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INHIBITION OF HUMAN PLATELET AGGREGATION BY DIHYDROPYRANO- AND DIHYDROFURANOCOUMARINS, A NEW CLASS OF cAMP-PHOSPHODIESTERASE INHIBITORS

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Abstract—Certain esters of dihydropyranocoumarin and dihydrofuranocoumarin alcohols have previously been shown to inhibit the cAMP-phosphodiesterase from bovine heart. We now report that these naturally occurring coumarins inhibit the high affinity (K_m = 1.1 μM) cAMP-phosphodiesterase from human platelets with activities that closely correlate with those obtained using phosphodiesterase from bovine heart tissue. Additionally the coumarins inhibit the aggregation of human platelets induced with ADP, adrenaline and collagen with activities comparable to those of dipyridamole. A lack of significant correlation between these metabolic and functional activities indicates that there exist, besides cAMP-phosphodiesterase inhibition, additional mechanisms of action for the platelet aggregation inhibitory effect of dihydropyrano- and dihydrofuranocoumarins.

We have recently shown that a number of acyloxydihydropyrano- and acyloxydihydrofuranocoumarins occurring naturally in plants belonging to the family Umbelliferae possess rather potent coronary vasodilatory, spasmolytic and cAMP-phosphodiesterase (cAMP-PDE) inhibitory properties [1]. The structure/activity relationships for the smooth muscle relaxationary and for the cAMP-PDE inhibitory activity appeared to be similar. Thus cAMP-PDE inhibition was suggested, as one of the major mechanisms of action for the coronary vasodilatory and spasmolytic activity of dihydropyrano- and dihydrofuranocoumarins.

Several studies have established that a rise in the intraplatelet cAMP level, e.g. by PDE inhibition, inhibits platelet activation [2–5]. In the present study we therefore examined the effects of 11 selected compounds belonging to this new class of PDE inhibitors on the cAMP hydrolysing enzyme of human platelets and on the ADP, adrenaline and collagen induced platelet aggregation.

MATERIALS AND METHODS

Substances. ^{3}H-cAMP sp. act. 35.5 Ci/mmoles (New England Nuclear Co., Boston, MA), 5′-nucleotidase (snake venom from *Ophiophagus hannah*, Sigma Chemical Co., St. Louis, MO), adenosine-5′-diphosphate (ADP) (Sigma Chemical Co.), adrenaline (Sigma Chemical Co.), collagen (collagen reagent, Hormon-chemie, Munich, West Germany) was diluted with SKF Horm buffer immediately before use, dipyridamole HCl (kindly supplied by Boehringer, Ingelheim, West Germany), theophylline and papaverine sulphate. The test substances I–XI, of which I–VI are dihydropyranocoumarins and VII–XI are dihydrofuranocoumarins, were dissolved in dimethylsulphoxide (DMSO) before application. Visnadin (I), pteryxin (II), isosamidin (III), samidin (IV), disenecioyl *cis*-khellactone (V), *cis*-khellactone (VI), peucenidin (VII), athamantin (VIII), archangelicin (IX), columbianadin (X), and vaginidiol (XI). The small volumes (less than 1% vol./vol.) of DMSO used did not interfere with any of the test systems.

Preparation of platelet homogenate. Freshly obtained human whole blood anticoagulated by 5 mM EDTA was centrifuged at 200 *g* for 10 min (4°). The supernatant platelet rich plasma (PRP) was centrifuged at 2500 *g* for 10 min (4°). The resulting platelet pellet was washed twice in buffer (40 mM Tris–HCl, 150 mM NaCl, 20 mM EDTA, pH 7.4). After centrifugation (2500 *g* for 10 min at 4°) the washed platelets were resuspended in lysosomal buffer (4.0 mM Tris–HCl, 5.0 mM EDTA, pH 7.4), to a final platelet count of 2×10^{12}/l, and finally sonicated for 10 sec on ice.

cAMP-phosphodiesterase (cAMP-PDE) assay. The cAMP-PDE activity was determined by measuring ^{3}H-5′-AMP from the enzymatic hydrolysis of ^{3}H-cAMP as originally described by Thompson and Appleman [6]. Two hundred microlitres of assay mixture, containing 50 mM Tris–HCl, 5.0 mM $MgCl_2$, bovine serum albumin 0.010% and adjusted to pH 8.0 at 20°, platelet homogenate and test substance were placed on ice. The reaction was initiated by the addition of a mixture containing ^{3}H-labelled and unlabelled cAMP (total concentration, 1.0 μM), and after incubation at 30° for 7 min terminated by heating at 100° for 1 min. After cooling, 50 μl of an aqueous solution of 50 μg 5′-nucleotidase was added. After 15 min of incubation at 0°, 1 ml methanol was added and the mixture was applied to a Dowex-1X8 (Cl^--form, 400 mesh, Pharmacia Fine Chemicals, Uppsala, Sweden) column (0.7 × 1.0 cm). Adenosine was eluted with 1 ml methanol. The radioactivity was determined after addition of 8 ml of scintillation

fluid for counting (Instagel II, Packard Instrument Co., IL). All experiments were performed in triplicate. In order to minimize the variation in initial velocity (V_{max}) the platelet homogenate concentration was adjusted so as to keep the hydrolysis of cAMP below 25% within 7 min of incubation in the absence of inhibitor. Each test substance was tested in four concentrations, and the concentration which caused 50% inhibition of the cAMP-PDE activity (I_{50}) using 1.0 μM cAMP as substrate was estimated from concentration effect curves. The I_{50} values listed in Table 1 are means of at least three determinations.

Enzyme kinetic analysis. K_m-values were determined from Lineweaver–Burk plots using rates of cAMP hydrolysis measured at 14 cAMP concentrations between 0.100 and 1500 μM.

Platelet aggregation assay. Human blood obtained from healthy volunteers was collected into 1/10 volume of 3.8% sodium citrate. Platelet rich plasma (PRP) was prepared by centrifugation of the blood at 150 *g* for 5 min at room temperature, and platelet poor plasma (PPP) was obtained by further centrifugation at 2000 *g* for 10 min. PRP was used without further adjustment of platelet concentration, due to a small variation in platelet count (420×10^9/l ± 28×10^9/l, mean ± S.E.M., N = 26). Platelet aggregation was studied at 37° with 900 rpm, stirring in 0.5 cm cuvettes using a turbidimetric device (Payton aggregometer, Scarborough, Ontario, Canada). Test substances were added to the platelet suspension 10 min before addition of the aggregating agent ADP (8 μM), adrenaline (1.0 μg/ml) or collagen (2.0 μg/ml). In all experimental series these concentrations of aggregating agents caused irreversible aggregations in the absence of inhibitor.

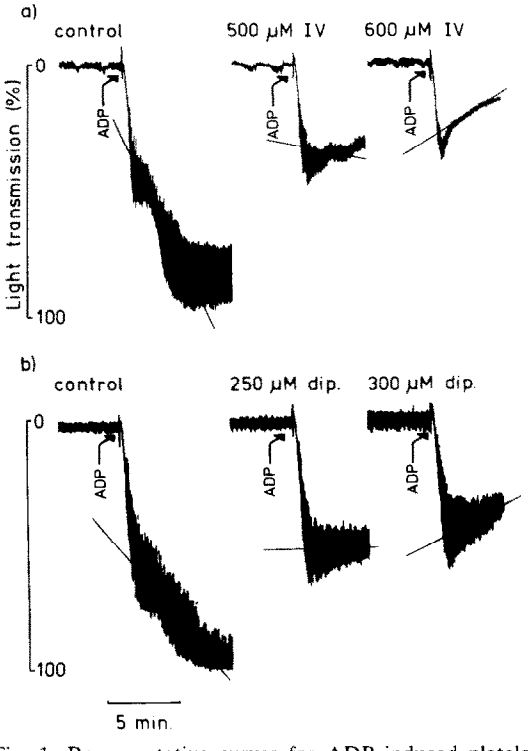


Fig. 1. Representative curves for ADP induced platelet aggregation with estimation of D^* values. (a) D^* for samidin (IV) in this experiment is 520 μM. (b) D^* for dipyridamole (dip.) in this experiment is 250 μM. ADP concentration: 8 μM.

Aggregation curve analysis. For aggregation induced by adrenaline or collagen in presence of DMSO (4 μl) without inhibitor, maximum levels of the aggregation curves were reached after 5.6 ± 0.8 min. (mean ± S.E.M., N = 15) and after 4.2 ± 0.6 min. (mean ± S.E.M., N = 17) respectively. Accordingly, the extent of aggregation induced by adrenaline and collagen was expressed in terms of height of the curve after 5.6 min and 4.2 min respectively as a basis for calculation of I_{50} values (dose of inhibitor causing 50% inhibition of the aggregation). In experiments where aggregation was induced by ADP, the maximum height of the curve attained during the primary phase of aggregation was taken as a measure of this primary response, whereas the extent of the secondary phase of aggregation was expressed in terms of the slope of the aggregation curve just after the primary response. Thus, the inhibitory activity of test substances on the ADP induced aggregation was expressed as the concentration, D^*, which just prevented the development of the secondary phase of aggregation (slope = 0.00, see Fig. 1) and as the percentage inhibition of the primary response at this concentration, D^*.

This method of expressing aggregatory activities has been selected, because it allows a more accurate way of distinguishing between effects on the primary and the secondary phase of ADP induced aggregation than conventional methods.

In each type of experiment the inhibitors were tested in at least four concentrations and the activities determined as means of at least three determinations. Furthermore, due to some variation in aggregatory behaviour between samples from different donors, dipyridamole was included as a standard inhibitor in all experimental series and all inhibitory activities of the test substances corrected correspondingly.

Log P analysis. Log P values of the diols (VI and XI) were found by examining the partition of the compounds between 1-octanol and aqueous buffer (pH = 7,4). Log P values for the esters (I–V and VII–IX) were calculated by addition of Hansch substituent constants (π) [7] to the partition coefficient of the parent diol.

Statistics. Statistical comparison was performed by means of Pearsons product moment correlation coefficients [8] using logarithmically transformed data.

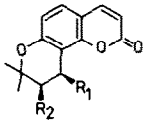
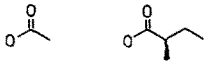
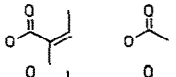
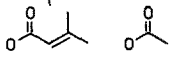
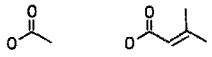
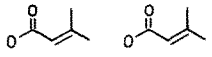
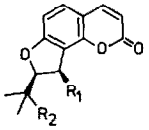
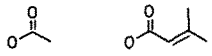
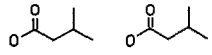
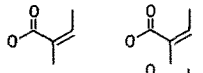
RESULTS

The kinetic analysis of the cAMP hydrolytic capacity of platelet homogenate indicates the existence of two apparent K_m-values of 1.1 μM and 290 μM and associated V_{max}-values of 0.0060 pmoles/min/10^9 platelets/l and of 0.19 pmoles/min/10^9 platelets/l. These values are consistent with those previously reported for human platelets [9, 10]. In the present study we examined the effect of the coumarins at the low level using 1 μM cAMP as substrate. The cAMP-PDE inhibitory activity of the tested compounds

are shown in Table 1. The most potent coumarins maximally inhibited the enzyme activity with 83–89%. In comparison, papaverine gave a maximal PDE inhibition of 95%. The activities determined in the present study, using platelet homogenate are fully concordant ($r = 0.96$, $N = 7$, $P < 0.001$) with results previously obtained with the same substances using a bovine heart PDE preparation [1].

All the coumarins tested acted as inhibitors of the secondary phase of ADP induced aggregation, but affected the primary response only to a minor extent (Table 1). Additionally, all the compounds tested inhibited the adrenaline induced aggregation, and a significant correlation exists between their inhibitory activities on adrenaline and ADP induced aggregation ($r = 0.79$, $N = 11$, $P = 0.0012$). In contrast the test substances showed a divergent inhibitory activity on the collagen induced aggregation. Thus no significant correlation exists neither between the collagen and the ADP nor between the collagen and

Table 1. Structures of dihydropyranocoumarins (I–VI) and dihydrofuranocoumarins (VII–XI) and their inhibitory activities against cAMP-phosphodiesterase from human platelets and platelet aggregation.

COMPOUND	STRUCTURAL FORMULA	INHIBITION OF cAMP-PDE	INHIBITION OF PLATELET AGGREGATION			
		I_{50} (μM)	ADP, sec. D^x (μM)	ADP, prim. % at D^x	Adr. I_{50} (μM)	Coll. I_{50} (μM)
						
	R_1 R_2					
I		160	640	9	550	530
II		160	770	20	550	500
III		75	190	9	330	500
IV		93	540	13	380	450
V		60	260	4	250	>800
VI	OH OH	>400	300	23	240	>800
						
	R_1 R_2					
VII		130	590	5	410	>800
VIII		>400	450	20	460	750
IX		>400	310	10	290	490
X	H 	260	240	5	140	>800
XI	OH OH	>400	260	22	260	390
Papaverine		2	30	73	30	25
Theophylline		320	980	35	1300	960
Dipyridamole		49	250	18	240	230

the adrenaline induced aggregations ($r = 0.25$, $N = 7$, $P = 0.31$ and $r = 0.55$, $N = 7$, $P = 0.11$).

The hydrophilic–lipophilic nature of the coumarins was expressed in terms of their partition between 1-octanol and water. Thus the log P values were found to be 1.6 and 1.5 for the two diols (VI and XI) and between 5.1 and 6.6 for the diesters (I–V and VII–IX).

DISCUSSION

Certain acyloxydihydropyrano- and acyloxydihydrofuranocoumarins have recently been shown to inhibit the activity of cAMP-PDE from bovine heart 1. In the present study we have demonstrated that this new class of PDE-inhibitors additionally inhibits the cAMP hydrolysing enzyme from human platelet homogenate with activities that fully correlate with those obtained using bovine heart tissue ($r = 0.96$, $N = 7$, $P < 0.001$). Due to the rather high lipophilicities of the coumarins, which allows them to penetrate the platelet membrane [11], they can be expected to exert a corresponding PDE inhibitory activity in intact platelets. It is well documented [12, 13] that a raised intraplatelet cAMP level, e.g. by cAMP-PDE inhibition, leading to calcium sequestration inhibits the activation of platelets. This mechanism of action has been proposed for the platelet aggregation inhibitory activity of some PDE inhibitors such as methylxanthines, flavonoides, cilostamide and other [4, 14, 15]. Presently it is substantiated that the cAMP-PDE inhibitory coumarins also possess a platelet antiaggregatory activity. It is evident, however, that a lack of significant correlation between the PDE and the aggregation inhibitory activities implies the existence of additional platelet inhibitory mechanisms. Thus, as part of a previous discussion concerning structure/activity relationships of dioxygenated dihydropyrano- and dihydrofuranocoumarins as inhibitors of cAMP-PDE [1] it was established that acylation of one or both hydroxyl groups in VI and XI is a prerequisite for obtaining cAMP-PDE inhibitory activity, which is clearly not valid with respect to the antiaggregatory activity of the coumarins.

The rather low antiplatelet activity of these substances as compared with the PDE inhibitory activity is a well-known phenomenon for compounds interfering with the cAMP/PDE systems examined by *in vitro* experimentations [16, 17]. Thus it may be assumed that these activities would increase considerably by analogous investigations *in vivo* [18–21].

The aggregation of human platelets induced by ADP occurs in two sequential phases. The coumarins almost exclusively affect the secondary phase of aggregation, which is associated with a release of aggregating components [22] from intraplatelet granules. This release reaction is predominantly mediated by calcium mobilization [23], and thus inhibited by prostaglandin or leukotriene biosynthesis inhibition, cAMP enhancement, calcium antagonization and maybe also by platelet activating factor (PAF) antagonization. It can therefore not be discounted that the coumarins beside the cAMP-PDE inhibition also influences the calcium flux by another biochemical mechanism. It is of interest in this connection that the dihydropyranocoumarin, (±)-praeruptorin A structurally related to the coumarins I–VI has been described to inhibit the transmembrane calcium influx without influencing the cAMP level in taenia coli tissue of guinea-pigs [24].

In summary, a group of structurally interrelated coumarins of plant origin, formerly shown to be cAMP-PDE inhibitors, has now been demonstrated to be platelet inhibitors *in vitro*. They may be useful as new templates for the development of better synthetic drugs, as their structures are basically different from those of other known types of platelet inhibitors.

As no significant correlation has been found to exist between their PDE-inhibitory and platelet inhibitory activities, however, their mechanism of action deserves further study.

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