INHIBITION OF PLATELET AGGREGATION AND OF cAMP-DEPENDENT PLATELET CYCLIC NUCLEOTIDE PHOSPHODIESTERASE BY 3-HYDROXYPYRIDINE DERIVATIVES

V. E. Kagan, N. B. Polyanskii, K. O. Muranov, A. A. Shvedova, L. D. Smirnov, and K. M. Dyumaev

UDC 615.273.53:547.823].015.4:612.111.7.015.1.577.152.311

KEY WORDS: platelet aggregation; phosphodiesterase; 3-hydroxypyridines.

Derivatives of 3-hydroxypyridine (3-HP) have recently begun to be used in clinical medicine for the treatment of hemorrhagic diseases [4, 5]. Yet the mechanisms of their action on the hemostasis system have not been explained. Platelet aggregation, which plays a key role in the cascade of potentiating mechanisms responsible for blood clotting, is a process which depends essentially on three factors: the intracellular Ca^{++} concentration, the intracellular cyclic nucleotide level, and the formation of prostaglandins, prostacyclin, and thromboxanes [11, 12]. The authors showed previously that 3-HP derivatives are able to inhibit phosphodiesterase (PDE) and thus to influence the cyclic nucleotide levels [2, 3].

3-HP derivatives also are inhibitors of free-radical oxidation [4] and, consequently, they inhibit synthesis of precursors of thromboxane A_2 from arachidonic acid [7].

The aim of this investigation was to study the action of 3-HP derivatives on platelet aggregation and on cAMP-dependent platelet cyclic nucleotide PDE activity, and to reveal any possible connection between these processes.

EXPERIMENTAL METHOD

Platelets were isolated from donors' blood by the method in [9]. Platelet aggregation was produced in a medium of the following composition: Tris-HCl 15 mM, NaCl 134 mM, glucose 5 mM, pH 7.4 (37°C). Platelet aggregation was initiated by thrombin (final concentration in incubation medium 1.5 unit/ml) and recorded as the change in scattering of light of the suspension, using an aggregometer from Chrono-Log Corp. (USA). The rate of aggregation was estimated as the tangent of the angle of the tangent to the curve corresponding to the rapid phase of aggregation (Fig. 1). The effectiveness of action of the test substances was estimated as their concentration causing 30% inhibition of aggregation (K^{30}_{inh}). The concentration of the test substances in the incubation medium varied between 10^{-5} and 10^{-2} M. The platelet fraction rich in cAMP-dependent PDE was isolated by the method in [8]; PDE activity was determined by the method in [6] in incubation medium: Tris-HCl 40 mM, $MgSO_4$ 5 mM, pH 8.0 (37°C). The protein content was 2-10 μg, the concentration of 3-HP derivatives between 10^{-4} and 10^{-2} M, and the cAMP concentration 10^{-7}-10^{-6} M; [^{3}H]-cAMP was present in the incubation medium with an activity of 0.05-0.15 μCi. Experiments to determine reversibility of the action of the inhibitors on PDE were carried out by the method in [3]; the protein content after transfer of aliquots from the preincubation medium into the incubation medium was 1.6-2 μg and the concentration of inhibitor $2 \cdot 10^{-3}$ M. Neither cAMP nor [^{3}H]-cAMP was present in the preincubation medium.

3-HP derivatives were synthesized in the Institute of Chemical Physics, Academy of Sciences of the USSR [4]. Preparations in the form of bases and hydrochlorides were used in the experiments. Bases were added to the incubation medium in the form of alcoholic solutions (the ethanol concentration in the incubation medium for measurement of platelet aggregation did not exceed 2%, for determination of PDE activity — 4%). Ten 3-HP derivatives with substituents in different positions (Table 1) were used; cAMP and AMP were from Reanal, Hungary; Tris and EDTA from Serva, Sweden; [^{3}H]-cAMP was from Amersham Corporation, England; aspirin from Lachema, Czechoslovakia; pyridoxol and pyridoxal phosphate were from the Krasnodar Bio-

Biological Faculty, M. V. Lomonosov Moscow State University. Institute of Chemical Physics, Academy of Sciences of the USSR, Moscow. Institute of Pharmacology, Academy of Medical Sciences of the USSR, Moscow. (Presented by Academician of the Academy of Medical Sciences of the USSR A. V. Val'dman.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 97, No. 4, pp. 416-418, April, 1984. Original article submitted May 3, 1983.

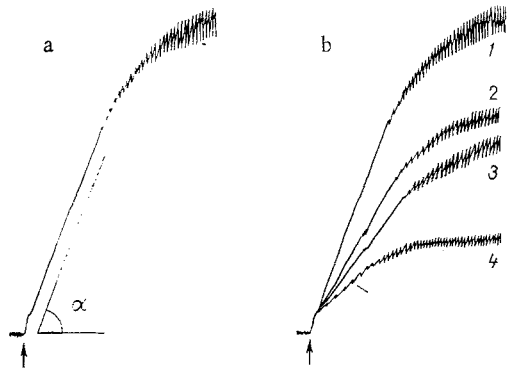

Fig. 1

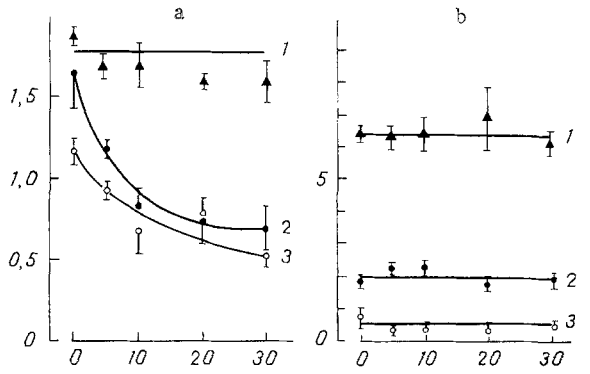

Fig. 2

Fig. 1. General appearance of aggregogram curve (a) and of aggregogram curves during measurement of action of 2-benzyl-3-HP (b). tan α) Rate of rapid phase of aggregation; 1) control with ethanol; 2-4) 10^{-4}, $3 \cdot 10^{-4}$, and 10^{-3} M 2-benzyl-3-HP, respectively. Arrow indicates injection of thrombin.

Fig. 2. Determination of reversibility of inhibition of platelet PDE activity. a: 1) Control; 2) derivative dimethyl-3-HP (K-1); 3) 2,6-dimethyl-3-HP. b: 1) Control; 2) TP; 3) IBMX. Abscissa, duration of preincubation of enzyme with inhibitor (in min); ordinate, activity (in nmole/min/mg protein).

TABLE 1. Inhibition of Platelet Aggregation and Inhibition of cAMP-Dependent Platelet Cyclic Nucleotide PDE by 3-HP Derivatives and Their Antiradical Activity

Compound	K_{inh}^{30}, M	K_i, M	Degree of inhibition relative to control, %	
			concentration of cAMP, $\cdot 10^{-7}$	concentration of cAMP, $\cdot 10^{-6}$
3-HP	—	—	0	0
2-Benzyl-3-HP	10^{-4}	—	0	0
2-tert-Butyl-3-HP	$2{,}5 \cdot 10^{-3}$	—	0	0
2,6-Dimethyl-3-HP	$2{,}6 \cdot 10^{-3}$	$5—8 \cdot 10^{-3}$	43±2	66±2
2-Ethyl-6-methyl- 3-HP	$2{,}5 \cdot 10^{-2}$	$4—10 \cdot 10^{-3}$	53±2	73±5
2,4,6-Trimethyl-pyridine	—	—	—	—
2-tert-Butyl-6-methyl-3-HP	-	$9—15 \cdot 10^{-3}$	37±10	47±10
6-Methoxy-2-benzene-3-HP		0	0	0
3-Aminopyridine derivative		0	0	0
Dimethyl-3-HP (K-1)		$3{,}5—4 \cdot 10^{-3}$	75±1	65±4
Pyridoxol		—	0	0
Pyridoxal		—	0	0
Pyridoxal phosphate		—	0	0
TP	10^{-3}	$1{,}5—2 \cdot 10^{-4}$	97±1	97±1
IBMX	$3{,}9 \cdot 10^{-4}$	$4—8 \cdot 10^{-4}$	96±4	87±4
Aspirin	10^{-3}			
4-Methyl-2,6-di-tert-butylphenol	$1{,}6 \cdot 10^{-5}$			

chemical and Reducing Preparations Combine; theophylline (TP), isobutylmethylxanthine (IBMX), and 4-methyl-2,6-di-tert-butylphenol were from Sigma, USA. The remaining substances were of "chemically pure" grade and were from Reakhim. The protein concentration was determined by the method in [10].

EXPERIMENTAL RESULTS

Of all the 3-HP derivatives studied, 2-benzyl-3-HP had the strongest inhibitory action on platelet aggregation (Table 1). K^{30}_{inh} for 2-benzyl-3-HP was comparable with K^{30}_{inh} for IBMX and was one order of magnitude greater than its value for TP and aspirin. Typical platelet aggregation curves in the presence of different concentrations of this compound are given in Fig. 1.

It follows from the results that 2,6-dimethyl-3-HP, 2-ethyl-6-methyl-3-HP, and derivative dimethyl-3-HP (K-1) are effective as PDE inhibitors (from 43 to 75% of inhibition), 2-tert-butyl-6-methyl-3-HP is less effective (inhibition about 40%), and the remaining 3-HP derivatives, including all physiologically important derivatives (pyridoxol, pyridoxal, pyridoxal phosphate), have virtually no effect on platelet PDE activity (Table 1).

All 3-HP derivatives effective as PDE inhibitors have alkyl groups in positions 2 and 6.

The results of experiments to determine reversibility of the action of inhibitors 2,6-dimethyl-3-HP and dimethyl-3-HP (K-1) derivative are given in Fig. 2 (for other 3-HP derivatives the graph of enzyme activity as a function of preincubation time with the inhibitor was similar in appearance). PDE activity in the presence of classical inhibitors (TP, IBMX) was independent of preincubation time, evidence that inhibition of PDE by these substances is reversible in character. In the presence of 3-HP derivatives, however, PDE activity decreased with an increase in preincubation time of the enzyme with the inhibitor, i.e., 3-HP inhibits PDE irreversibly.

Investigation of the kinetic characteristics of platelet PDE showed that, under the influence of 2-ethyl-6-methyl-3-HP, of the dimethyl-3-HP derivative K-1, and also of TP, K_m for cAMP increased from $(0.8\text{-}1.3)\cdot10^{-6}$ M, from which it follows that modification of the enzyme by the inhibitors mentioned above causes a change in affinity of enzyme for substrate. Since 3-HP derivatives inhibit PDE noncompetitively (Table 1), it can be postulated that 3-HP binds irreversibly with hydrophobic amino acids surrounding the active center of PDE, and modifies the tertiary structure of the enzyme, reducing its affinity for the substrate. **Methylxanthines (TP, IBMX) probably act** in the same way also, the difference being that they inhibit PDE reversibly.

It has been shown previously that 3-HP derivatives can inhibit PDE from rabbit heart and PDE from the outer segments of the retinal rods reversibly [3, 5]. The authors consider that the difference between the results obtained in the investigations cited and those now described is attributable to the fact that the **microenvironments of the** active center of platelet PDE, on the one hand, and of PDE-II from rabbit heart and PDE from the outer segments of the retinal rods, on the other hand, differ substantially.

Inhibition of platelet PDE is connected not only with a decrease in affinity of the enzyme for the substrate (an increase in K_m), but also with a decrease in V_{max} (compared with the control) under the influence of TP and the dimethyl-3-HP derivative K-1.

Comparison of the results given in Table 1 shows that inhibition of platelet aggregation in the presence of 3-HP derivatives does not correlate with their activity as PDE inhibitors ($r = -0.2$). It can be tentatively suggested that the action of 3-HP derivatives on platelet aggregation is connected with their ability to influence the synthesis of prostaglandins, which exert their action through the cyclic nucleotide system [1].

The authors are grateful to Professor E. B. Burlakova for useful discussion of the results.

LITERATURE CITED

1. D. Metzler, Biochemistry. Chemical Reactions in the Living Cell [Russian translation], Vol. 2, Moscow (1980), p. 553.
2. N. B. Polyanskii, L. D. Smirnov, A. A. Shvedova, et al., in: Cyclic Nucleotides [in Russian], Minsk (1982), p. 119.
3. N. B. Polyanskii, L. D. Smirnov, A. A. Shvedova, et al., Vopr. Med. Khim., No. 1, 123 (1983).
4. L. D. Smirnov and K. M. Dyumaev, Khim.-farm. Zh., No. 4, 28 (1982).
5. L. V. Spirina, A. A. Shvedova, and N. V. Makarskaya, in: Abstracts of Proceedings of the 4th All-Union Congress of Ophthalmologists [in Russian], Moscow (1982), pp. 449-450.
6. V. A. Tkachuk, V. G. Lazarevich, M. Yu. Men'shikov, et al., Biokhimiya, No. 9, 1622 (1978).

7. F. E. Dewhirst, Prostaglandins, 20, 209 (1980).
8. H. Hidaka and T. Asano, J. Biol. Chem., 251, 7508 (1976).
9. A. Hotebbliss, J. Jordan, F. Hirata, et al., Biochem. Pharmacol., 30, 2089 (1981).
10. O. H. Lowry, N. J. Rosebrough, A. L. Farr, et al., J. Biol. Chem., 193, 265 (1951).
11. A. J. Marcus, J. Lipid Res., 19, 793 (1978).
12. B. B. Vargaftig, M. Chignard, and J. Benveniste, Biochem. Pharmacol., 30, 263 (1981).

CYTOPHOTOMETRIC STUDY OF CHANGES IN GLUTAMATE DEHYDROGENASE AND GABA TRANSAMINASE IN THE CEREBRAL CORTEX DURING METRAZOL KINDLING

I. N. Moiseev, A. A. Shandra, and L. S. Godlevskii

UDC 616.853-092.9-02:615.21]-07:616.831.31

KEY WORDS: epilepsy; glutamate dehydrogenase; transaminase.

Kindling is a model of epilepsy created by repeated weak electrical stimulation of brain structures [13-15]. It has been shown that kindling can also be induced by repeated injections of subthreshold doses of various chemical convulsants [7, 10]. The pathogenetic mechanisms of kindling have not been adequately studied. It was decided to investigate enzyme activity of neurons and glia in animals with developed kindling.

The aim of this investigation was to study activity of glutamate dehydrogenase (GDH) and GABA transaminase (GABA-T) in the cerebral cortex of mice during metrazol-induced kindling.

EXPERIMENTAL METHOD

Experiments were carried out on inbred F_1 hybrid mice (CBA × C57BL/6, BAC/C) and on non-inbred albino mice weighing 18-22 g. Each group consisted of at least 15 animals. Metrazol was injected intraperitoneally in a dose of 30 mg/kg daily for three weeks in a volume of 0.1 ml under the same conditions, at the same time of day, in a room with the same intensity of illumination and with the same noise effect. After injection of metrazol the animals were kept in a glass chamber and were under observation for 30-40 min. Behavior seizures were assessed in points, as follows: 0) no seizure; 1) shaking of the head or twitching of individual trunk muscles; 2) repeated clonic spasms of the trunk; 3) clonic spasms of the forelimbs; 4) clonicotonic convulsions with the animal falling on its side, followed by postictal depression; **and 5) repeated** severe tonicoclonic convulsions or lethal convulsions. Animals of the control group received injections of physiological saline under the same conditions. The animals were decapitated 24 h after the last injection of metrazol and the brain was re-

TABLE 1. Enzyme Activity in Sensomotor Cortex of Control Mice and during Kindling (in conventional optical density units, M ± m)

Experimental conditions	Enzyme	Neurons	Glia
Control	GDH	39,45±0,89	22,59±0,59
Experiment		33,41±0,72*	18,03±0,44*
Control	GABA-T	49,47±0,9	35,78±0,65
Experiment		44,27±0,95*	36,99±0,81

Legend. *P < 0.05 compared with control.

Department of Histology and Embryology and Department of Pathological Physiology, N. I. Pirogov Odessa Medical Institute. (Presented by Academician of the Academy of Medical Sciences of the USSR D. S. Sarkisov.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 97, No. 4, pp. 418-420, April, 1984. Original article submitted July 12, 1983.

 0007-4888/84/9704-0422$08.50