MOLECULAR-BIOLOGICAL PROBLEMS OF THE CREATION OF DRUGS AND STUDY OF THE MECHANISM OF THEIR ACTION

INFLUENCE OF UNITHIOL, d-PENICILLAMINE, AND CYSTEINE ON THE BIOLOGICAL EFFECTS OF BRADYKININ AND THE ACTIVITY OF CARBOXYPEPTIDASE N AND PEPTIDYL DIPEPTIDASE

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The activity of the kinin system of the organism is regulated by a complex mechanism of kinin-forming and kinin-decomposing enzymes. Among the kinin-decomposing enzymes — kininases — carboxypeptidase N (arginine carboxypeptidase, kininase I: EC 3.4.12.7), which is present in blood plasma [1, 2], and peptidyl dipeptidase (kininase II, angiotensin-converting enzyme; EC 3.4.15.1), localized chiefly in the endothelium of the microvessels of the lungs, kidneys, and other internal organs, are the most important [3]. The indicated enzymes cleave to strictly determined peptide bonds in the molecule of bradykinin and other kinins, which leads to inactivation and cessation of the action of these biologically active peptides, which participate in the humoral regulation of the level of arterial pressure, the permeability of the microvessels, the smooth muscle tone, etc. It is of vital importance that peptidyl dipeptidase not only inactivates bradykinin, but also catalyzes the conversion of inactive angiotensin I to the active pressor factor — angiotensin II [4, 5]. The key position of peptidyl dipeptidase in the metabolism of the kinin and renin–angiotensin systems permits the use of this enzyme as an object for the influence of pharmacological preparations in order to regulate the activity of the indicated systems, in particular, to change the intensity and duration of the biological action of kinins.

Among preparations exerting an inhibiting effect on the kininase activity, the group of thiol compounds (derivatives of 2,3-dimercaptopropanol, cysteine, d-penicillamine, captopryl, etc.), which intensify and prolong the spasmogenic and hypotensive effects of kinins [6-9], are attracting attention. We were interested in making a comparative study of the influence of unithiol, d-penicillamine, and cysteine on the activity of human carboxypeptidase N and peptidyl dipeptidase, as well as on certain vascular effects of kinins.

EXPERIMENTAL

The influence of thiol preparations on the activity of pulmonary kininases has been studied on isolated lungs of guinea pigs of both sexes, weighing 250-300 g [10]. Perfusion of the vessels of the pulmonary circulation was carried out through the pulmonary artery with Krebs solution containing or not containing unithiol, d-penicillamine, or cysteine in concentrations from $1 \cdot 10^{-7}$ to $1 \cdot 10^{-3}$ g/ml, with the aid of an MR-1M pump at a rate of 6-8 ml/min. The degree of inhibiting effect of the preparations was evaluated by measuring the residual activity of bradykinin, passed through isolated lungs before and after 5 min perfusion of them with a solution of the inhibitor in the corresponding concentration. The activity of bradykinin contained in the outflowing perfusate was estimated according to the contraction of an isolated segment of the guinea pig ileum and compared with the reaction to bradykinin ($1 \cdot 10^{-9}$-$1 \cdot 10^{-8}$ g/ml), introduced directly into the beaker with the organ. The intestinal tonus was recorded with a balance pen; the action of each concentration of the thiol preparations was studied on 5 to 6 animals. The experimental results were treated statistically with a calculation of the arithmetic mean data and the standard error.

The total kininase activity (TKA) of human blood plasma was measured according to the rate of inactivation of bradykinin: to 875 µl of 0.05 M Tris-HCl buffer, pH 7.8, we added 0.1 ml of a 0.01% solution of bradykinin (10 µg) and 25 µl of blood plasma; the sample was incubated at 37°C (water bath); 0.3 ml aliquots were collected after 10, 20, and 30 min and added

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to 0.7 ml of a $4 \cdot 10^{-3}$ M solution of 1,10-phenanthroline to inactivate carboxypeptidase N. The bradykinin content in the sample after its 50-fold dilution with Jelon's solution was measured by a biological method on an isolated rat uterine horn [11].

The control sample, containing 10 μg bradykinin in 1 ml of 0.05 M Tris-HCl buffer, pH 7.8, was incubated at 37°C, and then the bradykinin content was determined as indicated above. The blood plasma TKA was calculated according to the difference of the bradykinin content in the control and experimental samples and expressed in micrograms of inactivated bradykinin in 1 min in 1 ml of blood plasma. Under these conditions the TKA of human blood plasma was 6.4 μg of inactivated bradykinin per ml per min.

In a measurement of the bradykinin inactivating activity of a partially purified preparation of carboxypeptidase N, 0.1 ml of a solution of carboxypeptidase N (100 μg) was added to a bradykinin solution (10 μg in 0.9 ml of 0.05 M Tris-HCl buffer, pH 7.8), the sample was incubated at 37°C, and then all the operations were performed as indicated above for blood plasma TKA. Under these conditions 1 μg of the carboxypeptidase N preparation inactivated 1.8 μg of bradykinin in 1 min.

To study the influence of unithiol and d-penicillamine on blood plasma carboxypeptidase N and TKA, 25 μl of blood plasma was mixed with 775 μl of 0.05 M Tris-HCl buffer, pH 7.8, or, correspondingly, with 100 μg carboxypeptidase N in 0.8 ml of 0.05 M Tris-HCl buffer, pH 7.8, 0.1 ml of a solution of one of the thiol preparations was added in concentrations of $0.5 \cdot 10^{-4}$, $1 \cdot 10^{-4}$, $2 \cdot 10^{-4}$, and $5 \cdot 10^{-4}$ M for unithiol and $0.5 \cdot 10^{-3}$, $1 \cdot 10^{-3}$, $2 \cdot 10^{-3}$, and $5 \cdot 10^{-3}$ M for d-penicillamine; the samples were kept at 37°C for 10 min to inhibit the enzyme, then 0.1 ml of bradykinin solution (10 μg) was introduced, and then the kininase activity was measured as described above for blood plasma TKA. The inhibitory effect of thiol preparations on kininases was estimated according to the inhibition (in percent) of the activity of the corresponding inhibitor concentration, which was calculated according to the formula

$$\frac{V_0 - V_i}{V_0} \cdot 100,$$

where V_0 is the reaction rate without the inhibitor; V_i, rate of inactivation of bradykinin in the presence of a given inhibitor concentration; 100 is the initial activity of the enzyme. The values obtained were plotted on a graph, and the value of I_{50} — the inhibitor concentration (in M) lowering the enzyme activity by 50% — was calculated.

The effect of thiol preparations on the activity of peptidyl dipeptidase from beef kidneys was determined according to the cleavage of the tripeptide Z-Phen-His-Leu,* which is the C-terminal fragment of angiotensin I [12]. The dipeptide His—Leu that is split out reacts with o-phthalic dialdehyde in alkaline medium, forming a product, the fluorescence of which was measured on an Opton spectrofluorometer (excitation at 370 nm, fluorescence at 500 nm). The content of His—Leu in the investigated samples was calculated according to the fluorescence of a standard solution of His—Leu. The peptidyl dipeptidase activity was expressed in micromoles of His—Leu liberated as a result of hydrolysis in 1 min per mg enzyme protein. In an investigation of the inhibiting effect of thiol preparations on the activity of peptidyl dipeptidase, the enzyme (0.1 μg) was preliminarily incubated in 1.9 ml of medinal buffer, pH 7.4, with one of the inhibitors for 15-20 min at 37°C, and then 0.1 ml of 1 mM solution of the substrate Z-Phen—His—Leu was added, followed by fluorometry.

The effect of thiol preparations on the intensity and duration of the depressor reaction, developing after intravenous injection of bradykinin in doses of 0.1-0.5 μg/kg, was studied on male rats weighing 250-300 g, anesthetized with urethane (1 ml of a 10% solution per 10 g of weight intraperitoneally). The arterial pressure was registered with a mercury manometer in the common carotid artery [13]. Bradykinin and thiol preparations, dissolved in isotonic sodium chloride solution, were introduced through a catheter into the jugular vein in volumes of 0.1-0.3 ml. In the indicated doses bradykinin induced a brief (15-20 sec) lowering of the arterial pressure by 10-15 mm Hg. The change in the magnitude and duration of the depressor reaction to bradykinin against a background of administration of thiol compounds was evidence of an inhibition of the inactivation of exogenous bradykinin by plasma and pulmonary kininases.

The effect of thiol preparations on the magnitude and duration of the edematous response induced by the injection of 0.1 ml of a 0.01% solution of bradykinin under the plantar aponeurosis of the right hind limb was studied on male rats weighing 130-150 g. The volume of the limb was measured oncometrically before injection of bradykinin and after 30 min, 1, and 2 h. Thiol preparations in a dose of 50 mg/kg (1/30 LD_{50} of d-penicillamine, the most toxic

of the compounds studied) in the form of aqueous solutions were injected intraperitoneally 15 min before the injection of bradykinin. The animals of the control group received 0.5 ml of distilled water intraperitoneally. The action of each preparation was studied on 10 rats. The results of the measurements were treated statistically with a calculation of the values of the mean and standard error.

Bradykinin triacetate from Reanal (Hungary), 1,10-phenanthroline from Chemapol (Czechoslovakia), hippuryl-L-argininic acid (laboratory sample), 0.38% citrate human blood plasma, carboxypeptidase N [partially purified preparation, specific activity with respect to hippuryl-L-argininic acid 0.45 μmole (mg·min0], and d-penicillamine — 3,3-dimethylcystamine (All-Union Scientific-Research Institute of Antibiotics, Moscow), as well as unithiol — sodium 2,3-dimercaptopropanesulfonate (industrial sample), were used in the work. Rats of the Wistar line (65 animals) were obtained from the nursery of the Academy of Medical Sciences of the USSR, Kryukovo Division; noninbred guinea pigs (35 animals) were obtained from the same nursery, Lytkino Division.

RESULTS AND DISCUSSION

The spasmogenic activity of a solution of bradykinin, passed through the vessels of the pulmonary circulation, on the ileum of the guinea pig was decreased by 73 ± 15% in comparison with the activity of bradykinin added directly to the beaker with the isolated organ. Preliminary perfusion of the lungs with a solution of thiol preparations lowered the degree of inactivation of bradykinin and, as a result, decreased the spasmogenic reaction of the intestine. In this case EC_{50} (the concentration of the preparations inhibiting the kininase activity of the lungs by 50%) was $5 \cdot 10^{-5}$ g/ml for penicillamine, $1.7 \cdot 10^{-6}$ g/ml for unithiol, and $3 \cdot 10^{-4}$ g/ml for cysteine (Fig. 1).

The results of the biochemical experiments confirmed the inhibiting action of thiol preparations on kininases I and II and on the whole coincided with the data obtained in the investigation of their effect on the kininase activity of the lungs. Figure 1 presents data on the inhibition of the kininase activity of a partially purified preparation of carboxypeptidase N and the TKA by unithiol and d-penicillamine. The degree of inhibition was expressed as a function of the concentration of each of the preparations. Just as in experiments on isolated guinea pig lungs, unithiol, in comparison with d-penicillamine, is a stronger inhibitor of carboxypeptidase N and TKA, which follows from the value of I_{50}, correspondingly equal to $1 \cdot 10^{-5}$ and $4 \cdot 10^{-5}$-$5 \cdot 10^{-5}$ M for unithiol and $1 \cdot 10^{-4}$ and $4 \cdot 10^{-4}$-$5 \cdot 10^{-4}$ M for d-penicillamine.

The same order of the activity was established in a study of the influence of the investigated thiol preparations on the activity of peptidyl dipeptidase from beef kidneys as well. In these experiments, which showed that mercapto compounds inhibit the activity of the indicated enzyme, unithiol was the most active; in a concentration of $1 \cdot 10^{-4}$ M it practically entirely blocked the peptidyl dipeptidase activity, while d-penicillamine in this concentration decreased its activity by 24%, and cysteine by only 5% (Fig. 2B). Higher concentrations of d-penicillamine and cysteine gave a more pronounced inhibitory effect (Fig. 2B).

In experiments on anesthetized and intact rats, the thiol preparations studied intensified and prolonged the vascular effects of bradykinin — the depressor reaction and the increase in the permeability of the microvessels. It was established in this case that they all intensify and prolong the depressor effect of bradykinin, beginning with doses of 0.5-0.1 mg/kg. However, when the dose was increased, the intensification of the bradykinin-potentiating action of the preparations differed. Thus, unithiol in doses of 5 and 10 mg/kg intensified the reaction of the arterial pressure to bradykinin by a factor of 2.5 and 3.2, respectively, while d-penicillamine in these doses changed it by a factor of 2.1 and 2.9, and the effect of cysteine was unchanged within the indicated dose range. When the dose of the preparations was increased to 20 mg/kg, a further intensification of the action of d-penicillamine, which increased the depressor responses to the injection of bradykinin by more than fivefold, was observed; the effect of cysteine was increased to a lesser degree (2.5-fold), while the bradykinin-potentiating activity in unithiol, on the contrary, was lowered to the level corresponding to the action of 1 mg/kg of this preparation. In doses of 30-100 mg/kg the activity of d-penicillamine and cysteine was also decreased, without, however, reaching the initial values (Fig. 3).

Together with the intensification of the depressor effect of bradykinin, the thiol preparations studied prolonged the reaction of lowering of the arterial pressure that they induced in rats, from 15-20 to 40-200 sec, depending on the dose used, i.e., by a factor of 2-10. The

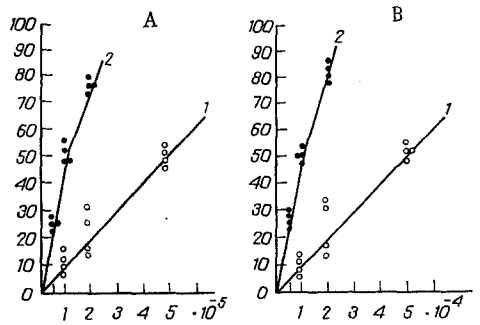

Fig. 1

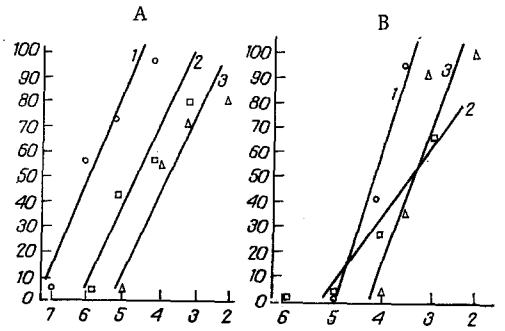

Fig. 2

Fig. 1. Inhibition of the kininase activity (bradykinin inactivating) by unithiol (A) and d-penicillamine (B). 1) Human blood plasma; 2) partially purified preparation of carboxypeptidase N. Along x axes: concentration of inhibitor in sample (in M); along y axes: inhibition of kininase activity (in %).

Fig. 2. Influence of thiol compounds on the kininase activity of guinea pig lungs (A) and the activity of peptidyl dipeptidase from beef kidneys (B). Along x axes: concentration of thiol preparations (in M); along y axes: inhibiting effect of the compounds (in %). 1) Unithiol; 2) d-penicillamine; 3) cysteine.

duration of the bradykinin-potentiating action of individual preparations *in vivo* depended on the dose used, but it did not exceed 1-2 h.

Thiol preparations prolonged the effect of bradykinin on the microcirculation, changing the dynamics of the edema of the limb in rats induced by injection of this polypeptide. In animals of the control group, bradykinin produced an increase in the volume of the limb, which reached a maximum by 30 min after its injection and gradually returned to the initial level by 60 min of observation. Preliminary administration of thiol preparations, while not significantly affecting the magnitude of the bradykinin edema, changed its dynamics prolonging the microcirculatory reaction to bradykinin for a period of more than 180 min (Fig. 4). With respect to activity in these experiments, unithiol surpassed d-penicillamine and cysteine.

Thus, the experiments showed that such thiol preparations as unithiol, d-penicillamine, and cysteine inhibit the activity of various kininases and, in connection with this, prolong and intensify the depressor effect of bradykinin and the increase in the permeability of the microvessels induced by it. The data obtained are evidence that thiol compounds are capable of effectively inhibiting the activity of the kinin-decomposing enzymes, lowering the rate of catabolism of kinins in the organism, and changing the degree of expression of their pharmacological effects. Noteworthy is the monotypic nature of the action of individual investigated thiol preparations, which inhibit both carboxypeptidase N and peptidyl dipeptidase, which are metal-dependent peptidases. The differences in the action of individual preparations are basically quantitative, and evidently they reflect the strength of the complex-forming properties of these compounds. On all the experimental models used, unithiol, containing two SH groups, surpassed d-penicillamine and cysteine with respect to inhibiting effect on the activity of kininases; the relative low activity of the cysteine sample used, in addition, could be determined by its partial oxidation of the SH group in air.

The ability of thiol compounds to inhibit the activity of kinin-decomposing enzymes permits them to be used as agents for regulating the activity of the kinin system of the organism, as well as the renin—angiotensin system, associated with it through peptidyl dipeptidase (an angiotensin-converting enzyme).

Investigations of recent years have shown [14, 15] that in hypertension stage II-III, a weakening of the depressor function of the kinin system is observed [14, 15]. At the same time, the role of activation of the renin—angiotensin system in the pathogenesis of certain forms of hypertension has been convincingly demonstrated on extensive clinical material [16-18, etc.]. In view of this, the use of inhibitors of kininases, which intensify the depressor function of the kinin system with simultaneous inhibition of the formation of the pressor factor — angiotensin II — seems a promising line in the therapy of certain forms of hypertension [19-20]. The aforementioned is supported by successful attempts to use unithiol in the complex therapy of hypertension [21, 22], as well as data on the antihypertensive activity of

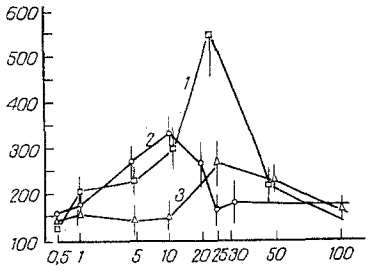

Fig. 3

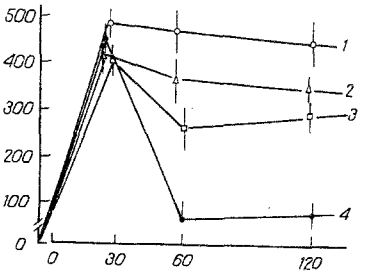

Fig. 4

Fig. 3. Increase in the depressor reaction to bradykinin by thiol preparations. Along x axis: doses of preparations (in mg/kg); along y axis: increase in depressor reaction (in % of the initial reaction ± standard error). 1) d-Penicillamine; 2) unithiol; 3) cysteine.

Fig. 4. Effect of thiol preparations (50 mg/kg internally) on the dynamics of edema of the limb in rats induced by bradykinin. Along x axis: time (in min); along y axis: value of response (in μl ± standard error). 1) Unithiol; 2) d-penicillamine; 3) cysteine; 4) control.

the preparation captropryl (SQ-14225) – D-3-mercapto-2-methylpropanoyl-L-proline, proposed in recent years for medical use [23, 24]. It also seems advisable to seek new selective inhibitors of kininases in order to create effective drugs for the treatment of hypertension.

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