EFFECT OF 3-HYDROXYPYRIDINES ON CYCLIC NUCLEOTIDE LEVELS AND INHIBITION OF PLATELET AGGREGATION IN MAN

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Emoxipine, a compound belonging to the 3-hydroxypyridine class (3-HP), has recently been successfully used in clinical ophthalmology as an effective mode of treatment of intraocular hemorrhages of vascular genesis [3]. The molecular mechanisms of action of this compound have not been finally elucidated. The writers showed previously that 3-HP derivatives are able to inhibit phosphodiesterases (PDE) specific both for cGMP and for cAMP in platelets, and also to inhibit platelet aggregation [1]. On the basis of these results it has been suggested that one mechanism of action of emoxipine is by inhibiting platelet aggregation as a result of inhibition of PDE activity, with a consequent increase in the intracellular cyclic nucleotide concentration.

To test this hypothesis experimentally, the action of several 3-HP derivatives was studied on the cyclic nucleotide levels in platelets and on platelet aggregation.

EXPERIMENTAL METHOD

Platelets were isolated from blood obtained form healthy blood donors [5], after which they were preincubated for 5 min in medium of the following composition: 15 mM Tris-HCl, 134 mM NaCl, 5 mM glucose (pH 7.4), in the presence of the test substances in different concentrations (Table 1). The volume of the incubated mixture was 1 ml. Aggregation in the medium was initiated by addition of thrombin (1.5 U/ml), and 1 min later the reaction was stopped by the addition of 1 ml of ethanol, followed by heating to 100°C on a water bath for 15 min. After heating, the samples were treated with a further 1 ml of ethanol and centrifuged (3000 rpm) and the supernatant was evaporated in vacuo at 50°C. Cyclic nucleotides in the samples were assayed by means of the appropriate kits (cyclic AMP RIAKIT and Cyclic GMP RIAKIT; Amersham Corporation, England). To measure radioactivity, 1215 Rack-Beta II and Mark II counters were used. Aggregation was recorded on an aggregometer (Chrono Log, USA) connected to an automatic writer (LKB, Sweden). The cell concentration in the medium was 200,000/ml. The rate of aggregation was estimated quantitatively as the tangent of the angle of slope of the tangent to the region of the curve correpsonding to the rapid phase of aggregation. Inhibition of aggregation with different concentrations of 3-HP was expressed as a percentage of the control. 3-HP derivatives with substituents in different positions were synthesized at the Institute of Pharmacology, Academy of Medical Sciences of the USSR [2]. Preparations were used in the experiments in the form of bases, which were added to the incubation medium in the form of alcoholic solutions (the ethanol concentration in the incubation medium was 2%). The HCl, EDTA, and glucose were obtained from Sigma, USA and the remaining reagents from Reakhim (USSR), and were of the chemically pure grade.

EXPERIMENTAL RESULTS

Results of experiments to study the action of 3-HP derivatives on the cyclic nucleotide levels in the platelets and also on the ability of the platelets to aggregate are summarized

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TABLE 1. Changes in Cyclic Nucleotide Levels (in pmoles per sample) and Inhibition of Aggregation of Thrombin-Treated (1.5 U/ml) Platelets in the Presence of 3-HP Derivatives

Compound	cAMP		cGMP		Inhibition of aggregation, %	
	concentration of 3-HP, M					
	$5 \cdot 10^{-3}$	10^{-3}	$5 \cdot 10^{-3}$	10^{-3}	$5 \cdot 10^{-3}$	10^{-3}
3-HP	2,0±0,1	1,3±0,2	0,24±0,01	0,23±0,02	0	0
2-Benzyl-3-HP	1,5±0,0	1,6±0,1	0,21±0,04	0,18±0,04	55	50
2-tert-Butyl-3-HP	1,1±0,1	1,3±0,1	0,20±0,09	0,19±0,03	32	16
2,6-Dimethyl-3-HP	1,5±0,1	1,3±0,1	0,07±0,04	0,15±0,07	50	0
2-Ethyl-6-methyl-3-HP	3,3±0,61	1,7±0,2	0,20±0,03	0,20±0,03	12	0
3-Dimethylcarbamoyl-HP	6,7±0,1	5,0±0,2	0,16±0,10	0,10±0,05	50	20
Aspirin	0,9±0,1	0,9±0,1	0,17±0,05	0,10±0,05	80	25
IBMX (concentrations $5 \cdot 10^{-4}$ and 10^{-4} M for determination of cAMP and cGMP respectively	5,8±0,1	3,1±0,1	0,68±0,02	0,41±0,02	32	18

Legend. cAMP concentration in the control was 1.2 ± 0.1 pmole, cGMP 0.13 ± 0.01 pmole per sample, inhibition of aggregation – 0.

in Table 1. The 3-HP derivatives studied, in concentrations of 10^{-3} and 5×10^{-3} M (these concentrations were chosen on the basis of data in [1]) had equally little effect on the cGMP concentration in the platelets. This effect was much weaker than that of lower concentrations of isobutylmethylxanthine (IBMX). Meanwhile the cAMP concentration and the rate of platelet aggregation were sensitive to different degrees to the action of the various 3-HP derivatives. On the basis of the results the substances tested can be divided into three groups. The 1st group includes 3-HP unable to inhibit platelet aggregation and leading to only a very small increase in their cAMP concentration. The 2nd group included 2-benzyl-3-HP, 2-tert-butyl-3-HP, and 2,6-dimethyl-3-HP, which were able to inhibit platelet aggregation effectively but did not significantly raise the cAMP level in the cell. The 3rd group included 2-ethyl-6-methyl-3-HP and 3-dimethylcarbamoyl-HP, which can effectively inhibit platelet aggregation, significnatly raise the cAMP level in the cell, and which are thus powerful inhibitors of PDE of cyclic nucleotides, as was shown previously [1].

Substances classed in the first two groups, incidentally, raised the cAMP level in the platelets, but not significantly: by 1.5-2 times compared with the control (Table 1). Since 3-HP possessing no antiaggregating activity were classed in these groups, it can be postulated that the increase in the cAMP concentration caused by compounds of these two groups in the cell was too small to affect the aggregation process. It can consequently be concluded that the effect of 2-benzyl-3-HP, 2-tert-butyl-3-HP, and 2,6-dimethyl-3-HP on platelet aggregation is realized through mechanisms unconnected with an increase in the intracellular cAMP concentration.

This conclusion was confirmed by the results of a study of the action of aspirin on the cyclic nucleotide levels in the platelets and on inhibition of platelet aggregation: aspirin effectively inhibits their aggregation but does not increase the cAMP concentration in them.

Substances which we included in group 3 (2-ethyl-6-methyl-3-HP and 3-dimethylcarbamoyl-HP) increased the cAMP concentration in the platelets by 3-6 times, and this correlated with the degree of inhibition of platelet aggregation by these compounds (Table 1).

It can thus be concluded that the action of 3-HP derivatives on platelet aggregation is not linked with a single mechanism of inhibition of PDE activity and increase in the intracellular cAMP concentration, although the possibility cannot be ruled out that inhibition of PDE activity and the consequent increase in the intracellular cAMP concentration are essential for the antiaggregating action of 2-ethyl-6-methyl-3-HP and of 3-dimethylcarbamoyl-HP.

As was pointed out above, the 3-HP derivatives which we studied changed the cGMP level in the platelets about equally, although only very slightly. Under these circumstances correlation was not found ($P = 0.05$) between the change in the rate of platelet aggregation and the change in the intracellular cGMP concentration in the presence of 3-HP derivatives. IBMX caused virtually equal changes in the cAMP and cGMP levels in the case of thrombin-in-

duced aggregation: the cGMP concentration was increased by 5 and 3 times, and the cAMP concentration by 4.8 and 2.6 times in concentrations of $5 \cdot 10^{-4}$ and 10^{-4} M, respectively.

It is generally considered that the process of platelet aggregation is determined by the ratio between the Ca^{++} and cAMP levels in the cell; these substances are secondary messengers in the process of signal transmission from the cell surface to the intracellular structures concerned in the processes of aggregation and disaggregation [4]. Meanwhile the question of the role of cGMP in platelet aggregation has not been settled. It is suggested that cGMP in platelets, just as in cell of other types, can behave as a cAMP antagonist [6].

The results of this investigation are evidence that the ability of 3-HP derivatives to raise the cGMP level, but not significantly, has probably no relation to their ability to inhibit platelet aggregation.

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EFFECT OF MINERALOCORTICOIDS ON RAT BRAIN Na,K-ATPASE ACTIVITY

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Much progress has been made in the study of the cellular and molecular action of mineralocorticoids, but the mechanism of this action has not been finally explained. The ability of aldosterone and deoxycorticosterone to stimulate Na,K-ATPase activity in the kidneys [1], urinary bladder [11], and skin of the frog [4], and heart [12] is well known. However, this effect develops not sooner than 1-3 h after the action of the hormone. It has been suggested [9, 10] that the effect of mineralocorticoids on Na,K-ATPase is mediated through primary acceleration of nuclear metabolism and synthesis of specific proteins, which may perhaps be Na^+ carriers. According to this view, integrity of the cell is essential. More recently the attention of research workers has been drawn to the contribution of the plasma membranes to the mechanism of hormonal action. In this connection there has been some interesting work to study the effect of aldosterone on Na^+ transport through the anuclear erythrocyte membrane [3]. Aldosterone in vivo depresses the Na,K-ATPase activity of human erythrocytes. The action of the hormone on the erythrocyte membrane thus revealed is not connected with the process of genetic induction. Investigations on erythrocyte membranes have shown that the addition of hormone to them induces conformational changes in them [5].

There has been very little experimental research on plasma membranes, but such investigations could make an important contribution to the study of the mechanism of action of mineralocorticoids, for they enable the possible participation of the cytosol receptor in

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