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The Role of Phospholipase C Isozymes during Fibrinolysis and in Relation to Platelet Function in Goat Blood.

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ABSTRACT

Agonist-induced platelet activation involves different signaling pathways leading to the activation of phospholipase C isozymes like (PLC) β or PLCγ. Activated PLC produces inositol 1,4,5-trisphosphate and diacylglycerol, which trigger Ca^{2+} mobilization and the activation of protein kinase C, respectively. PLCβ is activated downstream of Gq-coupled receptors for soluble agonists with only short interaction times in flowing blood. In contrast, PLCγ2 becomes activated downstream of receptors that interact with immobilized ligands such as the collagen receptor glycoprotein (GP) VI or activated integrins. In this study, we evaluated the effect of fibrinolysis in relation to platelet function on the phospholipase C activity in the blood of the goat.

Keywords: phospholipase C, isozymes, fibrinolysis, platelet, goad blood

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INTRODUCTION

In many pathological conditions of the blood hemolysis is a common feature. The process of hemolysis consists of the release of hemoglobin from red blood cells (RBC) to the medium surrounding it. *In vivo* hemolysis is a pathologic event that occurs in several forms of anemia, as well as in response to both intoxications and infections. On the other hand, *in vitro* hemolysis is often used as a sensitive and reliable method to assay the lytic action on cell membranes of any physical or chemical agent. In this context the membranes of the RBC are used as a membrane model. It was found many years ago that erythrocytes become more sensitive to hemolysis induced by certain drugs or toxins when, after incubation at 37 °C, the cell suspension is cooled to 4 °C. This is also known as "hot-cold hemolysis". The molecular basis for this phenomenon is not exactly understood. Different explanations have been proposed over the last several years, but none has gained wider acceptance. The other prominent blood cells i.e the platelets are small anuclear cells that play a critical role in hemostasis. They adhere to the injured vessel wall and recruit other platelets to form a hemostatic plug that is critical in limiting blood loss and initiating vascular repair. On the other hand, platelet–platelet interactions can lead to inordinate thrombus growth, which is a major patho-mechanism in the development of acute ischemic disorders, including stroke and myocardial infarction [1,2].

Two major PLC isoforms are expressed in the blood, and they become activated by different signaling pathways. PLCβ becomes activated downstream of G-protein (Gq)-coupled receptors, which are mainly triggered by soluble agonists such as adenosine diphosphate (ADP) and TxA_2 or locally produced thrombin with only short interaction times in flowing blood. In contrast, PLCγ2 is activated by signaling pathways involving tyrosine phosphorylation cascades downstream of receptors that predominantly interact with immobilized ligands and may trigger sustained signaling events. The best characterized PLCγ2-activating receptors in platelets are the immunoreceptor tyrosine-based activation motif (ITAM)-coupled collagen receptor GPVI, FcγRIIa, ligand-occupied integrins, and possibly also GPIb [3,4]. In addition, the recently identified C-type lectin-like receptor-2 (CLEC-2) also strongly activates PLCγ2 in platelets, and mediates powerful cellular activation [5]. Fibrinolytics are life saving drugs in deep vein thrombosis and myocardial infarction. In this study we evaluated the effect of pituitary hormone, vasopressin and its analogue terlipressin on the PLC activity in the blood after fibrinolysis with streptokinase.

MATERIALS AND METHODS

Drugs and Chemicals used

Arginine vasopressin, terlipressin, streptokinase, sodium nitroprusside, Lithium chloride and all laboratory grade reagents and chemicals for biochemical procedures.

Hemolysis Assay

Goat blood was collected and stored in EDTA containing tubes. Goat blood was washed three times with 25 mM HEPES, 140 mM NaCl, and 0.1 mM EGTA, pH 7.2, buffer by centrifugation at 1250*g* for 12 min and brought to 1% hematocrit. Hemolysis experiments

were carried out at 37 °C. At selected times, aliquots were removed from the reaction mixture and placed in ice for 3-20 min (Based on sample size) in order to induce hot-cold hemolysis. After centrifugation at 1750*g* for 10 min, hemolytic activity was measured as the increase at 415nm (i.e., increase in hemoglobin content) of the supernatant.

Estimation of PLC activity

The PLC activity was determined by anion exchange chromatography. Briefly, the 0.8 ml of hemolysed blood sample was taken in tubes containing 3.2 ml of (HBSS) and 0.5ml of 10mM lithium chloride was added to it. Pretreatment with Streptokinase and sodium nitroprusside was done and incubated for 20 minutes at 37 ^{0}C. Vasopressin and Terlipressin (5 µg) were later added and incubated at 37ºC for 20 mins. After incubation, 0.5ml 10% Trichloroacetic acid was added to the solution. The volume was made to 5 ml with distilled water finally and 0.1 ml of NaOH added. The contents were poured in the column containing Dowex resin and later eluted with 5 ml of 0.8M Ammonium formate + 0.1M formic acid. Finally the elutant was collected in a beaker and 0.5ml PA-resorcinol was added alongwith 0.5ml triethanolamine and Optical Density was measured at 400 nm using spectrophotometer.

RESULTS

The results of our study are shown in Table 1. The pituitary peptide analogues vasopressin and terlipressin were able to stimulate the phospholipase C activity significantly. The fibrinolytic drug streptokinase slightly increased the PLC activity in the blood. On the other hand, sodium nitroprusside was able to attenuate the PLC activity induced by the vasopressin analogues.

DISCUSSION

The vasopressin analogues produced an increase in the phospholipase C activity in the blood as shown in the Table 1. This effect was however further increased by the action of fibrinolysis. However the guanylate cyclase activator, sodium nitroprusside caused an inhibition of the inositol triphosphate levels. Vasopressin is a ligand for the three types of receptors: V1a,V1b, and V2. These receptors belong to a large family of G protein–coupled receptors [6]. The signal transduction in V1 receptors involves the activation of phospholipases C (Refer Figure 1), D, and A_2, the production of inositol 1,4,5-triphosphate and diacylglycerol, the stimulation of protein kinase C, and the mobilization of intracellular Ca^{+2} [6].

There is growing evidence of the importance of phospholipases in the blood. A significant role of PLCγ2 in platelet activation *in vitro* and *in vivo* has been demonstrated in previous studies and impaired thrombus formation at sites of superficial vascular damage in a model of laser-induced injury in mice lacking the enzyme. In the later study, Nonne *et al* [7] demonstrated that thrombus formation partially involves PLCγ2 activation, depending on the severity of the vascular lesion. At sites of deep laser injury, PLCγ2 deficiency can be functionally compensated for by thrombin/Gq/PLCβ-dependent activation, leading to the formation of large, stable thrombi. These results demonstrate that strong Gq/PLCβ

stimulation is sufficient to induce powerful thrombotic activity of platelets that can lead to vessel occlusion. Amazingly, however, lack of PLCγ2 leads to impaired primary hemostasis, suggesting that PLCγ2-dependent signaling in platelets is important for sealing of a wound, although this defect can be partially compensated by PLCβ, as revealed by moderately increased bleeding times in *Plcg2*$^{-/-}$ mice as compared with β_3-deficient or GPIbα-deficient mice. An interesting study in this perspective is that of the role of sphingolipids in hemolysis and the result on the PLC activity. This would seem at odds with the above results, but in fact the studies by Okino and Ito [8] refer to hemolysis at constant 37 °C. As discussed above, hemolysis at constant temperature consists, at least in its early stages, of the gradual release of hemoglobin, rather than of cell breakdown. In fact, it was recently showed that sphingosine, one of the products of ceramidase activity, causes also release of vesicular and cell contents [9] and is even more active than ceramide in this respect. Thus, if hemolysis at constant 37 °C can be interpreted as a gradual efflux of hemoglobin, conversion of ceramide into sphingosine via ceramidase can only help the phenomenon [10].

Phospholipase C – IP_3/Protein kinase C Signaling Pathway

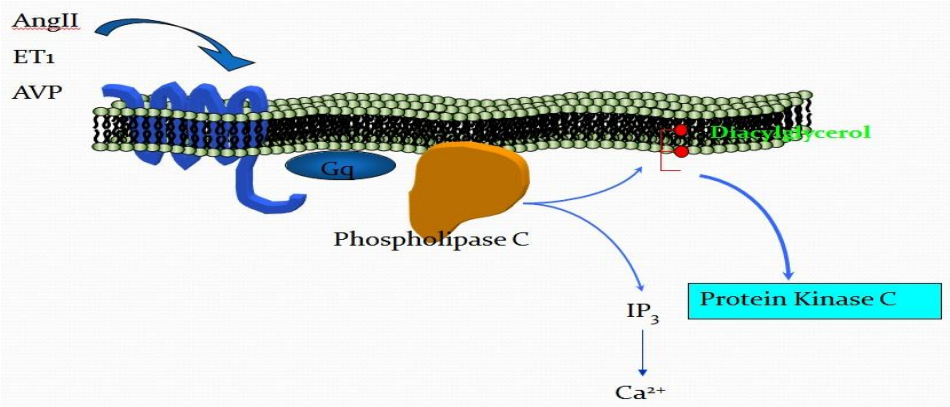

Figure 1

In our study, we evaluated the effects of the fibrinolytic agent streptokinase and the production of inositol trisphosphate levels. As shown in the results section, it is quite evident that the fibrinolytic drug caused a stimulation of the Phospholipase C activity and there was also a decrease in the phospholipase C activity as evidenced by the pretreatment with sodium nitroprusside (Table 1). This decrease in the PLC activity can be attributed to the activation of Protein kinase G (PKG) by sodium nitroprusside which could phosphorylate PLC and inhibit the agonist-induced IP_3 production in the hemolysed blood. A role for the sphingomyelinase enzyme and sphingolipids can be speculated based on the earlier observations [11].

Ealier studies has shown by using phosphopeptide mapping and site-directed mutagenesis, the two key phosphorylation sites which are Ser^{26} and Ser^{1105} for the regulation of PLC by PKG. There are also reports of the phospholipase C interacting with KCNQ ion channels by depleting the PIP_2 stores [12] and such an interaction in the blood remains unexplored. On the other hand the fibrinolytic drug, Streptokinase in this study seem to have activated the effects on the G protein cascade and possibly have actions on

the stimulatory G proteins, Gq and G 11 to mediate their stimulating actions on phospholipase C activity. In conclusion, it can be stated that the role of fibrinolysis on the agonist stimulated PLC activity by vasopressin and terlipressin can have implications for the blood dyscrasias and more comprehensive studies are required on this research topic.

Table 1: The effect of drugs on the vasopressin and terlipressin stimulated phospholipase C activity in the goat blood.

Serial No.	Pretreatment	Treatment	IP_3 generated (µg/ml)	% change
1	Nil	Vasopressin (6µg)	1.96 µg	NA
2	Nil	Terlipressin (6µg)	2.18 µg	NA
3	Streptokinase (15000 i.u)	Vasopressin (6µg)	2.15 µg	9.6
4	Streptokinase (15000 i.u)	Terlipressin (6 µg)	2.39 µg	9.7
5	Sodium Nitroprusside (5 mg)	Vasopressin (6µg)	1.89 µg	3.71
6	Sodium Nitroprusside (5 mg)	Terlipressin (6µg)	1.82 µg	16.51

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