Release of a Slow-reacting Substance from Rabbit Platelets

JEAN MICHEL MENCIA-HUERTA, LAHLOU HADJI, and JACQUES BENVENISTE,
Institut National de la Santé et de la Recherche Médicale
U 200, 92140 Clamart-Paris, France

ABSTRACT Washed rabbit platelets stimulated with platelet-activating factor, thrombin, or arachidonic acid, released a slow-reacting substance (SRS), whereas platelets aggregated by adenosine diphosphate did not. Production of platelet-derived SRS was neither affected by indomethacin nor aspirin but was reduced by large doses of eicosatetraynoic acid, an inhibitor of the cyclooxygenase and lipoxygenase. L-cysteine enhanced markedly the release of SRS from platelets. This SRS activity, which was antagonized by FPL 55712 and inactivated by arylsulfatase, followed the same elution pattern on Amberlite, silicic acid, and reverse phase high-pressure liquid chromatography columns as that described for the SRS from other origins. SRS activity released from platelets preincubated with [^{14}C]arachidonic acid exhibited the same retention time as radioactivity in reverse phase high-pressure liquid chromatography. The release of a SRS from platelets is consistent with their implication in the pathogenesis of asthma and other lung diseases.

INTRODUCTION

Intravenous injection of platelet-activating factor—now identified as a 1-O-alkyl-2-acetyl-*sn*-glyceryl-3-phosphorylcholine (PAF-acether)[1] (1–6) into anesthetized guinea pigs causes a marked transient thrombocytopenia, accompanied by hypotension and by bronchoconstriction. PAF-acether-induced bronchoconstriction is unaffected by aspirin but is suppressed by pretreatment of guinea pigs with rabbit antiguinea pig platelet antiserum and prostacyclin, (7). Besides their function in hemostasis and coagulation, platelets play an important role in inflammation, particularly in lung pathology, through the numerous substances that they secrete (8, 9), like vasoactive amines and thromboxane A_2, which are potential inducers of bronchoconstriction. PAF-acether-induced bronchoconstriction is neither blocked by antihistamine and antiserotonin drugs, nor by aspirin and indomethacin, the blockers of the cyclooxygenase pathway. One of the intermediate mediator for the PAF-acether-induced bronchoconstriction could be slow-reacting substance (SRS) (10). However it has not yet been found as originating from platelets. The purpose of our work was to determine whether platelets were capable of releasing SRS under various stimuli and more specifically during PAF-acether-induced aggregation.

[1] *Abbreviations used in this paper*: AA, arachidonic acid; ETYA, eicosatetraynoic acid; LTC, leukotriene C; LTD, leukotriene D; PAF-acether, platelet-activating factor; RP-HPLC, reverse phase high-pressure liquid chromatography; TG, Tyrode's solution containing gelatin; TG-indo, Tyrode's solution containing gelatin and indomethacin; SRS, slow-reacting substance.

METHODS

Preparation of platelets. Washed rabbit platelets were prepared by differential centrifugations, according to our described adapation of Ardlie's method (2, 11). Platelets were washed twice and resuspended in modified calcium-free Tyrode's solution at pH 6.5 containing 10 mM Tris (hydroxymethyl)-aminomethane (E. Merck, Darmstadt, West Germany), and 0.25% gelatin (Difco Laboratories, Detroit, Mich.) (2) Aspirin-treated platelets were prepared by adding 0.1 mM aspirin (Aspegic, Lab. Egic, Amilly, France) between the first and the second centrifugation washes. In some experiments platelets were preincubated for 1 h at 37°C with 2.5 μCi of [^{14}C]arachidonic acid (AA) (Amersham Corp., Buckinghamshire, England) (55.5 mCi/mM) and then washed three times before use. Cells were counted on a hemocytometer and contamination by white cells (essentially lymphocytes) was less than one in 10^6 platelets. In separate experiments, this concentration of leukocytes did not release any SRS.

Release of SRS. Release experiments were carried out in Tyrode's buffer pH 7.4 with added calcium ions (1.3 mM) and gelatin (TG). In some experiments, aspirin-treated platelets were used or indomethacin (Sigma Chemical Co., St. Louis, Mo.) (5 μM) was added to TG (TG-indo). Aspirin or indomethacin-treated platelets did not aggregate in the presence of 10 μM AA. Moreover, thin layer chromatography (plates/silica gel 60 F 254 (E. Merck), solvent, chloroform/methanol/acetic acid/water, 90:8:1:0.8) of the diethyl ether extract from ^{14}C-AA-stimulated platelets confirmed that formation of prostaglandin E_2, prostaglandin $F_{2\alpha}$, and thromboxane B_2 was inhibited by 98% as compared with untreated platelets (data not shown). Aggregating substances were used at doses just below those inducing maximal aggregation: 10 μM ADP, 10 μM AA (Sigma Chemical Co.), hog leukocyte

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PAF-acether purified by silicic acid and high pressure chromatography (0.72 nM) (4). Eicosatetraynoic acid (ETYA) (a gift from B. B. Vargaftig, Institut Pasteur, Paris) was diluted in dimethyl sulfoxide and then to suitable concentrations (1–100 μM) in TG-indo. Equivalent dilutions of dimethyl sulfoxide were used in control experiments. The release experiments were performed with 2.5×10^8 platelets in 1 ml TG or TG-indo. In some instances, 20 mM L-cysteine (Sigma Chemical Co.) was added 150 s before the activating substance (12). After 10-min incubation (except in the kinetics experiments) in a shaking water bath at 37°C, the reaction was stopped by centrifugation at 4°C (1,450 g/10 min), and the supernatants were tested for SRS activity, either immediately or after storage at −20°C. Aggregation was assessed in an aggregometer (Icare, Marseille, France) using 1×10^8 platelets stirred at 1,100 rpm in 300 μl TG or TG-indo.

Determination of SRS activity. SRS activity was assayed according to Stechschulte et al. (13) on a guinea pig ileum immersed in 4 ml Tyrode's solution gassed with 99% O_2 and containing 0.5 μM atropine methyl bromide (ICN K&K Laboratories Inc., Plainview, N. Y.), 1 μM pyribenzamine chloride (ICN), and 0.42 μM methysergide maleate (Sandoz Laboratories, Rueil-Malmaison, France). Contractions were recorded on a physiograph MK3 (Narco Bio-Systems, Inc., Houston, Tex.). 1 U SRS was defined according to Orange et al. (14) as being equivalent to the ileum contraction due to 5 ng histamine (Sigma Chemical Co.) free base. At the doses used in these experiments, the aggregating agents did not induce any ileum contractions.

Characterization of SRS. The reversal of the SRS-induced contracture by the antagonist FPL 55712 (kindly supplied by Dr. P. Sheard, Fisons Laboratories, Loughborough, England) was studied on all SRS samples (15). SRS from 2.5×10^9 platelets in 10 ml TG-indo stimulated by PAF-acether or thrombin was extracted by addition of 3 vol of absolute ethanol at 4°C for 30 min. After evaporation to dryness under vacuum, 50 ml 0.1 N NaOH was added and after 30 min at 37°C the pH of the samples was brought to neutrality by addition of 1 N HCl. SRS was then chromatographed on "preconditioned" (14) amberlite XAD_2 (Serva Feinbiochemica GMBH and Co., Heidelberg, West Germany), bioassayed, and further purified on a silicic acid column following Orange's procedure (14). Amberlite-desalted SRS was also incubated with arylsulfatase type V (Sigma Chemical Co.) as described (16, 17). Briefly, duplicate samples of 20 U of amberlite-desalted SRS in 80% ethanol were evaporated to dryness under nitrogen stream and resuspended in 0.5 ml acetate buffer (10 mM) pH 5.0. One of the samples received 5 U arylsulfatase in 0.5 ml acetate buffer, and the other one was supplemented with the same buffer but without the enzyme. After 1 h at 37°C the samples were directly bioassayed on the guinea pig ileum. In all these experiments, platelet-derived SRS was compared with SRS obtained from rat peritoneal cells stimulated by 1 μg/ml ionophore A 23187 (Calbiochem-Boehring Corp., American Hoechst Corp., San Diego, Calif.) (18). Platelet-derived SRS was pooled from several experiments and further characterized by its retention time on high-pressure liquid chromatography (Waters Associates, Inc., Milford, Mass.) using a reverse phase column (Bondapak C 18) (RP-HPLC). Elution was carried out with two types of methanol-water gradients as in (19, 20). Tritiated prostaglandin F_2 and prostaglandin E_2, [^{14}C]AA (Amersham Corp.), and synthetic leukotrienes C and D (LTC and LTD, respectively) kindly provided by Dr. Rokach (Merck Frost Laboratories, Pointe Claire, Dorval, Quebec, Canada), were used as standards, in the RP-HPLC procedures. The same procedures were applied to the SRS released from [^{14}C]AA-labeled platelets. Radioactivity was measured in a scintillation counter (Intertechnique, Plaisir, France) using lipoluma (Kontron Analytique, Zurich, Switzerland) as scintillation fluid.

RESULTS

Release of SRS activity from platelets. PAF-acether, thrombin, and AA released SRS from aggregated platelets (Table I). In contrast, ADP (10 μM) caused platelet aggregation but there was no release of SRS activity above controls. Higher doses of ADP (100 μM) also failed to liberate SRS from platelets (not re-

TABLE I
Release of SRS from Platelets

Treatment	SRS release*		
	TG	TG-Indo	TG-indo + L-cysteine‡
None	0.11±0.26 (−)	0.45±0.35 (−)	2.61±3.82
PAF-acether	2.72±0.93 (+)§	2.21±0.98 (+)§	19.43±3.72‖
Thrombin	2.56±0.20 (+)§	2.34±1.62 (+)§	28.00±5.26‖
AA	1.48±0.84 (+)§	1.13±1.18 (−)§	14.65±3.47‖
ADP	0.31±0.15 (+)**	0.43±0.26 (+)**	2.91±4.28**

Platelets (2.5×10^8) in 1 ml TG or TG-indo were incubated for 10 min at 37°C with either PAF-acether (0.72 nM), thrombin (0.15 U), AA (10 μM), or ADP (10 μM). In parenthesis is indicated the presence (+) or the absence (−) of platelet aggregation.

* Results are expressed in arbitrary SRS U (14) as the mean±1 SD of five experiments.

‡ L-cysteine (20 mM) was added 150 s before the activating substances.

§ $P < 0.05$, ‖ $P < 0.005$, ** not significant, as compared with control values (t test).

ported in the Table). Indomethacin (5 μM)—a dose that abolished the AA-induced aggregation of platelets—had no effect on the release level of platelet-derived SRS by PAF-acether, thrombin, and AA. Platelets pretreated with aspirin yielded similar amounts of SRS as untreated platelets. Kinetics experiments showed that the release of SRS paralleled aggregation with a maximum at 3–5 min after addition of the activators, and remained constant for at least 15 min (Fig. 1). L-cysteine (20 mM), added 150 s before the activating agents caused by itself a small spontaneous release of SRS and markedly potentiated the SRS release from platelets by PAF-acether, thrombin, or AA (Table I). Large doses of ETYA partially inhibited the release of platelet-derived SRS induced by PAF-acether, thrombin, or AA in TG-indo (Table II).

There are several indications that serotonin was not the guinea pig ileum-contracting substance: (*a*) ileum contraction induced by up to 10 μM serotonin was blocked by the concentration of the specific antagonist methysergide present in the bath fluid, (*b*) the amount of serotonin obtained from 2.5×10^8 lyzed rabbit platelets was unable to contract the ileum. Furthermore, in control experiments using radiolabeled serotonin, we observed that 10% of serotonin remained in the SRS fraction after the amberlite step, out of which only 16% was present after the silicic acid column. At this stage no serotoninlike-contracting activity could be detected in the SRS samples, even using the sensitive rat uterus assay. These data completely rule out serotonin as the contracting agent in our final SRS preparation, (*c*) serotonin exhibited a tachyphylactic effect on the guinea pig ileum that was not observed with platelet-derived SRS.

Identification of platelet-derived SRS activity. In the preceding experiments, the contractions of guinea pig ileum induced by platelet-derived SRS were reversed by FPL 55712 (0.1 μg/ml). Two samples of the amberlite-desalted SRS activity (20 U each) from thrombin-stimulated platelets were inactivated upon incubation with arylsulfatase by 41 and 51% as compared with control tubes incubated without the enzyme. The desalted active material from PAF-acether and thrombin-stimulated platelets was resistant to incubation for 30 min at 37°C in 0.1 N NaOH. It was applied to a silicic acid column that was developed by stepwise addition of eight organic solvents exactly as described (14). SRS activity (either from platelets or peritoneal cells) was eluted only with ethanol/concentrated ammonia/water (6:3:1 vol/vol). Furthermore, platelet and peritoneal cell-derived SRS exhibited similar RP-HPLC elution patterns that were identical to those reported for guinea pig lung in (19) (Fig. 2). Fig. 3 (top) shows the elution pattern of platelet-derived SRS chromatographed on RP-HPLC as in (20). In this system of elution the retention times of the standards were prostaglandin F_2:38 min, prostaglandin E_2:40 min, LTC:46 min, LTD:54 min, AA:94 min. It is clear that platelet SRS comigrated with LTC and LTD standards. However, when SRS was obtained from [^{14}C]AA-labeled platelets, radioactivity was eluted in RP-HPLC as a single peak the retention time of which (54 min) coincided with one peak of SRS activity.

TABLE II
Effect of ETYA on the Release of SRS from Platelets

ETYA M	Release of SRS		
	PAF/acether	Thrombin	AA
none	3.13±0.51*	2.76±0.31	1.81±0.31
10^{-4}	1.45±0.18	1.46±0.24	0.79±0.12
10^{-5}	2.20±0.23	2.28±0.16	1.20±0.23
10^{-6}	3.05±0.63	2.85±0.22	1.63±0.36

Platelets (2.5×10^8) were incubated in 1 ml TG-indo with or without ETYA which was added immediately before the activators (doses as in Table I).
* Results are expressed in SRS U as the mean±1 SD of three experiments.

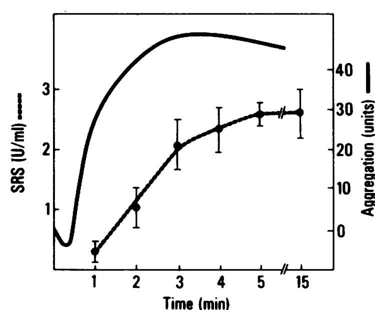

FIGURE 1 Kinetics of platelet-derived SRS as compared to platelet aggregation (mean±1 SD of five experiments).

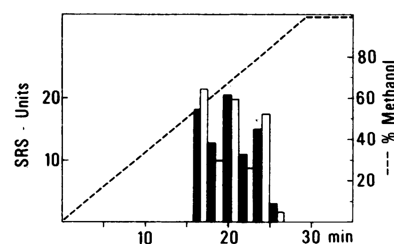

FIGURE 2 Elution pattern of platelet-derived SRS (dotted bars) and SRS from peritoneal cells (open bars) on RP-HPLC. Elution was carried out with a continuous methanol-water gradient (19).

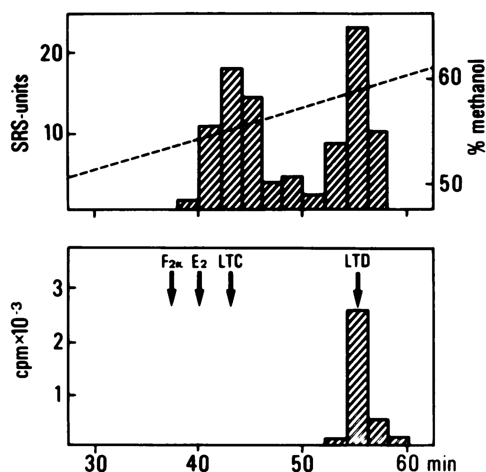

FIGURE 3 RP-HPLC elution pattern (20) of platelet-derived SRS. Top, retention time as detected by ileum contracting activity. Bottom, retention time of radiolabeled SRS.

DISCUSSION

SRS released by rabbit platelets upon challenge with PAF-acether, thrombin, and AA shared the characteristics of SRS from other origins (12–21), following the same RP-HPLC retention time pattern as the SRS from rat peritoneal cells for both the elution systems used (19, 20).

SRS release from platelets was slow as compared with that of serotonin induced by PAF-acether, which is 55% complete within 10 s (22). This lag period was consistent with a *de novo* synthesis, as reported for SRS from human lung (23). The kinetics of release, however, were compatible with the involvement of platelet-derived SRS in anaphylactic reactions. Extrapolating from results of Orange and Moore (12), the amount of SRS obtained from platelets was low as compared with that from lung or peritoneal cells. Indeed, in our bioassay 2.5×10^6 rat peritoneal cells released 32 ± 6 SRS U by action of 1 μg/ml ionophore. However, this low yield might be compensated in vivo by the high number of blood platelets. Moreover, we used concentrations of the platelet-stimulating agents inducing submaximal aggregation, and high doses of the activators could increase the yield of SRS. This release was potentiated by L-cysteine, in agreement with the results for SRS from peritoneal cells and lungs (12, 24). However, L-cysteine has also been shown to potentiate the release of thromboxane A_2 from rabbit platelets (25), raising the question whether L-cysteine has a general effect on platelet metabolism or acts more directly as a precursor for the SRS molecule (26).

Several peaks of activity were detected after RP-HPLC. They could be due to storage in deep freeze, an effect that has already been observed (19). However, SRS appears to belong to several molecular species derived from AA (27–32). Furthermore, SRS-like substances have been obtained from eicosatrienoic acid and eicosapentaenoic acid (33). Its chromatographic characteristics indicate that the SRS activity recovered from stimulated platelets is also a mixture of several related molecules, comigrating with LTC and LTD. The precise structure of the platelet-derived SRS remains to be elucidated because it has been previously established that platelets form only 12-hydroperoxy-eicosatetraenoic acid metabolites (34). However, given the high specific biological activity of LTC or LTD, it could be possible that platelets also form trace amounts of 5-hydroperoxy-eicosatetraenoic, undetectable by conventional methods, which are then converted into SRS. Interestingly Parker et al. (32) have reported the formation by rat basophilic leukaemia cells of a 5-hydroxy,12-glutathionyl SRS molecule. Thus AA metabolites with different position of the amino acid moiety than LTC and LTD could exhibit biological activity similar to that of the latter compounds. Further identification of the platelet-derived SRS is underway in our laboratory. This should permit us to explain why only the substance eluted from RP-HPLC as LTD was labeled when platelets were previously loaded with [^{14}C]AA.

SRS release by PAF-acether, AA, or thrombin-stimulated platelets appears to be unrelated to the cyclooxygenase pathway, because a concentration of indomethacin or aspirin that suppressed platelet aggregation by AA and formation of cyclooxygenase products was unable to inhibit SRS formation. Furthermore, RP-HPLC retention time of the prostaglandins was different from that of the major peaks of SRS. The increment in SRS production by indomethacin has been reported (26, 35, 36). However this was not observed with the concentration of indomethacin that we used in the platelet system. This result may be due to a difference in indomethacin concentration or to a peculiarity of platelet SRS formation. In contrast, the inhibition of ETYA suggests that involvement of the lipoxygenase pathway in the release of SRS from platelets. However the required concentrations of ETYA were above those used by others (17, 31), raising the question of the specificity of this inhibition.

PAF-acether is released from alveolar macrophages from various mammalian species, including man, during phagocytosis of insoluble particles (37–39) or hyperimmunized rats upon antigenic challenge (40). PAF-acether is a mediator of the allergic reaction because it is released from IgE-sensitized rabbit basophils (2). Moreover, its injection into guinea pigs induces platelet-dependent, noncyclooxygenase-medi-

ated bronchoconstriction (7). In this paper we have presented proof that PAF-acether, AA, or thrombin release SRS from rabbit platelets. Lung SRS has been described in various species, making it likely that our present findings will also be confirmed in platelets from guinea pig and man. PAF-acether from alveolar macrophages could thus be implicated as an indomethacin-resistant bronchoconstriction inducer through the release of SRS by platelets. Although we still lack direct demonstration for the *in situ* release of SRS from PAF-acether-stimulated platelets aggregating in lung vessels, the platelet-derived SRS may be viewed as instrumental in the onset of asthma and other lung diseases implicating platelets.

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