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Supercooperativity in platelet aggregation: Substituted pyridyl isoxazoles, a new class of supercooperative platelet aggregation inhibitors

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Abstract

The phenomenon of supercooperativity in platelet aggregation is manifested by the occurence of clear-cut thresholds in dose–response relationships; in such cases the Hill coefficient has unusually high values. Approximation, by the Hill equation, of the relationship of the rate of arachidonate-induced platelet aggregation to the concentrations of either the inducer or inhibitors such as substituted pyridyl isoxazoles (synthesized by us), indomethacin, and pinane thromboxane A_2, demonstrated that the Hill coefficients ranged from 30 to 100. 3-(3-Pyridyl)-5-phenylisoxazole, which exhibited maximal anti-aggregatory activity among the synthesized compounds, inhibited neither cyclooxygenase nor thromboxane synthase. The compounds affected the signal transduction pathway at/or posterior to the stage of thromboxane A_2 reception.

Key words: Platelet aggregation; Inhibition; Supercooperativity; Substituted pyridyl isoxazole

1. Introduction

Platelet aggregation is the most important defensive mechanism preventing the organism from blood loss upon vessel wall damage [1]. The platelets are bridged into aggregates by intercellular GP IIb/IIIa fibrinogen – GP IIb/IIIa bonds [2,3]. The exposure of GP IIb/IIIa, the platelet fibrinogen receptor, is the result of a series of successive biochemical processes, initiated by aggregation inducers. Binding of the inducers (TxA_2, ADP, serotonin, collagen, epinephrine, vasopressin, thrombin, platelet-activating factor) by their specific platelet receptors is an important element in this chain of events [4–10]. Such receptor– ligand interactions trigger off a variety of G-protein-mediated effects, e.g. activation of phospholipases (A_2 and/or C) and inhibition of adenylate cyclase [11–14]. Intracellular enzyme systems change the concentration of regulators and secondary messengers (inositol tris-phosphate, diacylglycerol, TxA_2, cyclic AMP, Ca^{2+}, H^+, etc.) [13,15–17] which constitute the signal transduction cascade.

Hence, generation of aggregatory responses is the result of transduction of chemical signals through multistage pathways. Although the exact number of stages is unknown, each stage is obviously characterized by a certain relationship between input and output signals. Approximation of such relationships by the Hill equation allowed us to analyze a generalized model of signal transduction [18,19]. The relationships of cell response (F) to inducer (A) or inhibitor (I) concentrations, which were obtained in the context of this model, can also be approximated by the Hill equation with a high degree of precision (Eqs. **(1)** and **(2)**, respectively):

$$F/F_{max} = \frac{([A]/K_A)^{h_A}}{1 + ([A]/K_A)^{h_A}} \quad (1)$$

$$F/F_{max} = \frac{1}{1 + ([I]/K_I)^{h_I}} \quad (2)$$

where K_A is the EC_{50}, K_I is the IC_{50}.

It follows from the analysis [18] that, if the relationship between input and output signals at a given step of signal transduction is characterized by a very high value of the Hill coefficient (i.e. the step is supercooperative), the Hill coefficient (h_A) for the relationship of F to [A] (Eq. **(1)**) will also take an extremely high value (i.e. the relationship will be supercooperative). If an inhibitor I affects the signal transduction pathway at a stage preceding the supercooperative step, the expression for h_I will incorporate the Hill coefficient value of the supercooperative step, and h_I for the relationship of F to [I] (Eq. **(2)**) will also be very high (i.e. the relationship will be supercooperative). In this latter case the inhibitor should be termed 'supercooperative inhibitor'. If an inhibitor affects the signal transduction pathway at or posterior to the supercooperative step, the Hill coefficient value of the supercooperative step will not enter Eq. **(2)**, and the relationship of F to [I] in this case will be of ordinary cooperativity (the value of h_I for such relationships is usually comparable to unity).

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Abbreviations: Tx, thromboxane; PTA_2, pinane thromboxane A_2; GP, glycoprotein; ADP, adenosine diphosphate; AMP, adenosine monophosphate; F, cell response; A, inducer; I, inhibitor; h, Hill coefficient; PRP, platelet-rich plasma; PPP, platelet-poor plasma; DMSO, dimethylsulfoxide.

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2. Materials and methods

2.1. Preparation of PRP

Blood was drawn from cubital veins of normal volunteers into 1/10 vol. of 3.8% trisodium citrate and centrifuged at 150 × *g* (room temperature) for 15 min.

Aggregation of platelets was measured as defined elsewere [19]. The aggregation rate (*F*) was calculated from the slope of each aggregation curve. In the results presented the aggregation rate is expressed as a fraction of the averaged maximal value obtained throughout a given experiment.

2.2. Reagents

ADP, indomethacin, hemin and arachidonic acid were purchased from Sigma (USA), PTA_2 from Calbiochem-Behring (USA), epinephrine from Serva (Germany).

Substituted pyridyl isoxazoles and isoxazolines were synthesized (to be published elsewere) by 1,3-cycloaddition of pyridyl nitrile oxides (generated in situ from pyridyl hydroxamoyl chlorides) to terminal alkynes and alkenes, respectively. Compounds (3a–c, 3g, 3i, 4a–c) were synthesized for the first time. The structures were assigned by ^{1}H NMR spectroscopy, mass spectrometry and element analysis.

3. Results and discussion

Our interest in substituted pyridyl isoxazoles as platelet-affecting agents [20–21] relies on the assumption that bicyclic structures (including isoxazoline and isoxasolidine moieties) are largely complementary to the active sites of eicosanoid-binding receptors and enzymes [22–25].

We synthesized and studied 3,5-substituted isoxazoles and isoxazolines, which contained pyridine fragments at position 3:

3(a–i) 4(a–d)

Where 3a (R = $-CH_2OH$, R′ = 2-Py), 3b (R = $-CH_2OH$, R′ = 3-Py), 3c (R = $-CH_2OH$, R′ = 4-Py), 3d (R = $-C_6H_5$, R′ = 2-Py), 3e (R = $-C_6H_5$, R′ = 3-Py), 3f (R = $-C_6H_5$, R′ = 4-Py), 3g (R = $-C(OH)(CH_3)_2$, R′ = 3-Py), 3h (R = -Cl, R′ = 4-Py), 3i (R = $-CH_2Br$, R′ = 2-Py), 4a (R = $-CO_2Et$, R′ = 3-Py), 4b (R = $-CO_2Et$, R′ = 4-Py), 4c (R = $-(CH_2)_5CH_3$, R′ = 3-Py), 4d (R = $-OCOCH_3$, R′ = 3-Py).

The compounds synthesized lacked proaggregatory activity up to 1 mM, but inhibited, within the range from 1 to 1000 μM, both arachidonate-induced platelet aggregation and the second phase of ADP-induced platelet aggregation (primary ADP-induced aggregation was not affected).

Judging by IC_{50} values for arachidonate-induced platelet aggregation, pyridyl isoxazoles can be arranged in the following order of decreasing activity:

3e > 3b > 3f ≥ 3c > 3d > 3a.

3-(3-Pyridyl)-5-phenylisoxazole (compound 3e) turned out to be the most active in this series. Fig. 1 shows the relationship (obtained with PRP samples of 8 individuals) of the rate of arachidonate-induced aggregation to the concentration of this compound, as plotted in dimensionless relative coordinates. This threshold-like relationship can be described by Eq. (2) using a cooperativity coefficient (h_I) equal to 30.

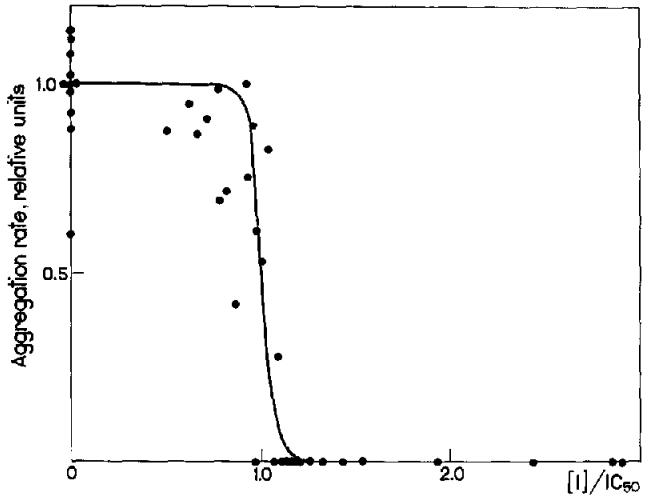

Fig. 1. Relationship of the rate of arachidonate-induced human platelet aggregation to the concentration of 3-(3-pyridyl)-5-phenylisoxazole. Arachidonic acid, 500 μM. The theoretical curve is plotted using Eq. (2) (h_I = 30).

In its turn, concentration-dependence of arachidonate-induced aggregation rate is described by Eq. (1) at h_A = 30 (Fig. 2). Thus, in both cases the Hill coefficient considerably exceeds unity. Similar relationships were obtained for the second phases of ADP- or epinephrine-induced aggregation [19]. The inhibition of arachidonate-induced platelet aggregation by indomethacin (a cyclooxygenase blocker) or PTA_2 (a TxA_2 receptor antagonist) is described by Eq. (2) at h_I = 100 (Fig. 3). Here, again, the Hill coefficient substantially exceeds unity.

As follows from the analysis [18], such supercoopera-

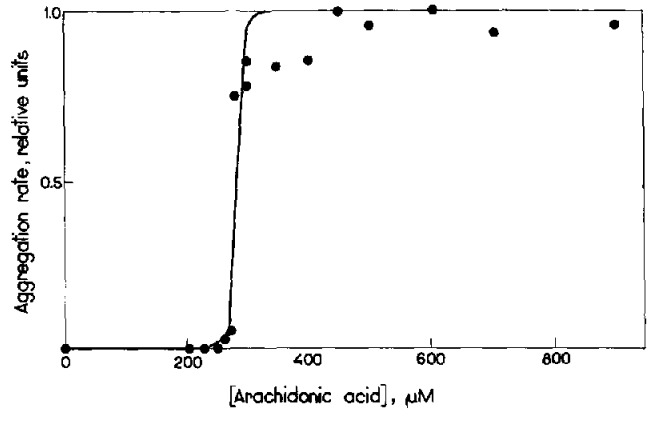

Fig. 2. Relationship of the rate of arachidonate-induced human platelet aggregation to the concentration of the inducer. The theoretical curve is plotted using Eq. (1) (K_A = 275 μM, h_A = 30).

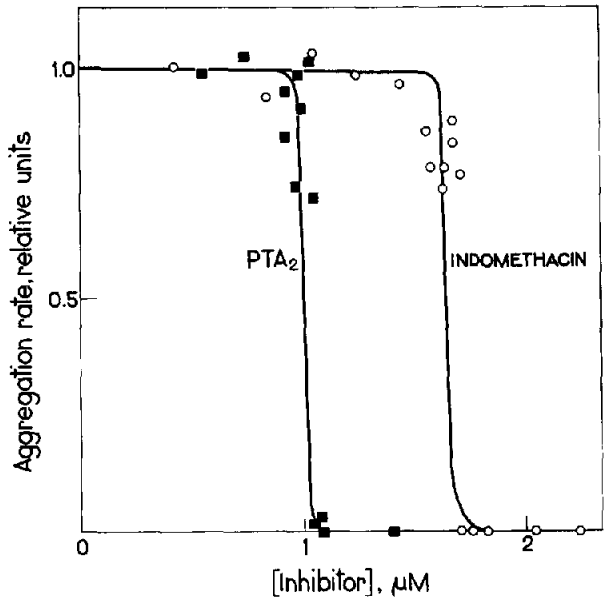

Fig. 3. Inhibition of arachidonate-induced human platelet aggregation by indomethacin or PTA_2. Arachidonic acid, 500 μM. The theoretical curves are plotted using Eq. (2) (Indomethacin, K_i = 1.62 μM, h_i = 100; PTA_2, K_i = 1.0 μM, h_i = 100).

tive relationships can arise from the existence of a supercooperative step within the signal transduction pathway. In the case of platelet aggregation, this step is located within the intracellular region of the pathway, since the extracellular stages (platelet collisions in the shear flow and aggregate formation) are characterized by ordinary cooperativity [26–28].

The supercooperative step is expected to be posterior to the stages of arachidonate oxidation and TxA_2 reception. It is noteworthy that supercooperative effects could be observed with indomethacin-sensitive (arachidonate-dependent) second phases of ADP- and epinephrine-induced platelet aggregation, whereas the first phases of these responses (arachidonate-independent) were characterized by ordinary cooperativity [19]. Hence, the phenomenon of supercooperativity may be associated with cyclooxygenase metabolism of arachidonate.

To elucidate the mechanism whereby 3e inhibited platelet aggregation, we assessed the effects of this compound on the activity of the enzymes of the arachidonate cascade. In these experiments we used two enzyme preparations, cyclooxygenase from sheep vesicular glands and the microsomal fraction of human platelets that contained both cyclooxygenase and Tx synthase. We were able to demonstrate that the tested compound inhibited neither cyclooxygenase, nor Tx synthase (up to 1,000 μM; data not shown).

Hence, the substituted pyridyl isoxazoles synthesized by us are supercooperative inhibitors of platelet aggregation and affect the signal transduction pathway at a stage preceding the supercooperative step. Compound 3e was similar in its kinetic characteristics to indomethacin and PTA_2, but inhibited neither cyclooxygenase nor Tx synthase. In a series of preliminary experiments we detected specific binding of the tritium-labeled 3e to the platelet surface. Therefore, TxA_2 receptors, which constitute the nearest possible target within the signal transduction pathway, might be affected by the isoxazole-containing compounds under study.

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