Acyl-kallikrein; a delivery system for the kinin-liberating enzyme

F. Markwardt, J. Stürzebecher and H. Müller

Institute of Pharmacology and Toxicology, Medical Academy Erfurt, DDR-5010 Erfurt (German Democratic Republic), 2 May 1983

Summary. Glandular kallikrein can be temporarily acylated at the active centre by 4-amidinophenyl benzoates. The time course of reactivation depends on the nature of the acyl group introduced. Benzoyl kallikrein was used as a delivery system for kallikrein in vivo. In rabbits, bolus injection of the acylated enzyme caused a long-lasting drop in blood pressure due to sustained kinin liberation.

During the development of synthetic, low molecular weight inhibitors of trypsin-like serine proteinases[1,2] a series of 4-amidinophenyl benzoates were found to act as temporary inhibitors of trypsin, plasmin, thrombin, kallikrein and urokinase[3-5]. 4-Amidinophenyl esters of arylcarboxylic acids behave like substrates of trypsin-like enzymes but acylation is much more rapid than deacylation. The result is accumulation of a comparatively stable acyl-enzyme. On deacylation the active enzyme is reactivated. In plasma, an acyl-enzyme behaves like an inert form of the enzyme and is protected from being inactivated by natural plasma inhibitors.

This principle has been made use of in the development of acyl-plasmin and acylated streptokinase-plasminogen activator complex as a delivery system for fibrinolytic agents in vivo[6,7]. We used this concept for preparation of an acylated glandular kallikrein. Due to the kinin-liberating effect in blood, kallikreins cause peripheral vasodilation.

Materials and methods. 4-Amidinophenyl esters of substituted benzoic acids were prepared according to Wagner and Horn[8]. Porcine pancreatic kallikrein (1374 KU/mg) was a gift from Professor H. Fritz, München. Its enzymatic activity, determined by titration with p'-nitrophenyl p-guanidino benzoate, was 30 nmol/mg.

Acyl-kallikrein preparation was carried out as follows. To 1.8 ml of a kallikrein solution (3–6 μmole/l) in Tris buffer (0.05 mole/l, pH 7.2) 0.2 ml amidinophenyl ester (2 mmole/l in ethanol) was added and the mixture was kept at 25 °C for 15 min.

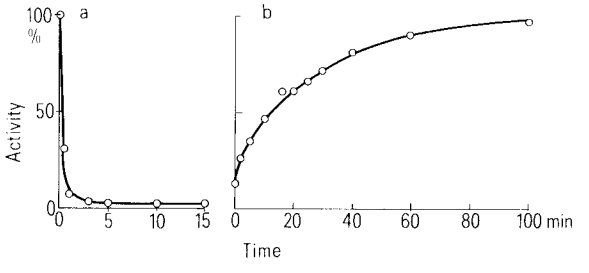


Figure 1. Time course of inactivation of kallikrein by 0.2 mmol/l 4-amidinophenyl benzoate (a) and reactivation of benzoyl kallikrein (b) at pH 7.2. The concentration of kallikrein or acyl-kallikrein was about 3 μmole/l.

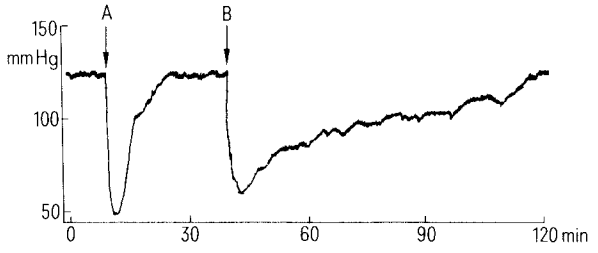


Figure 2. Effects of intravenous injection of 0.44 nmole/kg kallikrein (A) and benzoyl kallikrein (B) on mean blood pressure in rabbits.

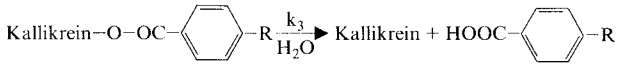
The pH was adjusted to 5.0 by addition of 1 mol/l HCl and the resulting solution was dialyzed against 0.154 mol/l NaCl (adjusted to pH 5.0) at 4 °C. Reactivation of acyl-kallikrein resulting from deacylation was carried out by incubating the dialyzed preparation (about 6 μmole/l acyl-enzyme) with Tris buffer (pH 7.2) in a proportion of 1:1 at 37 °C. The time course of inactivation or reactivation was obtained by measuring the resulting enzymatic activity of aliquots (0.05 ml) towards H-D-Val-Leu-Arg-pNA (S-2266, Kabi Diagnostica, Stockholm). In the assay mixture (1.1 ml) the substrate concentration was 0.05 mmole/l. The deacylation rate constant was calculated from the slope of a semilog plot.

The deacylation rate constants k_3 for all compounds used were determined spectrophotometrically as described by Tanizawa et al.[9]. Kallikrein (0.65–1.3 μmole/l) was incubated with 4-amidinophenyl ester (87 μmole/l) in Tris buffer (0.05 mole/l, pH 7.2) at 37 °C, and liberation of 4-amidinophenol was followed at 305 nm using a Unicam SP 800A double beam spectrophotometer. The effect of spontaneous hydrolysis was balanced out by using the same solution as reference with the omission of the enzyme. For calculation of k_3, ε_{305} 38,000 determined experimentally was used.

The pressure response to intravenous injection of kallikrein was studied in a group of 20 rabbits (Alaska) of both sexes and 2.8–3.2 kg b.wt. The animals were anesthetized by intraperitoneal injection of 1.2 g ethyl urethane/kg and a polyethylene catheter was inserted into the common carotid artery. The catheter was connected to a continuous infusion pump and to a Statham P 37 B pressure transducer. Mean arterial pressure was recorded on a 4-channel Mingograph 34.

Results and discussion. By the use of 4-amidinophenyl benzoates acyl residues were introduced into the kallikrein active site. The deacylation rate constant of the acyl-enzymes increases with the electron accepting potency of the benzoic acid substituent. Therefore, a variety of acyl-kallikrein intermediates with different deacylation rates can be designed (table). For in vivo studies the benzoyl kallikrein was chosen. The residual activity of the acyl-enzyme preparation was about 10% of that the original enzyme, while almost complete reactivation (> 98%) as a result of deacylation was observed on incubation of the acyl-enzyme at pH 7.2. The time course of inactivation and reactivation of benzoyl kallikrein is shown in

Deacylation rate constants k_3 and half-times of reactivation $t_{1/2}$ of various acyl-kallikreins at pH 7.2 and 37 °C



R	k_3, min^{-1}	$t_{1/2}$, min
OCH_3	0.0024	289
CH_3	0.0039	178
H	0.0128	54
F	0.0494	14
Cl	0.0666	10
CN	0.1068	6.5
NO_2	0.2508	2.8

figure 1. From the semilog plot of reactivation a value of 0.039 min^{-1} for k_3 was calculated.

The effect of kallikrein and benzoyl kallikrein on kinin liberation in vivo was followed by the drop in blood pressure in rabbits (an example is shown in fig. 2). Kallikrein administered intravenously causes a quick transient drop in systemic blood pressure. Besides sympathetic contraregulation and degradation of the pharmacologically active kinins, the inhibition of the administered kallikrein by natural plasma inhibitors may be of importance for the fast normalisation of blood pressure[10]. Compared to the situation with the non-acylated enzyme, the duration of the decrease in blood pressure following a bolus injection of acyl-kallikrein is longer and equals the time of reactivation (fig. 1). The time-course of the decrease in blood pressure corresponds to that of an infusion of the same dose of kallikrein within 40 min. The results indicate that acylated kallikrein is protected from being inactivated by plasma inhibitors up to restoration of enzymatic activity.

1 Markwardt, F., Haemostasis *7* (1978) 177.
2 Markwardt, F., Trends pharmac. Sci. *1* (1980) 153.
3 Markwardt, F., Wagner, G., Walsmann, P., Horn, H., and Stürzebecher, J., Acta biol. med. germ. *28* (1972) K 19.
4 Nozawa, M., Tanizawa, K., Kanaoka, Y., and Moriya, H., J. Pharm. Dyn. *4* (1981) 559.
5 Markwardt, F., Drawert, J., and Walsmann, P., Biochem. Pharmac. *23* (1974) 2247.
6 Smith, R.A.G., Dupe, R.J., English, P.D., and Green, J., Nature *290* (1981) 505.
7 Markwardt, F., Nowak, G., and Stürzebecher, J., in: Progress in Fibrinolysis, p. 249. Churchill Livingstone, London 1983.
8 Wagner, G., and Horn, H., Pharmazie *28* (1973) 427.
9 Tanizawa, K., Kasaba, Y., and Kanaoka, Y., J. Am. chem. Soc. *99* (1977) 4485.
10 Fritz, H., Fink, E., and Truscheit, E., Fedn Proc. *38* (1979) 2753.

0014-4754/84/040373-02$1.50 + 0.20/0

Dissociation between inhibition of phospholipid methylation and production of PAF-acether by rabbit platelets

L. Touqui, M. Chignard, C. Jacquemin, F. Wal and B.B. Vargaftig

Unité des Venins, Département de Physiopathologie expérimentale, Institut Pasteur, 28, rue du Dr. Roux, F-75015 Paris (France); INSERM U 200, 32, rue des Carnets, F-92140 Clamart (France), and Faculté des Sciences de Reims, B.P. 775, F-51062 Reims (France), 2 June 1983

Summary. Platelet-activating-factor (PAF-acether, 1-O-alkyl-2-acetyl-sn-glycero-3-phosphorylcholine) is formed by and released from rabbit platelets stimulated with thrombin, with the ionophore A23187, with collagen and with the platelet-stimulating glycoprotein convulxin. We here show that 3-deazaadenosine (C_3ado) and L-homocysteine (HCy), two inhibitors of platelet methylation, reduced the formation of PAF-acether and of its deacetylated product lyso-PAF-acether by rabbit platelets challenged with thrombin, under conditions where the accompanying aggregation was not significantly modified. In contrast, platelet aggregation induced by convulxin was completely and irreversibly blocked when C_3ado and HCy were associated. Aggregation by thrombin was not affected by the methylation inhibitors even when ADP was scavenged and thromboxane formation was suppressed. Our results indicate that phospholipid methylation, thrombin-induced platelet aggregation and formation of PAF-acether can be dissociated. The formation of PAF-acether by rabbit platelets can be blocked by mechanisms other than inhibition of phospholipase A2, since the latter is not affected by C_3ado and/or HCy.

PAF-acether is a phospholipid mediator released from various cell types upon appropriate stimulation, which induces platelet aggregation and the secretion of their granular constituents. Since platelet aggregation by PAF-acether does not require ADP release nor formation of thromboxane A2, PAF-acether was proposed as the mediator of a 3rd pathway of platelet aggregation[1-3]. 1-O-alkyl-2-acetyl-sn-glycero-3-phosphorylcholine is the presently accepted formula of this mediator[4-6] which is also formed by and released from rabbit platelets stimulated with the calcium ionophore A23187[1], with collagen, thrombin[2] or with convulxin, a platelet-stimulating glycoprotein extracted from the venom of *Crotalus durissus cascavella*[7-9]. Platelet phospholipid methyl transferases, which catalyse the formation of phosphatidylcholine (PC) from phosphatidylethanolamine (PE)[10,11], can be blocked by 3-deazaadenosine (C_3ado) and/or L-homocysteine (HCy)[12,13]. These reagents also suppress collagen- and convulxin-induced platelet activation, but do not reduce significantly aggregation due to ADP, to thrombin, to trypsin or to A23187[12-14].

Since it has been hypothesized that a PC analogue (1 alkyl-2-acyl-sn-glycero-3-phosphorylcholine) may be a precursor of PAF-acether[15] and of its deacetylated analogue lyso-PAF-acether in platelets[16], we decided to investigate whether the inhibition of the formation of PC from PE and of their respective l-alkyl analogues by methyl transferases would interfere with the formation of PAF-acether and how this would correlate with platelet aggregation.

Materials and methods. Measurement of phospholipid methylation. Blood was collected from the central ear artery of adult New Zealand white rabbits on a mixture of disodium and tetrasodium salts of EDTA (5 mM final concentration). Washed platelets prepared as described[2] were resuspended in tris-Tyrode buffer containing 1 mM $MgCl_2$, 2 mM $CaCl_2$ and 2.5 mg/ml of fatty acid-free bovine serum albumin (Sigma) at pH 7.4. Platelet suspensions were incubated at room temperature with 3-deazaadenosine (C_3ado, Southern Research Institute, Birmingham, USA) and/or L-homocysteine thiolactone (HCy, Sigma) or with their solvent in the presence of 160 µCi/ml of Me-^{3}H-L-methionine (2 µM, 80 Ci/mmol, NEN), which was added to the platelets 1 h before the potential inhibitors. The time-course of the incorporation of radiolabeled methyl into phospholipids was followed. At different intervals a 0.5-ml sample was removed from the incubates and added to 1 ml of chloroform-methanol (3:1 vol) at 4 °C. The mixture was processed according to Randon et al.[12], to measure the extent of phospholipid methylation.

Determination of the formation of PAF-acether by platelets. The platelet suspensions prepared as indicated above were incubated at room temperature with the potential inhibitors (C_3ado and/or HCy) or with their solvents. At different time-