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EFFECTS OF 2,2′-DIPYRIDYL AND RELATED COMPOUNDS ON PLATELET PROSTAGLANDIN SYNTHESIS AND PLATELET FUNCTION

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Summary

2,2′-dipyridyl, a chelator of ferrous iron and inhibitor of platelet aggregation, was studied together with several similar compounds to determine the mechanism of their effects on platelets. All of these compounds were more potent inhibitors of arachidonic-acid-mediated aggregation (IC_{50}, 0.17—1.8 mM) than of ADP-mediated aggregation (IC_{50}, 7.6—19.7 mM). At low concentrations required to inhibit arachidonic-acid-mediated aggregation, 2,2′-dipyridyl, 4,4′-dipyridyl and 2-chloropyridine specifically inhibited the platelet cyclo-oxygenase. The mechanism of inhibition of ADP-induced aggregation was investigated, but was not explained. At concentrations needed to inhibit ADP-induced aggregation, 2,2′-dipyridyl did not alter cell ultrastructure, serotonin or nucleotide content or interfere with release of [^{14}C]arachidonic acid or calcium movements. Therefore, our results indicate that 2,2′-dipyridyl and related compounds have two effects on platelets, both due to the unprotonated form. The inhibition of cyclo-oxygenase by low concentrations of these compounds is not due to bidentate iron chelation, since 4,4′-dipyridyl was almost as effective as 2,2′-dipyridyl, but is compatible with binding of these inhibitors to the iron in the heme of the cyclo-oxygenase.

Introduction

Platelet activation occurs through at least two pathways, one of which is dependent on the synthesis of prostaglandin endoperoxides and thromboxane A_2 [1,2]. In the study of the mechanism of synthesis of prostaglandin endo-

peroxides, considerable interest has focused recently on the role of iron in the cyclo-oxygenase [3,4], and evidence that iron, as heme, is required for its activity [5,6]. A few reports have suggested that certain compounds, which are well established chelators of iron, and others, which under certain circumstances may bind iron as unidentate chelators, could be effective in inhibiting platelet aggregation and platelet prostaglandin synthesis [7—9]. The agents tested included compounds representing unidentate pyridine derivatives with a single functional ring nitrogen atom and bidentate compounds having two ring nitrogen atoms. Of the bidentate compounds, 2,2′-dipyridyl with two functional nitrogen atoms located nearby could act as a true bidentate chelator whereas 4,4′-dipyridyl, although bidentate by definition, could not chelate the metal ion because the functional nitrogen atoms are at opposite ends of the molecule.

In the present study, we have evaluated a group of pyridine derivatives for their effects on platelet function. This group contains compounds which can interact with iron in a bidentate fashion, or a unidentate fashion, and which vary in lipid solubility and pK_a values and, therefore, may give useful information on the mechanism by which these compounds inhibit platelet reactions. Considerable experience with one of these compounds, 2,2′-dipyridyl, in other cells and in isolated mitochondria has suggested that it can cross lipid membranes and chelate iron intracellularly [10,11]. Previous work also suggests it can inhibit platelet aggregation [7]. Thus, experiments with 2,2′-dipyridyl and related compounds can be expected to be important to further understanding the role of iron in prostaglandin synthesis and in platelet function, and in facilitating the design of future, useful, platelet aggregation inhibitors.

Materials and Methods

Chemicals and reagents. 2,2′-dipyridyl (2,2′-bipyridine); 4,4′-dipyridyl dihydrochloride; pyridine; 2-chloropyridine; 3-chloropyridine; 4-chloropyridine; 2,4,6-trimethylpyridine and 3,5-dimethylpyridine were obtained from Aldrich Chemical Co., WI. 5-hydroxy[*side chain*-2-^{14}C]tryptamine creatinine sulfate was obtained from Amersham, Arlington Heights, Illinois. [1-^{14}C]arachidonic acid was obtained from New England Nuclear, Boston, MA. Unless otherwise noted, all other chemicals were obtained from Sigma Chemical Co., St. Louis, MO.

General. Platelets for these studies were obtained from healthy adult volunteers after informed consent. Blood drawn from an anticubital vein into siliconized glass or plastic syringes was mixed immediately with citrate/glucose buffer (0.1 M sodium citrate/0.13 M glucose pH 6.5; 9 blood: 1 anticoagulant). Platelet-rich plasma was separated by centrifugation at $100 \times g$ for 20 min at room temperature. The effect of pyridine derivatives on platelet morphology, platelet nucleotides and platelet serotonin was evaluated by adding various concentrations of these agents to samples of platelet-rich plasma and incubating the samples for 30, 60 and 120 min at 37° C. Samples were prepared by glutaraldehyde-osmic acid fixation for study in the electron microscope according to the methods established in this laboratory [12]. Samples for nucleotide analysis were analyzed using high-pressure liquid chromatography [13], while sero-

tonin content was assessed spectrofluorometrically [14]. Adenylate energy charge was calculated by the formula (ATP + 0.5 [ADP])/(ATP + ADP + AMP).

Platelet aggregation studies. Aggregation was monitored with a dual-channel Payton aggregometer at a stirring speed of 1000 rev./min and calibrated using platelet-rich plasma and platelet-poor plasma. Baseline of the chart recorder was adjusted with platelet-rich plasma and maximum light transmission with platelet-poor plasma. All platelet samples were prewarmed for 10 min at 37°C and inhibitors, where necessary, were added before incubation. Light transmission equivalent to that seen in platelet-poor plasma was considered to represent 100% aggregation. A series of concentrations of the pyridine derivatives were tested for inhibition of aggregation induced by a single concentration of each agent, where the concentration of aggregation agent gave greater than 90% aggregation. Inhibitory concentration (IC_{50}) values were the concentrations at which the inhibitor reduced the maximum aggregation to half of that which was obtained for the same aggregating agents with the same platelet-rich plasma with the buffer only added.

In order to determine if the effects of pyridine derivatives were reversible, platelets incubated in 10 mM solutions of the pyridine derivatives were diluted 1 : 1 (v/v) with the anticoagulant solution, pelleted by centrifugation for 20 min at 700 × *g*, and resuspended in Hanks' balanced salt solution at half the original platelet-rich plasma volume. An equal volume of citrated platelet-poor plasma was added and these washed platelet suspensions were tested for aggregation induced by ADP, epinephrine and arachidonic acid by the methods above.

Platelet serotonin secretion. Platelets in platelet-rich plasma were labelled with [^{14}C]-serotonin by a modification [15] of the method of Jerushalmy and Zucker [16], for the evaluation of the release reaction. Samples of prelabelled platelets were exposed to an aggregating agent on the aggregometer at 37°C until maximum aggregation was obtained. Aggregation was stopped with the addition of EDTA to a final 1% concentration, and the pellet and supernatant separated by rapid centrifugation at 4°C. Pellet and supernatant fractions containing the labelled serotonin were separated and counted in a scintillation counter.

Platelet metabolism of arachidonic acid. The ionophore A23187-stimulated release of [^{14}C]arachidonic acid from platelet phospholipids was measured using a modification [17] of the method of Bills et al. [18]. Metabolites formed during platelet oxidation of arachidonic acid by the lipoxygenase enzyme, leading to the formation of 12-L-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE), and the cyclo-oxygenase leading primarily to the formation of the 17 carbon atoms hydroxytrienoic acid (12-HHT) and thromboxane B_2, were followed using a modification [17] of the method of Hamberg and Samuelsson, [19] with [^{14}C]arachidonic acid as the substrate. Labelled arachidonic acid was diluted with unlabelled arachidonic acid (Nu Chek Prep) to a specific activity of 25—28 Ci/mol, made up as a sodium salt and diluted in 0.1 M Tris-HCl buffer, pH 7.0. 1 ml aliquots of each reaction mixture containing $2 \cdot 10^8$ platelets were incubated for 3 min at 37°C with 1 μg labelled arachidonic acid. At the end of the incubation period, 10 ml of ethanol was added to stop the reaction. The conversion of labelled fatty acid to hydroxy derivatives

and to thromboxane B_2 was followed using thin layer chromatography of the methyl esters on silica gel G with the organic layer of isooctane/water/ethyl acetate (100 : 100 : 50, v/v) as the eluting solvent (system A). Conversion of labelled arachidonic acid to thromboxane B_2 was also evaluated using thin-layer chromatography of the free acids on silica gel G with diethyl ether : methanol : acetic acid (135 : 5 : 3, v/v) as the eluting solvent (system B).

Assessment of a possible interaction of 2,2′-dipyridyl and calcium. The ability of 2,2′-dipyridyl to interact with calcium was evaluated in three different ways: (1) the absorbance spectra of dipyridyl, dipyridyl in 100 mM $CaCl_2$, and dipyridyl incubated first in 100 mM $CaCl_2$ before exposing the solution to 1 mM $FeSO_4$; (2) the influence of dipyridyl on the conversion of biconcave discoid erythrocytes to echinocytes and spheroechinocytes in the presence of the ionophore A23187 and calcium was evaluated as described previously [20]. Ionophore-induced changes in red blood cell morphology have been shown to result from calcium flux caused by the ionophore. Red blood cells in 10 mm 2,2′-dipyridyl were exposed to 1 μM ionophore to evaluate whether dipyridyl complexed the calcium or otherwise interfered with the action of ionophore, thereby blocking the membrane alterations induced by calcium uptake; (3) a platelet membrane fraction which actively sequesters calcium was prepared using a modification [21,22] of the methods of Robblee [23] and Kaser-Glanzmann et al. [24]. To assess the effect of 2,2′-dipyridyl on the release of calcium from inside the vesicles, the membrane fraction was equilibrated with $^{45}CaCl_2$ in the presence of 2 mM ATP for 10 min so that vesicles would accumulate calcium before addition of the calcium ionophore, A23187 [25,26].

Results

The influence of pyridine derivatives on platelet aggregation

All pyridine derivatives tested were capable of inhibiting platelet aggregation induced by either arachidonic acid, epinephrine or ADP. Of the three aggregating agents, arachidonic acid was the most sensitive to the influence of pyridine derivatives with a mean IC_{50} for the six compounds tested of 1.11 mM (Table I). Epinephrine was somewhat less sensitive with a mean IC_{50} of 4.0 mM, and ADP was much less sensitive with a mean IC_{50} of 10.5 mM. 2,2′-Dipyridyl was the most potent inhibitor of arachidonic acid aggregation. However, the difference between it and 4,4′-dipyridyl was small. Variations in the inhibition appeared unrelated to the pK_a of the pyridine derivative. There was a trend for increased inhibitory activity associated with increased lipid solubility. This trend approaches significance when the partition coefficients are correlated with the IC_{50} for arachidonic acid-induced aggregation ($r = 0.70$; $P < 0.05$). If only the pyridine derivatives are considered, that is dimethylaniline is deleted, this trend is more significant ($r = 0.85$; $P < 0.02$).

To determine if the pyridine derivatives irreversibly damaged or inactivated a component essential for platelet function, platelets, exposed to 10 mM 2,2′-dipyridyl, were washed to remove the compound and then restudied. These washed platelets showed no effects of their previous exposure to 2,2′-dipyridyl and aggregated normally in response to arachidonic acid, epinephrine and ADP (Fig. 1).

TABLE I

IC_{50} VALUES, ACID DISSOCIATION CONSTANTS AND PARTITION COEFFICIENTS FOR SELECTED PYRIDINE DERIVATIVES

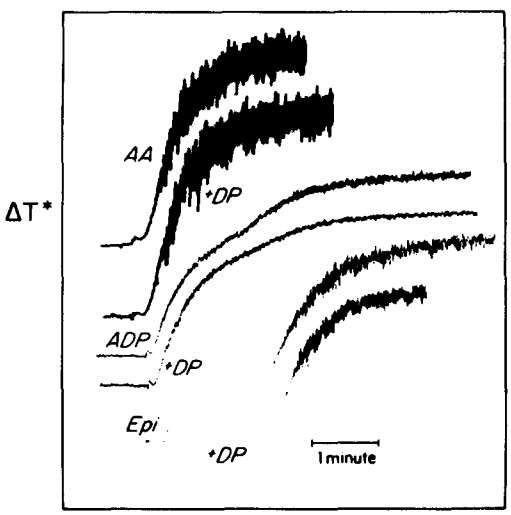
IC_{50} values are expressed as mean and S.E. (n = 3). n.a., not assayed.

Inhibitor	pK'_a *	P.C. **	IC_{50} values (mM)		
			ADP (5 μM)	Epinephrine (5.5 μM)	Arachidonate (770 μM)
2-Chloropyridine	0.72	3.9	8.9 ± 1.2	3.0 ± 0.6	1.2 ± 0.2
3-Chloropyridine	2.84	4.2	9.3 ± 1.1	3.2 ± 0.7	0.9 ± 0.1
4-Chloropyridine	3.88	2.4	9.1 ± 1.1	3.0 ± 0.6	1.8 ± 0.1
2,2′-Dipyridyl	4.44	4.6	8.5 ± 1.0	1.4 ± 0.5	0.17 ± 0.04
4,4′-Dipyridyl	4.8	4.6	n.a.	n.a.	0.26 ± 0.17
Dimethylaniline	5.07	8.7	n.a.	2.2 ± 0.6	0.52 ± 0.07
Pyridine	5.17	0.5	19.7 ± 1.7	12.3 ± 1.8	1.8 ± 0.8
3,5-Dimethylpyridine	6.14	3.2	7.6 ± 0.6	0.9 ± 0.5	0.9 ± 0.1

* pK'_a values taken from Ref. 27.
** Partition coefficient between isooctane and 150 mM NaCl, pH 7.

The influence of pyridine derivatives on platelet ultrastructure serotonin and nucleotide content

Platelets incubated with 10 mM 2,2′-dipyridyl for up to an hour showed no significant changes in ultrastructure from platelets incubated with buffer alone. In both the control and experimental samples the platelets retained their discoid appearance with granules and dense bodies scattered randomly within



* ΔT—change in light transmission

Fig. 1. The reversibility of 2,2′-dipyridyl inhibition of platelet aggregation. Platelets were incubated with buffer or 2,2′-dipyridyl 10 mM for 10 min at 37°C. At this time 2,2′-dipyridyl completely inhibited arachidonic acid and epinephrine aggregation, and inhibited ADP-mediated aggregation by about 90%. Samples were then washed once, and retested. After one wash, dipyridyl-treated platelets aggregated normally to greater than 70 μM arachidonic acid (AA), 5 μM adenosine diphosphate (ADP) and 5.5 μM epinephrine (Epi).

the cytoplasm, microtubules remaining in a circumferential band just beneath the cell membrane (Fig. 2). As 2,2′-dipyridyl at high concentration (10 mM) had some effect on the ATP level (Table II, Experiment A), studies with a lower concentration of 2,2′-dipyridyl were conducted. At this concentration (1 mM) 2,2′-dipyridyl had no significant effect on adenine nucleotide levels and available adenylate energy charge (Experiment B).

The influence of pyridine derivatives on platelet arachidonic acid metabolism

The release of arachidonic acid from platelet phospholipids as stimulated by the calcium ionophore, A23187 (10 μM), was unaffected by 2,2′-dipyridyl (buffer alone, 15.7% ± 1.8; with 10^{-2} M dipyridyl, 15.9% ± 4.3; ± S.D. of six replicates). In contrast, pyridine derivatives showed a concentration-dependent inhibition of the conversion of arachidonic acid to HHT and thromboxane B_2 (Table III). Concomitantly, there was an increase in formation of the product of the lipoxygenase pathway (12-HETE). The inhibition of prostaglandin synthesis by 2,2′-dipyridyl is similar to that seen using aspirin or aminotriazole where cyclo-oxygenase inhibition is accompanied by increased 12-HETE production. Inhibition in this situation is due to failure of endoperoxide and thromboxane A_2 synthesis, rather than to accumulation of 12-HETE [8,27]. 2,2′-Dipyridyl

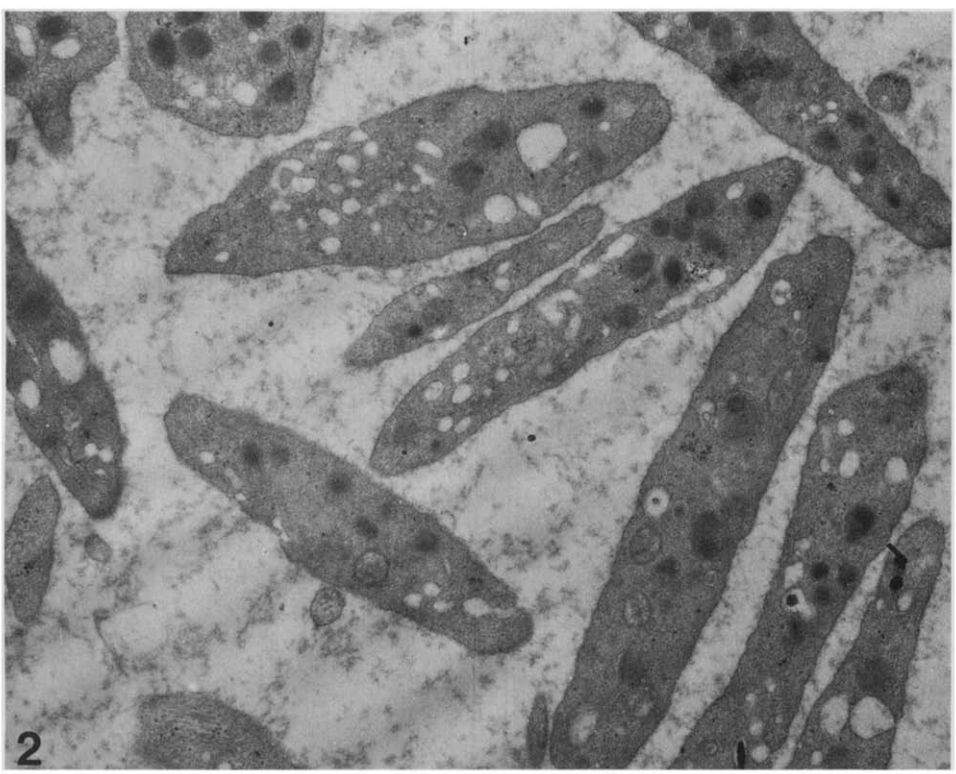


Fig. 2. Platelets from a sample of platelet-rich plasma incubated with 10 mM 2,2′-dipyridyl for 120 min at 37°C. These platelets have retained their discoid shape. Microtubules are present just beneath the cell membrane as in resting cells, and granules are scattered randomly within the platelet interior. Stained with uranyl acetate and lead citrate (Mag × 16 500).

TABLE II

THE INFLUENCE OF PYRIDINE DERIVATIVES ON PLATELET CONTENT OF NUCLEOTIDES AND SEROTONIN CONTENT

Values are expressed as mean ± S.E. (n = 3). PRP, platelet-rich plasma.

		Concentrations (μmol/10^{11} platelets)				Cyclic AMP (pmol/ml PRP)	Serotonin (ng/10^9 cells)
		ATP	ADP	AMP	AEC		
Experiment A	Control platelets	4.7 ± 0.5	3.0 ± 0.2	0.59 ± 0.03	0.74 ± 0.004	29 ± 0.8	927 ± 11
	Platelets + 2,2′-dipyridyl 10^{-2} M	4.1 ± 0.1	3.2 ± 0.1	0.85 ± 0.06	0.69 ± 0.005	30 ± 0.9	917 ± 18
	Platelets + 2-chloropyridine 10^{-2} M	4.0 ± 0.1	3.0 ± 0.1	0.91 ± 0.04	0.69 ± 0.004	28 ± 0.8	896 ± 14
Experiment B	Control platelets	5.1 ± 0.4	3.0 ± 0.3	0.46 ± 0.1	0.77 ± 0.004	—	—
	Platelets + 2,2′-dipyridyl 10^{-3} M	5.3 ± 0.2	3.1 ± 0.1	0.48 ± 0.02	0.78 ± 0.005	—	—

TABLE III

THE INFLUENCE OF 2,2′-DIPYRIDYL AND ANALOGS ON PLATELET ARACHIDONIC ACID TRANSFORMATION

The percent of product is percent label converted to each metabolite. Results are the mean of duplicate determinations.

	Percentage of product			Inhibition of HHT and thromboxane B_2 (%)
	12-HETE	12-HHT	Thromboxane B_2	
Washed platelets + buffer + [^{14}C]arachidonic acid	24.9	21.3	24.2	
Washed platelets + 10^{-2} M 2,2′-dipyridyl + [^{14}C]arachidonic acid	71.0	0.5	1.1	96.5
Washed platelets + 10^{-3} M 2,2′-dipyridyl + [^{14}C]arachidonic acid	57.0	4.0	4.7	80.9
Washed platelets + 10^{-4} M 2,2′-dipyridyl + [^{14}C]arachidonic acid	21.1	19.2	18.5	17.1
Washed platelets + 10^{-2} M 4,4′-dipyridyl + [^{14}C]arachidonic acid	68.0	1.0	2.4	92.6
Washed platelets + 10^{-3} M 4,4′-dipyridyl + [^{14}C]arachidonic acid	36.4	8.3	14.5	49.9
Washed platelets + 10^{-4} M 4,4′-dipyridyl + [^{14}C]arachidonic acid	24.5	20.5	23.5	3.3
Washed platelets + 10^{-2} M 2-chloropyridine + [^{14}C]arachidonic acid	69.1	1.2	1.5	94.1
Washed platelets + 10^{-3} M 2-chloropyridine + [^{14}C]arachidonic acid	32.7	12.6	17.5	33.8
Washed platelets + 10^{-4} M 2-chloropyridine + [^{14}C]arachidonic acid	20.4	20.4	24.0	3.4

was the most potent, 4,4′-dipyridyl slightly less effective and 2-chloropyridine the least effective, as inhibitors of arachidonic acid induced-aggregation. The IC_{50} for inhibition of 12-HHT and thromboxane B_2 synthesis for all three compounds was in the range of 0.2—2 mM, similar to the range of IC_{50} seen for arachidonic acid-induced platelet aggregation. No increased production of prostaglandin E_2 and prostaglandin D_2 was seen, showing that at concentrations which inhibited arachidonic acid aggregation the effect was due to inhibition of cyclo-oxygenase and not a selective inhibition of the thromboxane synthetase enzyme [28].

The influence of pyridine derivatives on platelet secretion

Platelets which had been incubated with pyridine derivatives at a concentration of 10 mM, showed a marked and significant inhibition of secretion with all aggregating agents tested including arachidonic acid, epinephrine, ADP, collagen and the calcium ionophore A23187 (Table IV).

Since high concentrations of 2,2′-dipyridyl caused inhibition of secretion induced by the ionophore, A23187, as well as ADP, the effect of lower concentration (1 mM) of 2,2′-dipyridyl was tested against these aggregating agents (Table V). At this concentration 2,2′-dipyridyl blocked arachidonic acid and ADP induced aggregation and secretion of serotinin. However, there was no

TABLE IV

EFFECT OF 2,2'-DIPYRIDYL (10 mM) ON THE RELEASE OF [^{14}C]SEROTONIN FROM HUMAN PLATELETS

	cpm × 10^3		Percent release *	
	Pellet	Supernate	Without inhibitor	With inhibitor
Control	9.2	0.48	4.9 ± 0.12	—
Collagen (30 μg/ml)	4.7	3.9	45.9 ± 0.81	5.8 ± 0.29
Epinephrine (5.5 μM)	4.1	3.6	46.9 ± 0.91	5.2 ± 0.15
ADP (5 μM)	4.7	3.2	40.6 ± 1.0	5.7 ± 0.31
Arachidonic acid (390 μM)	3.8	4.1	51.6 ± 1.7	5.3 ± 0.17
Ionophore A23187 (1 μM)	3.5	4.5	56.1 ± 2.3	6.0 ± 0.28

* Mean ± S.E. (n = 3).

inhibitory effect on the aggregation or release of serotonin induced by the ionophore A23187.

The interaction of 2,2'-dipyridyl with calcium

Since it is generally assumed that calcium modulates the contraction-secretion process in platelets, as well as platelet aggregation [29], the ability of dipyridyl to chelate calcium was studied. Ultraviolet spectral studies of dipyridyl challenged with a high concentration of calcium followed by subsequent exposure to ferrous iron showed that the ability of the compound to complex with the iron was unaltered. Studies with red blood cells showed that 2,2'-dipyridyl did not block the discocyte to echinocyte transformation of the cells induced by calcium ionophore, suggesting that dipyridyl did not block the calcium flux induced by ionophore. Results of the studies with isolated membrane vesicles showed that dipyridyl had no inhibitory effect on the selective uptake of calcium by the membrane vesicles or its release by ionophore A23187 (Table VI). Results of these studies suggest that dipyridyl does not inhibit platelets by

TABLE V

EFFECT OF 2,2'-DIPYRIDYL (1 mM) ON THE RELEASE OF [^{14}C]SEROTONIN FROM HUMAN PLATELETS

	cpm × 10^3		Percent release *
	Pellet	Supernatant	
Control	132.0	12.3	8.5 ± 0.1
Arachidonic acid (450 μM)	62.8	79.4	55.8 ± 1.4
Dipyridyl (1 mM) Arachidonic acid (450 μM)	130.0	13.4	9.4 ± 0.3
ADP (3 μM)	90.0	53.8	37.3 ± 1.6
Dipyridyl (1 mM) ADP (3 μM)	131.9	11.6	8.0 ± 0.5
Ionophore A23187 (5 μM)	25.4	118.2	82.2 ± 0.9
Dipyridyl (1 mM) Ionophore A23187 (5 μM)	29.0	113.5	79.5 ± 0.7

* Mean ± S.E. (n = 3).

TABLE VI

THE INFLUENCE OF 2,2′-DIPYRIDYL ON UPTAKE AND RELEASE OF CALCIUM FROM A PLATELET MEMBRANE FRACTION

Data expressed as mean ± S.D. of three determinations.

	Calcium uptake (ng/mg protein per 10 min)	Percent calcium release induced by 5 μM A23187
Buffer alone	27.3 ± 1.1	86.9 ± 1.7
Dipyridyl, 0.1 mM	26.9 ± 1.5	83.3 ± 5.6
Dipyridyl, 1 mM	27.6 ± 1.4	88.5 ± 1.2
Dipyridyl, 10 mM	25.3 ± 0.7	91.4 ± 2.1

interfering with calcium-mediated processes. However, the possibility that calcium release mediated in platelets by more physiological agents than ionophore A23187 could be affected by dipyridyl was not ruled out in this study.

Discussion

The present investigation has evaluated the influence of pyridine derivatives on platelet function and found significant inhibitory effects. Initial studies on platelet aggregation showed that arachidonic acid was much more sensitive to the inhibitory influence of these agents than was ADP. Studies of the mechanism by which these agents prevented arachidonic acid aggregation showed that at the inhibitory concentration (1 mM) they were not damaging platelets or reducing the pool of ATP, but specifically inhibited the conversion of arachidonic acid to 12-HHT and thromboxane B_2. 2,2′-Dipyridyl was more potent than the derivative containing a single pyridine ring, but only slightly more potent that 4,4′-dipyridyl which could not function as a bidentate chelator. A re-evaluation of the results obtained by Vargaftig et al. using other chelators [7] suggests the same conclusion. The bidentate chelator, 1,17-phenanthroline, was only 1.9 times more effective than 1,7-phenanthroline, which can only function in an unidentate fashion. The specificities of these bidentate chelators and their relative potency cannot be used to identify the metal ions involved in arachidonic acid metabolism as indicated before [7], since their inhibition may not be exerted through bidentate interactions. The lack of bidentate chelation is compatible with liganding to heme iron because one ligand site is available on the iron atom exposed on either side of the planar porphyrin structure.

Heme has also been implicated in arachidonic acid metabolism by cyclo-oxygenase since it alone restores both cyclo-oxygenase and peroxidase activity to the purified enzyme [3—6]. Furthermore, studies from our laboratory have emphasized a critical role for the ferrous form of iron in arachidonic acid oxidation [9,26—32]. Based on these findings a model has been proposed to explain the heme-arachidonic acid interaction [33]. In the model, arachidonic acid is visualized as attaching with its carboxylic acid residue to one ligand of the Fe^{2+} in heme, and then curling around the outside of the protophorphyrin to interact at its C_{11} carbon atom with O_2 attached to the other available Fe^{2+} ligand position. If this model is correct, unidentate and bidentate Fe^{2+} chelators would be expected to inhibit the heme interaction since a chelator would

need only one functional position to interfere with heme-arachidonic acid interaction.

Comparison between the relative potency and other physical properties of the different compounds tested indicates inhibition primarily results from unprotonated, lipid-soluble species not limited to pyridine derivatives. In these experiments at pH 7.4, the concentration of the unprotonated species of each derivative was nearly equal to its total concentration; even dimethylpyridine with the highest pK_a was predominantly unprotonated. However, the protonated species of each derivative varied markedly in proceeding from 2-chloropyridine, with the lowest pK_a, to dimethylpyridine. The lack of a marked parallel increase in potency implicates the unprotonated species in the inhibition. Lipid solubility appears a factor since pyridine is less effective but more water soluble. Certainly the inhibition is not specific for pyridine since dimethylaniline is just as effective. This interpretation of the results that concludes that the inhibition is primarily due to the lipid-soluble, unprotonated species, applies not only to the aggregation stimulated by arachidonate but also ADP- and epinephrine-induced aggregation.

From the platelet aggregation studies, it is apparent that dipyridyl and related compounds in high concentrations must be able to exert an inhibitory effect independent of their action on arachidonic acid metabolism. Thus, dipyridyl in high concentrations inhibited ADP and ionophore A23187-mediated aggregation, platelet responses which are largely independent of prostaglandin endoperoxide and thromboxane biosynthesis. The mechanism of this effect was explored, and like the influence on arachidonic-acid-mediated aggregation, the effect was not due to a permanent alteration, for platelets exposed to these concentrations of dipyridyl could be washed and the responsiveness to ADP restored. This generalized depression of cell responsiveness suggested that dipyridyl might be suppressing some aspect of calcium flux. However, in our limited studies no interaction of dipyridyl with calcium was found. The mechanism of the effect of dipyridyl and related compounds on ionophore A23187-induced aggregation and secretion is unknown. However, since dipyridyl interferes with a number of cellular metalloproteins [34—36] it is possible that Fe^{2+} in heme might be critical to these responses. We cannot rule out a less specific effect on the lipid membrane, however.

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