EXPERIMENTAL BIOLOGY

Effect of Recombinant Forms of Urokinase Plasminogen Activator on Platelet Aggregation and Intracellular Calcium Accumulation

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Urokinase caused plasmin-dependent inhibition of platelet aggregation in platelet-rich plasma, while its proteolytically inactive form had no such effect. Both forms potentiated the increase in platelet calcium concentration induced by aggregation inductors and facilitated aggregation of washed platelets. In contrast to full-length urokinase molecule, its aminoterminal fragment inhibited platelet aggregation and the corresponding elevation of intracellular calcium. These data suggest that urokinase exerts a plasmin-independent effect on platelet activity. This effect depends on urokinase structure.

Key Words: *urokinase; platelets; aggregation; intracellular calcium*

The main biological function of the fibrinolysis system is the lysis of fibrinous thrombi with plasmin formed from plasminogen under the influence of urokinase and tissue activators [3,4,12]. Urokinase also modulates cell migration and proliferation as well as the intracellular signal transduction via its own receptors (glycosylphosphatidylinositol-anchored protein) expressed in most cell types (smooth muscle cells, monocytes, hepatocytes, *etc.*) [2,5,6].

The effect of the fibrinolysis system on platelet activity is largely mediated via plasmin-dependent modifications of plasma membrane components and factors involved in platelet aggregation [10,11]. Platelets have plasminogen binding sites on their surface, but contain no classical urokinase receptor. At the same time, they possess an urokinase-binding protein, differing from typical urokinase receptor in most cell types by its kinetic characteristics, molecular weight, and antigen specificity [8]. The function of this protein remains unknown, but the effect of urokinase on platelets can be mediated via this protein.

In this study we analyzed the effects of urokinase on platelet activity independent of its catalytic component and plasminogen activation.

MATERIALS AND METHODS

Platelet aggregation in platelet-rich plasma (PRP) and plasma-free platelet suspension was measured with a Biola 230-LA dual-channel laser aggregometer as described previously [1].

To prepare PRP, blood was stabilized with 130 mM sodium citrate (pH 7.4, 9:1 v/v ratio) and centrifuged at 190*g* for 15 min. After PRP separation the blood was centrifuged at 2000*g* for 15 min to obtain platelet-depleted plasma. To isolate plasma-free platelets, PRP was centrifuged at 650*g* for 10 min, resuspended in bufer A, containing 10 mM HEPES-Na (pH

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6.55 at 37°C), 150 mM NaCl, 2.7 mM KCl, 0.37 mM NaH_2PO_4, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 5 mM glucose, 0.35% bovine serum albumin (fraction V), 50 U/ml heparine, 0.034 U/ml apyrase.

Calcium concentration in platelet cytoplasm was measured with Fura-2 fluorescent probe on a Shimadzu 5000 spectrofluorimeter at excitation and emission wavelengths of 340-380 and 500 nm, respectively, and calculated as described elsewhere [7].

Suspensions of Fura-2-containing platelets were prepared by mixing the blood with anicoagulant (6:1) containing 85 mM sodium citrate, 66.6 mM citric acid, and 100 mM glucose followed by centrifugation at 190*g* for 15 min. PRP was incubated for 50 min

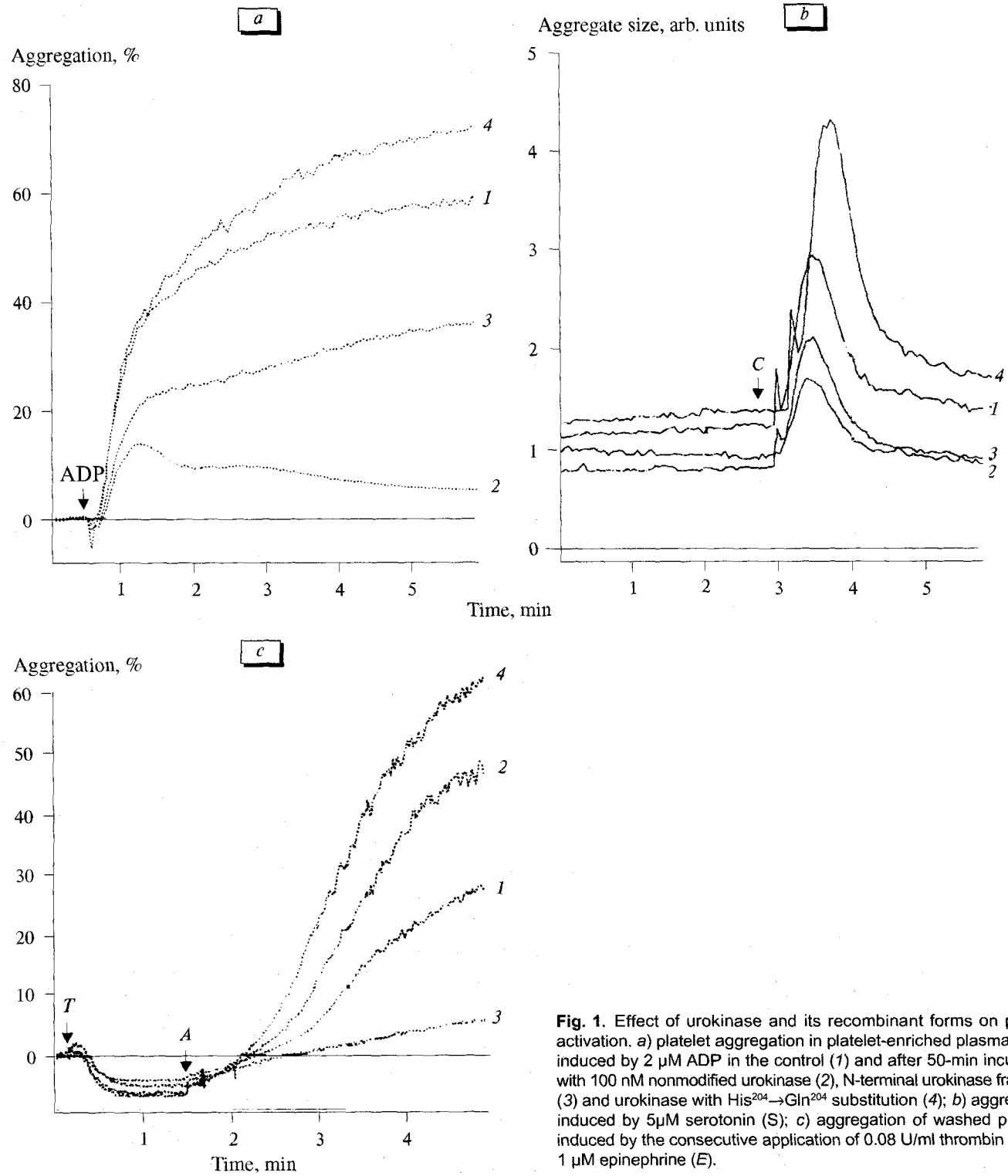

Fig. 1. Effect of urokinase and its recombinant forms on platelet activation. *a*) platelet aggregation in platelet-enriched plasma (PRP) induced by 2 μM ADP in the control (*1*) and after 50-min incubation with 100 nM nonmodified urokinase (*2*), N-terminal urokinase fragment (*3*) and urokinase with $His^{204} \rightarrow Gln^{204}$ substitution (*4*); *b*) aggregation induced by 5μM serotonin (S); *c*) aggregation of washed platelets induced by the consecutive application of 0.08 U/ml thrombin (*T*) and 1 μM epinephrine (*E*).

with 1 μM Fura-2- and centrifuged at 650g for 10 min. The sediment was resuspended in bufer A and adjusted to the initial volume. The effect of urokinase on platelet activity in PRP and plasma-free platelet suspension was assessed after 50-min incubation.

The high-molecular-weight recombinant single-strand form of urokinase, its catalytically inactive recombinant form with histidine to glutamine substitution in the active center ($His^{204}\rightarrow Gln^{204}$), and its N-terminal fragment (NTF) containing 1-156 amino acid residues were kindly provided by Laboratory of Genetic Engineering of the Institute of Experimental Cardiology of the Russian Cardiology Research-and-Production Complex. Adenosin diphosphate (ADP), serotonin, sodium citrate dihydrate, Fura 2-AM, bovine serum albumin, and apyrase were purchased from Sigma.

In each experiment, platelet aggregation and intracellular Ca^{2+} concentration were measured 3 times. The data were expressed as the mean±standard deviation.

RESULTS

Urokinase inhibited the ADP- or serotonin-induced platelet aggregation in PRP (Fig. 1, *a, b*). The decrease in amplitude and rate of platelet aggregation depended on the time of preincubation and was prevented by serine protease inhibitor aprotinin. [1]. The inhibitory effect of urokinase on platelets is determined by its ability to convert plasminogen in plasmin by proteolysis [9]. In its turn, plasmin splits membrane and plasma components involved in the aggregation process.

Recombinant urokinase without proteolytic activity because of $His^{204}\rightarrow Gln^{204}$ substitution within the active center did not inhibit the aggregation process. Moreover, when applied in high concentrations, it even potentiated ADP- and serotonin-dependent aggregation (Fig. 1, *a, b,* Table 1).

N-terminal urokinase fragment 1-156 (NTF) suppressed platelet aggregation although its inhibitory effect was less pronounced than that of nonmodified molecule (Fig. 1, *a, b*, Table 1). The principal difference between the inhibitory effects of NTF and urokinase is that NTF devoid of the catalytic center is unable to convert plasminogen into plasmin.

The increase in intracellular Ca^{2+} concentration is a key event in platelet activation by aggregation inductors. Preincubation of platelets with nonmodified urokinase did not suppress, but even enhanced ADP-induced Ca^{2+} entry (Fig. 2, Table 1). Similar effect was observed in the presence of catalytically inactive urokinase. NTF inhibited platelet calcium response in parallel with its effect on the aggregation process.

For evaluation of direct (not mediated by plasmin) modulation of platelet aggregation by urokinase, we studied the effects of its recombinant forms on washed platelet suspensions. The aggregation was stimulated with 0.08 U/ml thrombin followed by 1 μM epinephrine (Fig. 1, *c*). In our experiments, thrombin changed the shape of platelets and reduced translucence of the cell suspension, while epinephrine caused slow platelet aggregation. Urokinase and its inactive form accelerated aggregation of washed platelets, while NTF inhibited it (Fig. 1, *c*, Table 1).

The data indicate that urokinase can modulate platelet activity without plasmin activation. This effect can be realized through direct interaction of urokinase and its N-terminal fragment with platelets. A mem-

TABLE 1. Effect of Urokinase and Its Recombinant Forms on the Parameters of Aggregation and Ca^{2+} Exchange in Platelets ($M\pm\sigma$)

Parameters, % of control		Control	Urokinase forms, nM					
			nonmodified		with His→Gln substitution in active center		N-terminal fragment	
			50	100	50	100	50	100
Aggregation in PRP								
ADP-dependent aggregation	amplitude	100±4	53±11	11±1	101±7	106±8	86±9	65±7
	rate	100±4	61±12	4±1	102±6	106±8	83±7	60±6
Serotonin-dependent aggregation	amplitude	100±5	66±6	53±4	91±6	131±7	83±5	64±3
	rate	100±5	57±6	43±11	96±5	143±4	94±3	70±2
Aggregation of washed platelets								
	rate	100±8	76±11	159±6	143±7	178±8	51±8	19±3
Platelet Ca^{2+} concentration, nM		103±6	127±9	146±12	109±10	120±8	160±13	136±12
ADP-stimulated Ca^{2+} entry		100±6	118±14	127±7	112±6	141±9	59±6	38±9

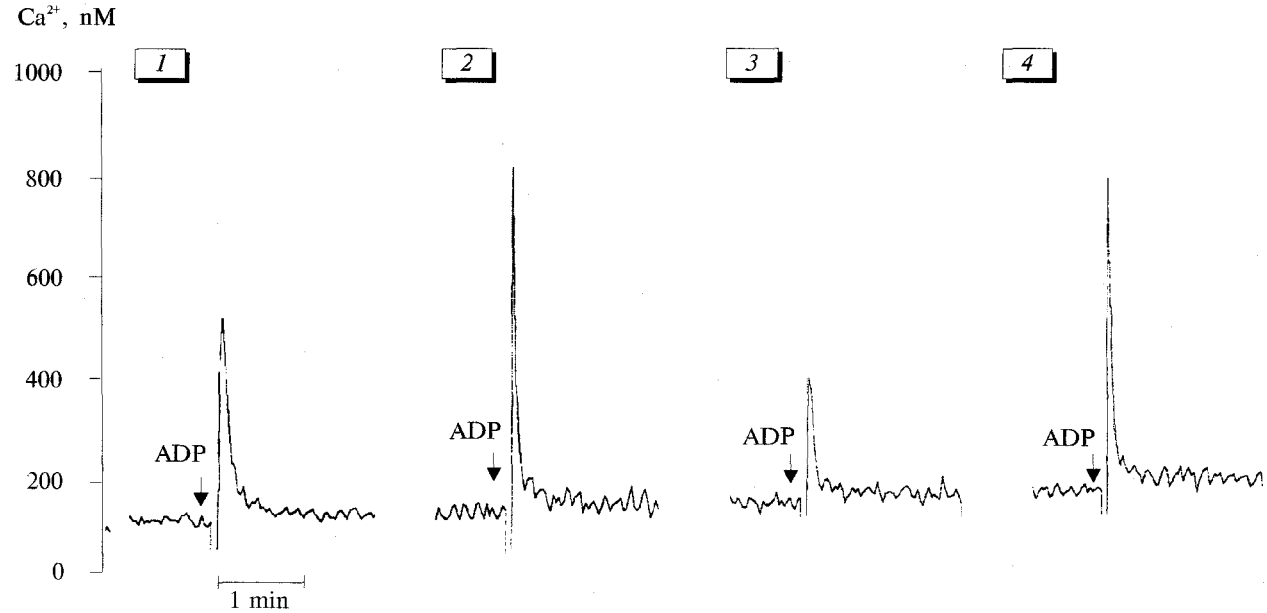

Fig. 2. Increase in platelet Ca^{2+} concentration induced by 1 μM ADP in the control (*1*) and after 50-min incubation of platelet-rich plasma with 100 nM nonmodified urokinase (*2*), N-terminal urokinase fragment (*3*) and urokinase with $His^{204} \rightarrow Gln^{204}$ substitution (*4*).

brane protein different from urokinase receptor of the majority of cell types [8] is the most likely candidate for this interaction.

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