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SEPARATION OF DIFFERENT RECEPTOR-MEDIATED EFFECTS OF A PROSTAGLANDIN H_2 ANALOGUE (U46619) ON HUMAN PLATELETS BY MEANS OF HUMAN GRANULOCYTIC ELASTASE AND CHYMOTRYPSIN

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Abstract—Previous investigations indicated two classes of thromboxane A_2/prostaglandin H_2 (TXA_2/PGH_2) receptors on human platelets and suggested that shape change and myosin light chain phosphorylation correlated with the occupancy of high affinity receptors while serotonin release was related to a putative low affinity binding component (Morinelli TA *et al.*, *Am J Physiol* **253**: H1035–H1043, 1987). The current study shows that chymotrypsin destroyed three receptor-mediated responses of platelets to U46619 (a TXA_2/PGH_2 agonist), i.e. shape change, myosin light chain phosphorylation and serotonin release. Human granulocyte elastase selectively inactivated platelet ability to release serotonin following stimulation with U46619, but it did not affect significantly shape change and myosin light chain phosphorylation. In conclusion, it is possible to separate different receptor-mediated effects of U46619 on human platelets by means of human granulocytic elastase and chymotrypsin.

In a previous study, we demonstrated [1] that shape change and myosin light chain phosphorylation are receptor-mediated effects of a stable thromboxane A_2/prostaglandin H_2 (TXA_2/PGH_2) analogue (U46619) on human platelets. Shape change and myosin light chain phosphorylation were effects found to be saturable and dose dependent; the concentrations of U46619 causing 50% of these effects (EC_{50}) were 0.035 and 0.057 μM respectively. On the other hand, the EC_{50} value for U46619 for serotonin release was 0.54 μM. Binding of [^{3}H]U46619 to intact platelets showed two components. Occupancy of high affinity sites correlated with platelet shape change and myosin light chain phosphorylation. Platelet release of serotonin was not correlated with occupancy of high affinity receptors. We proposed that it could be related to a putative low affinity binding component for [^{3}H]U46619 [1]. However, the hypothesis that there are two different classes of TXA_2/PGH_2 receptors which govern different functions of platelets could not be proven by means of the analysis of the binding data because non-specific binding of [^{3}H]U46619 is very high (70%) [1] and because ^{125}I-labeled PTA-OH, a TXA_2/PGH_2 antagonist, only binds to high affinity sites [2]. In our previous study [1], computer analysis of the binding isotherms of [^{3}H]U46619 to platelets could not exclude that the low affinity binding component represented non-specific binding.

McGowan and Detwiler [3] used chymotrypsin to differentiate between different effects that are coupled to thrombin receptors on the platelet surface, and Burch *et al.* [4] described tryptic proteolysis of solubilized TXA_2/PGH_2 receptors from platelet membranes.

In this paper we describe the effects of two enzymes, chymotrypsin and human granylocytic elastase, on the TXA_2/PGH_2 receptor and on three of the receptor-mediated responses of human platelets to U46619 (shape change, myosin light chain phosphorylation and serotonin release). Treatment of platelets with chymotrypsin destroyed all three responses of these cells to U46619. Human granulocytic elastase inactivated the ability of the platelets to release serotonin following stimulation with U46619, but did not affect significantly stimulation of platelet shape change and myosin light chain phosphorylation induced by this agonist.

MATERIALS AND METHODS

Reagents. U46619 (9,11-dideoxy,9α-11α-methanepoxy-$PGF_{2\alpha}$), a stable analogue of the prostaglandin endoperoxide PGH_2, was obtained from Cayman Chemical (Denver, CO). The TXA_2/PGH_2 antagonist, I-PTA-OH (9,11-dimethylmethano-11,12-methano-16-(3-indo-4-hydroxyphenyl)-13,14-dihydro-13-aza-15α-$\beta\omega$-tetranor-TXA_2) was obtained through the generosity of Dr Perry Halushka (Medical University of South Carolina, Charleston, SC). The above reagent was stored as stock solutions in ethanol:water (7:3). Dilutions from the stock solutions were made in ethanol:water (1:9). Further dilutions from this stock solution were made in 0.15 M NaCl.

Adenosine diphosphate (ADP), bovine serum

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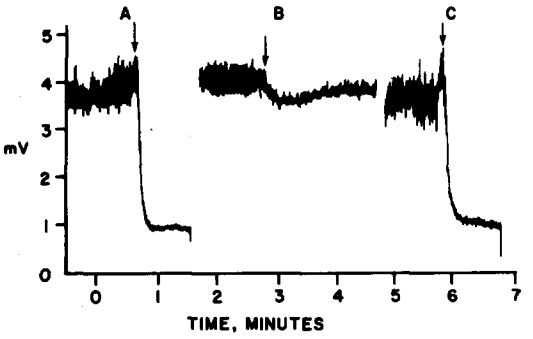

Fig. 1. Effect of preincubation of platelets with chymotrypsin (17 μg/ml) or elastase (7 μg/ml) on the shape change induced by U46619. Aliquots of 480 μl of the suspensions of intact platelets (A), chymotrypsin-treated platelets (B) and elastase-treated platelets (C) were stirred for 45 sec in the aggregometer. U46619 (20 μl) was added at a final concentration of 0.1 μM as indicated by the arrows. The platelet count in each suspension was 3×10^8/ml. The experiment is a representative one of ten similar experiments.

albumin (BSA), glucose, apyrase (grade VII), soybean trypsin inhibitor (SBTI), prostaglandin E_1 (PGE_1), and α-chymotrypsin (Type I-s) or TLCK-chymotrypsin were from the Sigma Chemical Co. (St Louis, MO). Human granulocytic elastase was obtained from Elastin Products, Inc. (Pacific, MO). Urea used for the electrophoresis of the alkaline gels was purchased from Schwarz-Mann (Cleveland, OH). Human thrombin was supplied by Dr J. W. Fenton, III, Albany, NY. All other chemicals were of reagent grade and purchased from Fisher Chemical (King of Prussia, PA).

Isolation of human platelets. Human platelets were isolated from whole blood taken from healthy donors, free of aspirin for at least 1 week prior to donation, who had signed an informed consent in agreement with the Declaration of Helsinki. Blood was collected into acid citrate dextrose (ACD, 1:8). Platelets were then isolated from whole blood using the method of Mustard *et al.* [5]. PGE_1 (0.4 μM) and apyrase (0.0125 units/ml) were added during the isolation, but were omitted from the final suspension of platelets. The final suspension normally contained platelets at a concentration of $3–5 \times 10^8$/ml in a 10 mM *N*-α-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-buffered Tyrode's solution (0.35% bovine serum albumin, 2 mM $CaCl_2$, 3 mM KCl, 0.1 mM $MgCl_2$ and 5 mM glucose), pH 7.4. The addition of BSA was necessary to stabilize the platelets and to maintain their viability over the length of the experiments. In experiments designed to measure myosin light chain phosphorylation, albumin was omitted.

Treatment of washed human platelets with chymotrypsin and elastase. Suspensions of washed platelets (4×10^8/ml) were incubated with TLCK-chymotrypsin or elastase for 45 min at 37°. At the end of the incubation, an equimolar concentration of soybean trypsin inhibitor was added to chymotrypsin-treated platelets and a 5-fold molar excess of

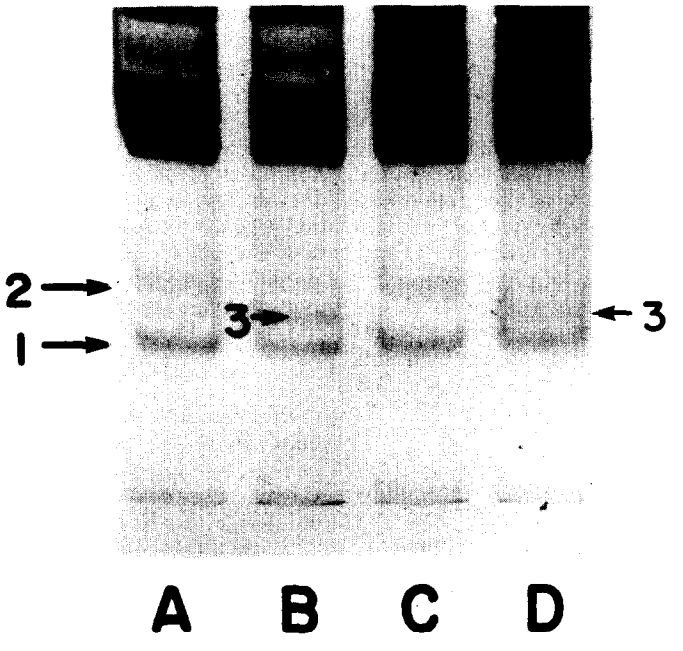

Fig. 2. Effect of elastase treatment of platelets on the phosphorylation of myosin light chain in platelets stimulated with U46619 in alkaline urea gels (A). Unstimulated platelets. (B) Platelets stimulated with U46619. (C) Platelets pretreated with elastase. (D) Platelets pretreated with elastase and then stimulated with U46619. The following bands are indicated by the arrows: (1) 17 kD band, (2) unphosphorylated 20 kD myosin, (3) phosphorylated 20 kD myosin. The experiment is a representative one of three similar experiments.

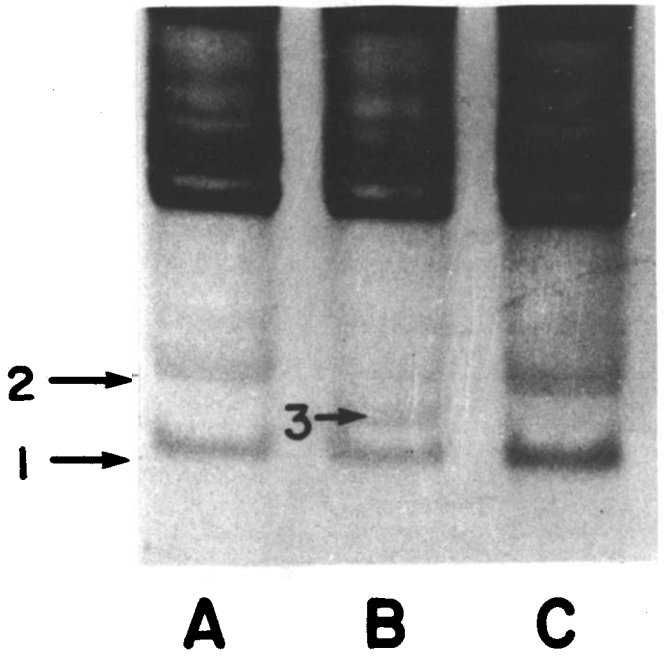

Fig. 3. Effect of chymotrypsin treatment of platelets on the phosphorylation of myosin light chain in platelets stimulated with U46619 in alkaline urea gels. (A) Unstimulated platelets. (B) Platelets stimulated with U46619. (C) Platelets pretreated with chymotrypsin and then stimulated with U46619. For other details, see the legend of Fig. 2. The experiment is a representative one of three similar experiments.

α_1-antitrypsin (supplied by Dr R. Pixley, Thrombosis Research Center, Temple University School of Medicine) was added to neutralize human granulocytic elastase. The platelets were then washed once in Tyrode-albumin buffer, pH 7.4, and resuspended in the same buffer. Platelets were counted electronically in a Coulter counter.

Measurement of platelet shape change induced by U46619. Platelet shape was measured as previously described [1]. Briefly, isolated human platelets (0.48 ml of 3×10^8/ml) were placed into a glass cuvette. A small metal stir bar was added, and the cuvette was placed into a platelet aggregometer (Payton Scientific, Scarborough, Ontario). The sample was then stirred at 1400 rpm and maintained at 37°. After approximately 30 sec, agonist was added (an appropriate volume of 0.15 M NaCl was added prior to agonist in order to adjust the final volume to 0.5 ml). As the platelets in suspension underwent shape change, there was a small decrease in the amount of transmitted light passing through the cuvette. This optical change was monitored with respect to time and the maximal extent of shape change was measured. Previous investigations [1] demonstrated that platelet shape change induced by 0.1 μM U46619 was not altered by the presence of a mixture of creatine phosphate–creatine phosphokinase, suggesting that platelet shape change induced by U46619 is not mediated by ADP. In a number of studies there was a good correlation between the shape change of intact platelets [6, 7] and chymotrypsin-treated platelets [8] detected by scanning electron microscopy and spectroscopic assay. In this study platelet shape change was evaluated only by the optical method.

Table 1. Release of [^{14}C]serotonin from intact and enzyme-treated platelets upon stimulation with 3 μM U46619

Incubation mixture	[^{14}C]Serotonin released (% of total)		
	Intact platelets	Chymotrypsin-treated platelets	Elastase-treated platelets
Platelets + buffer	3.3 ± 0.5	1.1 ± 0.14	3.3 ± 1.7
Platelets + U46619	25.1 ± 8.6	3.3 ± 1.9	5.1 ± 3.7

Suspensions of washed [^{14}C]serotonin-labeled platelets (3×10^8 per ml) were incubated with either chymotrypsin (17 μg/ml) or elastase (7 μg/ml) for 45 min at 37°. Both chymotrypsin- and elastase-treated platelets lost their ability to agglutinate with bovine von Willebrand factor. [^{14}C]Serotonin release was measured in the platelet supernatant fraction, as described in Materials and Methods. The percentage of [^{14}C]serotonin release was calculated in relation to total radioactivity present in the platelet suspension which amounted to 40,000–80,000 cpm/100 μl. Data are means ± SD from three experiments. The mean release of [^{14}C]serotonin from intact platelets stimulated by 2 units of thrombin was 69.8 ± 7.2%.

Myosin light chain phosphorylation. The phosphorylation of myosin light chain was measured by the method of Daniel *et al.* [9]. Platelets, suspended in albumin-free Tyrode's solution, were treated with U46619 to induce shape change. At the time of the maximal response, the reaction was stopped by the addition of an equal volume of 0.6 M perchloric acid.

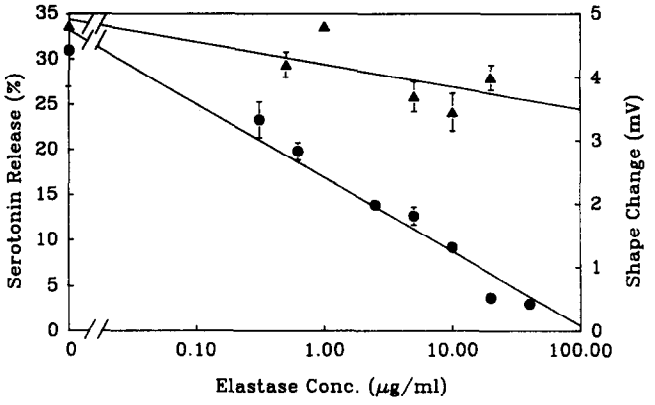

Fig. 4. Effect of preincubation of platelets with various concentrations of elastase on serotonin release (●) and platelet shape change (▲). Platelets were stimulated with 80 nM U46619 to induce shape change and with 22.4 μM U46619 to induce serotonin release. Data are means ± SD of three experiments.

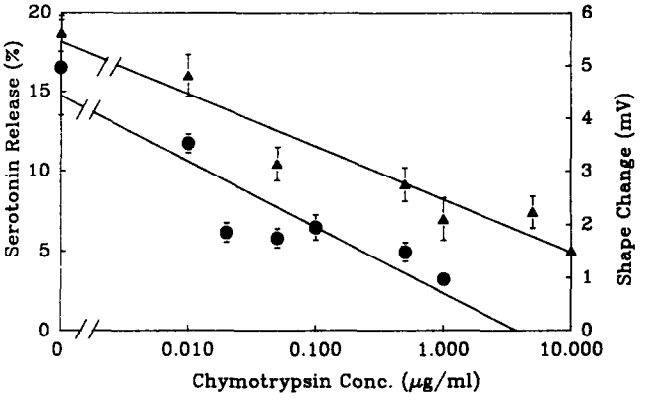

Fig. 5. Effect of preincubation of platelets with various concentrations of chymotrypsin on serotonin release (●) and platelet shape change (▲). For other details, see the legend of Fig. 4.

The pellets were centrifuged and washed with water. The pellets were run on alkaline-urea electrophoresis gels. After staining and destaining of the gels, we identified several bands including the phosphorylated and unphosphorylated 20,000 myosin light chain. The phosphorylated form of the light chain migrated faster than the non-phosphorylated form.

[^{14}C]-*Serotonin release.* [^{14}C]-Serotonin release from stimulated platelets was determined according to the method of Holmsen and Setkowsky-Dangelmaier [10]. After a 10-min stimulation of the platelets by U46619, the suspension (490 μl platelets + 10 μl of agonist) was transferred to an Eppendorf tube containing 100 μl of an ice-cold solution made of 1 vol. 37% formaldehyde, 8 vol. 0.15 M NaCl and 9 vol. of 50 mM EDTA. The samples were then spun in an Eppendorf table top centrifuge for 3 min (10,000 *g*). The supernatant fractions were removed and added to 5 ml of Scintiverse II for counting. A 490-μl sample of unstimulated platelets was added to the solvent for counting of total serotonin present. The percentage of serotonin release was then calculated in relation to total serotonin present.

RESULTS

The purpose of the first series of experiments was to compare the effects of chymotrypsin and elastase on platelet responses to U46619.

Figure 1 shows the effect of incubation of platelets with enzymes on the shape change induced by U46619. Platelets pretreated with elastase (7 μg/ml) and intact platelets showed a similar response to this agonist, whereas platelets preincubated with chymotrypsin (17 μg/ml) showed significantly less shape change upon stimulation with U46619. Stimulation of intact and elastase treated-platelets with U46619 resulted in the phosphorylation of myosin light chain (Fig. 2); however, this phosphorylation did not occur following addition of U46619 to chymotrypsin-treated platelets (Fig. 3). Pretreatment of platelets with elastase (Fig. 2) and with chymotrypsin alone (data not shown) did not result in phosphorylation of myosin light chain. Table 1 shows that preincubation of platelets with low concentration of chymotrypsin resulted in an almost complete inactivation of their capacity to release [^{14}C]serotonin upon stimulation with U46619. Release of serotonin induced by U46619 was inhibited also completely by a 100-fold molar excess of I-PTA-OH (data not shown).

Figure 4 shows the effects of pretreatment of platelets with various concentrations of elastase on the

shape change and serotonin release following stimulation with U46619. Over a wide range of concentrations of this enzyme, there was a gradual decrease of the platelet ability to release serotonin, whereas the ability to respond by shape change was decreased only slightly. On the other hand, pretreatment of platelets with various concentrations of chymotrypsin resulted in a parallel decrease of these responses to U46619 stimulation (Fig. 5).

DISCUSSION

Chymotrypsin and human granylocytic elastase show many similar effects on platelets. Both enzymes degrade glycoprotein Ib, interfere with platelet responses to thrombin and to von Willebrand factor, and expose fibrinogen receptors associated with the glycoprotein IIb/IIIa complex. They do not themselves cause the platelet release reaction [11–14]. In our experimental system, chymotrypsin incubated with human platelets appeared to inactivate the platelet responses to the stable PGH_2 analogue U46619, i.e. shape change, myosin light chain phosphorylation and serotonin release. On the other hand, human granulocytic elastase inactivated selectively the ability of human platelets to release serotonin upon stimulation with U46619, whereas the shape change response and myosin light chain phosphorylation remained unchanged.

The data presented in this paper are in agreement with our previous study [1] in which U46619 showed two different effects on human platelets. Shape change and myosin light chain phosphorylation occurred at low concentrations of U46619 (EC_{50} = 35–57 nM) and correlated with the occupancy of high affinity binding sites for [^{3}H]U46619. On the other hand, serotonin release, platelet aggregation and fibrinogen receptor exposure required higher concentrations of U46619 (EC_{50} = 0.54–1.3 μM). Both the present and the former studies separate two biological effects of this TXA_2/PGH_2 analogue on human platelets.

It is conceivable that the TXA_2/PGH_2 receptor is coupled with two different mechanisms of platelet activation involving either a calmodulin-dependent myosin light chain kinase [6] that leads to myosin light chain phosphorylation and platelet shape change or involving a protein kinase C and P_{47} phosphorylation that leads to serotonin and ADP release from platelet dense granules [15]. Separation of two effects of U46619 on platelets by means of enzyme treatment is compatible either with the existence of two different types of receptors (low and high affinity) or with two different transduction mechanisms. The relationship of these events to the heterogeneity of TXA_2/PGH_2 receptor and the molecular mechanism of the alteration of this receptor by chymotrypsin and by human granulocytic elastase require further studies.

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