receptor. The progressively increasing non displaceable binding of picotamide is unlikely to be due to a simple diffusion of the drug into the platelets because the non displaceable binding of [methylene^{3}H]-picotamide was saturable, reached a plateau and was specifically blocked by the addition of unlabelled picotamide. The non displaceable binding was found to be almost completely inhibited when the binding experiments were performed at 4°C. This fact and the observation that the K_M of the specific non reversible binding was in the same order of magnitude of the receptor binding K_D, are indicative of an internalization of the TxA_2 receptor-picotamide complex, although a simple non reversible receptor blockade cannot be excluded. Previous studies reported evidence of internalization of the TxA_2 receptor after the binding of ONO11120 (an antagonist of TxA_2) in human platelets (Modesti *et al.*, 1990) and U46619 (a TxA_2-mimetic) in cultured human leukaemic cells (Dorn, 1991).

In conclusion, picotamide binds to the TxA_2 receptor on human platelets with peculiar kinetics. After a first stabilizing period when the binding of picotamide is still reversible, a progressive stabilization of the binding takes place, resulting in an irreversible receptor blockade. For these characteristics picotamide is to be considered an inhibitor of platelet activity displaying both competitive and non competitive activity against thromboxane A_2-mediated responses.

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