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The Ca^{2+}-mobilizing potency of α-thrombin and thrombin-receptor-activating peptide on human platelets

Concentration and time effects of thrombin-induced Ca^{2+} signaling

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In single platelets and in suspensions of platelets, α-thrombin evokes dose-dependent, transient increases in cytosolic Ca^{2+} concentration, $[Ca^{2+}]_i$, which are more prolonged than the $[Ca^{2+}]_i$ transients evoked by other platelet agonists such as the thrombin-receptor-activating hexapeptide SFLLRN, thromboxane A_2 analog U46619, and ADP. As a quantity taking into account both the magnitude and length of the Ca^{2+} response, we defined the Ca^{2+}-mobilizing potency (CMP) of an agonist as the integrated rise in $[Ca^{2+}]_i$ during the time of the Ca^{2+} signal. It was observed that: (a) the CMP increased with the agonist concentration in a saturating way, its maximal value being about four-times higher with α-thrombin than with SFLLRN; (b) the high CMP of α-thrombin was for only a small part due to endogenous production of ADP or thromboxane, and was mainly a consequence of prolonged influx of external Ca^{2+}; (c) the CMP declined when α-thrombin was inactivated during the course of the Ca^{2+} signal; (d) CMP values increased with the agonist concentration upon sequential addition of increasing amounts of α-thrombin or SFLLRN; (e) when α-thrombin was gradually added to the platelets or formed by an *in situ* reconstituted prothrombinase system (with factor Xa, factor Va, and prothrombin), integrated Ca^{2+} responses were a function of the product of the α-thrombin concentration and the time of its presence. However, in these cases, the final CMP values were independent of the rate of α-thrombin addition or formation. We conclude that α-thrombin-induced Ca^{2+} signals in platelets rely largely upon Ca^{2+} influx, are not, or only slightly, subjected to homologous desensitization, and reflect the enzymatic capacity of α-thrombin to cleave protease-activated receptors. Thus, the high and prolonged Ca^{2+} signal induced by α-thrombin is due to continuous receptor cleavage without desensitizing effects of previously cleaved receptors.

Keywords: calcium mobilization; platelets; protease-activated receptor; α-thrombin; thrombin-receptor-activating peptide.

α-Thrombin is a potent platelet-activating agonist that is locally formed at hemostatic sites in the blood stream as a result of the coagulation process. In flowing blood, platelets are likely to be exposed to only short-lasting waves of this agonist because, once produced, α-thrombin is almost immediately inactivated by anti-thrombins in plasma with a velocity proportional to its concentration [1]. Accordingly, at hemostatic sites, both the local α-thrombin concentration and the time during which it is active may determine the activating effect on platelets.

The identity of the functional α-thrombin receptors on platelets is still debated. In 1991, a tethered ligand-linked receptor for α-thrombin was identified, which is now designated as the protease-activated receptor type 1. After cleavage by α-thrombin, this receptor activates itself through the newly exposed N-terminal ligand [2]. On the basis of experiments with synthetic thrombin-receptor-activating peptides such as SFLLRN, which bind to this receptor in the absence of proteolysis, it was initially concluded that the tethered ligand receptor mediates many or all of the effects of α-thrombin on human platelets [3–5]. However, later studies showed that these mimicking peptides were often less active than α-thrombin itself [6–9], suggesting that other α-thrombin receptors may also exist. Experiments with antibodies against glycoprotein Ib and with platelets from Bernard-Soulier patients, deficient in this glycoprotein, have suggested that (subsets of) glycoprotein Ib may act as high-affinity receptors for α-thrombin [10, 11]. On the other hand, it has been shown that the platelet-stimulating effect of α-thrombin is unrelated to the equilibrium binding of α-thrombin to platelets, but is solely due to its proteolytic effect on (receptor) substrates on the platelet membrane [12]. This would imply that not only the α-thrombin concentration, but also of the time of its action determines the degree of platelet activation. A second type of protease-activated receptor for α-thrombin has recently been cloned from human tissue [13], although it is still unclear whether this receptor is functional in platelets.

Regardless of the identity of the platelet receptors involved, α-thrombin is known to be a potent stimulator of phospholipase C-β and -γ isoforms, causing substantial and prolonged formation of inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] [3, 14–17] and a high increase in cytosolic $[Ca^{2+}]_i$ [9–11, 18–20]. α-Throm-

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Abbreviations. $[Ca^{2+}]_i$, cytosolic Ca^{2+} concentration; CMP, Ca^{2+}-mobilizing potency; Ins(1,4,5)P_3, inositol 1,4,5-trisphosphate; U46619, (5*Z*,9$_{\alpha}$,13*E*,15*S*)-9,11-(epoxymethano)pros-5,13-dien-1-oic acid.

Enzymes. Apyrase (EC 3.6.1.5); coagulation factor Xa (EC 3.4.21.6); phospholipase C (EC 3.1.4.3); thrombin (EC 3.4.21.5).

bin-evoked increases in inositolphosphates [3] and $[Ca^{2+}]_i$ [8, 18] are inhibitable by post-addition of thrombin active-site inhibitors, suggesting that the continuous presence of enzymatic α-thrombin is required for generation of these second messengers. In studies with transfected Rat 1 fibroblasts, equipped with labeled, recombinant protease-activated receptors for α-thrombin, it was observed that the rate and extent of second messenger generation in response to α-thrombin was related to the rate of proteolytic cleavage of the thrombin receptors [21]. Although the situation in platelets with several types of native thrombin receptors is likely to be more complex, the fibroblast model rightly points to the existence of both an α-thrombin concentration-dependent and time-dependent component in Ins(1,4,5)P_3 formation and Ca^{2+} mobilization. In agreement with this, we have earlier shown that individual platelets, when activated with α-thrombin, show spiking variations in $[Ca^{2+}]_i$ that are dose-dependent and often of prolonged duration [22, 23].

Taken together, in coagulating plasma with varying concentrations of α-thrombin, two time-dependent processes may play a role in α-thrombin-induced Ca^{2+} mobilization: (a) the duration of the exposure of platelets to α-thrombin, and (b) the length of the Ca^{2+} signal elicited by a certain concentration of α-thrombin at a certain time. To study the relevance of the time component, we measured the Ca^{2+} responses of platelets that were exposed to variable concentrations of α-thrombin or SFLLRN. The α-thrombin concentration was changed here by either continuous infusion of preformed α-thrombin or by gradual generation of α-thrombin using a reconstituted prothrombinase complex, consisting of factor Xa, factor Va and prothrombin [24, 25]. The Ca^{2+}-mobilizing potency (CMP) of α-thrombin or other agonists was then defined as the time integral of the rise in $[Ca^{2+}]_i$ over the time period where the agonist was active. The results show that α-thrombin has a high CMP in comparison to SFLLRN and other agonists. They also indicate that the α-thrombin-evoked Ca^{2+} signal, at any time, is essentially determined by the α-thrombin concentration at that time. However, the integrated Ca^{2+} signal (CMP) appears to be determined by the product of α-thrombin concentration and time of its action, suggesting this parameter reflects the extent of proteolytic cleavage of α-thrombin-degradable receptors.

EXPERIMENTAL PROCEDURES

Materials. Human prothrombin and α-thrombin (activity >3000 NIH U/mg protein; 1 U/ml equivalent to 11.4 nM) and clotting factor Xa and factor Va were purified to homogeneity, as described elsewhere [26, 27]. Human α-thrombin of the same purity was also bought from Sigma. ADP, apyrase, fibrinogen, dioleoylglycerophosphocholine, dioleoylglycerophosphoserine, prostaglandin E_1, and the thromboxane A_2/prostaglandin H_2 analog (5*Z*,9$_\alpha$,13*E*,15*S*)-9,11-(epoxymethano)pros-5,13-dien-1-oic acid (U46619) were also from Sigma. The thrombin-receptor-activating hexapeptide, SFLLRN, which was purified to homogeneity by HPLC, was obtained from IHB (Academic Hospital Leiden, The Netherlands). Human antithrombin III was purchased from Enzyme Research Laboratories; recombinant human hirudin was from Ciba-Geigy; FPR chloromethylketone (H-Phe-Pro-Arg chloromethylketone) was from Calbiochem. Fura-2 acetoxymethyl ester was bought from Molecular Probes. The α-thrombin-cleavable chromogenic substrate S2238 was from Chromogenix. Other chemicals were reagent grade and were obtained from sources as described before [19].

Preparation of platelets and loading with fura-2. Platelet-rich plasma was prepared from blood freshly obtained from healthy donors, who had not taken medication during the preceding two weeks [23]. Platelets in plasma were incubated with 3 μM fura-2 acetoxymethyl ester at 37°C for 45 min. Lysine acetyl salicylate (aspirin) was added at a concentration of 100 μM, where indicated. The platelets were then sedimented by centrifugation at 630 *g* for 15 min, washed twice in the presence of apyrase (0.1 U ADPase/ml), and resuspended in buffer A, pH 7.45 (37°C), containing 136 mM NaCl, 10 mM glucose, 5 mM Hepes, 2.7 mM KCl, 2 mM $MgCl_2$, and 0.05% (mass/vol.) bovine serum albumin [28]. Suspensions were adjusted to 1×10^8 platelets/ml.

Activation of platelets in suspension. Washed, fura-2-loaded platelets (2×10^8/2 ml buffer A) were slowly stirred in a cuvette placed in a thermostated (37°C) spectrofluorometer. Where indicated, α-thrombin or other agonists were added from concentrated stock solutions. In other experiments, α-thrombin or SFLLRN was slowly infused into the stirred platelet suspension. The flow was generated by a motor-driven pump (Harvard Apparatus, Vel, Belgium) pushing the piston of a 100-μl siliconized, pressure-lock syringe filled with a solution of agonist in buffer A, kept at 37°C. In some experiments, α-thrombin was generated from prothrombin *in situ*. In this case, fura-2-loaded platelets (2×10^8/2 ml) were incubated with 2 mM $CaCl_2$ and the desired concentrations of purified coagulation factors Va and Xa under slowly stirring at 37°C. Prothrombinase activation was started by the addition of 0.5 μM prothrombin.

Activation of single, immobilized platelets. Fura-2-loaded platelets were immobilized on fibrinogen-coated glass coverslips in the presence of 10 μM RGDS, apyrase (0.2 U ADPase/ml), and 0.1 μM prostaglandin E_1, as described before [23, 29]. The bound platelets were bathed in 0.5 ml buffer A supplemented with 10 μM RGDS, apyrase (0.2 U ADPase/ml), and $CaCl_2$ (2 mM) at room temperature. Under these conditions, >95% of the platelets immobilized on fibrinogen remained low in $[Ca^{2+}]_i$ for at least 10 min. Agonists and antagonists were given in volumes of 0.1 ml from concentrated stock solutions in bathing medium. Thrombin was generated *in situ* by the addition of 0.24 nM factor Va, 0.1 nM factor Xa, 0.5 μM prothrombin, and 2 μM phospholipid vesicles composed of 9:1 (mol/mol) phosphatidylcholine/phosphatidylserine.

Measurements of cytosolic $[Ca^{2+}]_i$. In suspensions of fura-2-loaded platelets, changes in $[Ca^{2+}]_i$ were measured in a thermostated (37°C) cuvette chamber using a Shimadzu RF-5001PC spectrofluorometer, basically as described before [28]. Briefly, samples were alternately illuminated with 340-nm and 380-nm light passing from an excitation monochromator (bandpasses of 5 nm), and the fluorescent light of 510 nm was recorded after background subtraction. Ratio values of fluorescence at 340 nm/380 nm excitation were obtained 30−120 times/min. The calibration of fluorescence ratio to values of $[Ca^{2+}]_i$ was according to Grynkiewicz et al. [30].

In single cell experiments, changes in fura-2 fluorescence were measured as usual [23, 31], except that a Quanticell fluorometric video imaging system was employed (Applied Imaging, Sunderland, Tyne & Wear, UK). Excitation light was passed alternately through filters of 340 nm and 380 nm wavelengths, and the fluorescence at 505 nm was observed by an intensified charge-coupled device camera (Photonic Sciences, Robertsbridge, Sussex, UK), working at standard video rate and connected to an inverted microscope. Fluorescence images were digitalized and averaged, background images were subtracted, and averaged ratio images (obtained every 2 s) were stored onto a 650-MByte read/write optical disk. Geometric regions matching individual cells were defined and analyzed for changes in the fluorescence ratio. Calibration of 340 nm/380 nm fluorescence ratio values to $[Ca^{2+}]_i$ was not performed, because of the

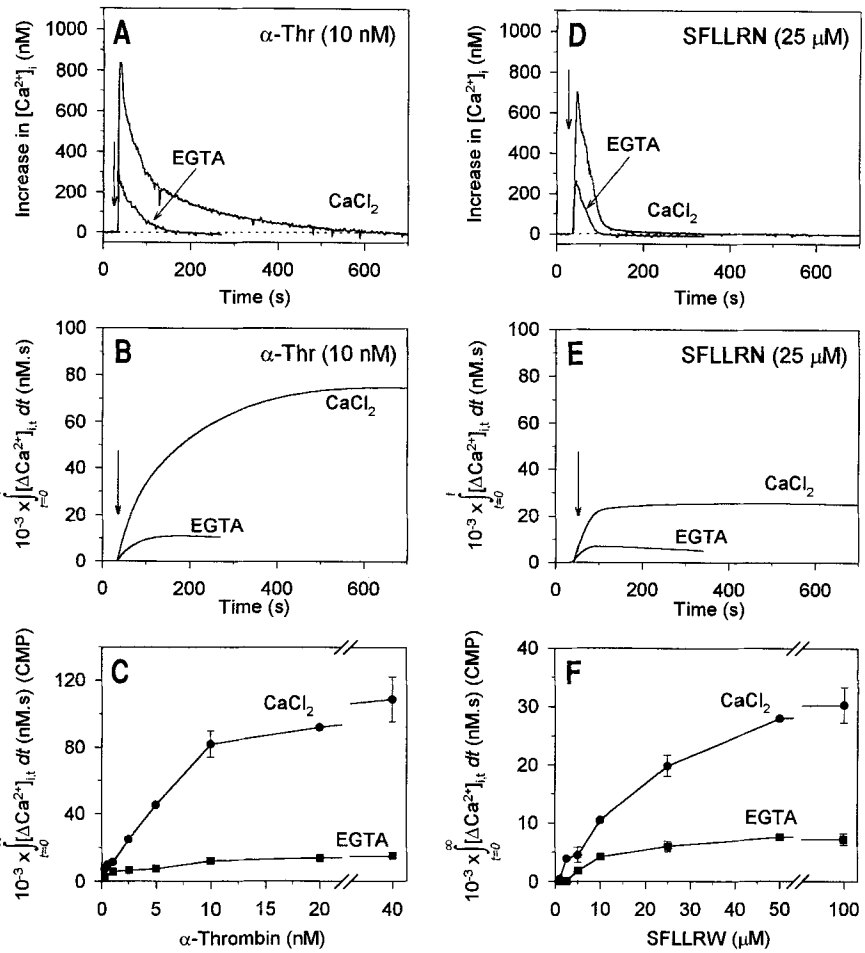

Fig. 1. Dose-dependent effect of CMP of α-thrombin and SFLLRN. Fura-2-loaded platelets in suspension (2×10^8/2 ml) were activated with 10 nM α-thrombin (α-Thr) (A, B) or 25 μM SFLLRN (D, E) in the presence of 1 mM $CaCl_2$ or 1 mM EGTA. (A, D) Traces are given of changes in cytosolic $[Ca^{2+}]_i$, after correction for the leakage of fura-2 dye out of the platelets. (B, E) Time plots are constructed of areas under the $[Ca^{2+}]_i$/time curves by determining the integral, $\int\Delta[Ca^{2+}]_{i,t}dt$. The CMP is the value reached by this integral at the end of the Ca^{2+} response. Data are representative for at least three experiments. (C, F) Dose-dependency of CMP of α-thrombin and SFLLRN in the presence of $CaCl_2$ or EGTA. Data are mean values ± SE (n = 3).

small and variable amount of fluorescence per cell and the rapid bleaching of the fluorescent signals [23].

Determination of the Ca^{2+}-mobilizing potential. The CMP of a platelet agonist was defined as the integral of the increase in $[Ca^{2+}]_i$ over the time period where the agonist is active, $\int\Delta[Ca^{2+}]_{i,t}dt$. To calculate this parameter for experiments with fura-2-loaded platelets in suspension, time plots were constructed of agonist-evoked increases in $[Ca^{2+}]_i$ relative to basal $[Ca^{2+}]_i$ (i.e. the level before addition of agonist). These plots were corrected for leakage of fura-2 and bleaching of the dye, by subtracting the time-dependent, apparent changes in basal $[Ca^{2+}]_i$, as observed in control incubations without agonist (about 4 nM Ca^{2+}/min). Complete return of $[Ca^{2+}]_i$ at the end of incubations with agonists was checked, if necessary, by the addition of prostaglandin E_1, which activates Ca^{2+} extrusion from the cytosol, but does not influence basal $[Ca^{2+}]_i$. Failure to return to basal $[Ca^{2+}]_i$ was in most cases due to lysis of some of the platelets, as was verified by measuring the release of lactate dehydrogenase [28].

For the Ca^{2+} responses of single fura-2-loaded platelets, time integrals of increases in 340 nm/380 nm fluorescence were assessed without correction for leakage of probe or fluorescence quenching.

Measurement of α-thrombin activity. In incubations where α-thrombin was added or generated, the concentration of proteolytically active α-thrombin was determined by taking samples of 50 μl and measuring the chromogenic activity using the α-thrombin substrate, S2238 [31, 32].

RESULTS

Factors determining the CMP of α-thrombin and SFLLRN. In suspensions of fura-2-loaded platelets, agonists for GTP-binding-protein-coupled receptors such as α-thrombin, thrombin-receptor-activating peptide SFLLRN, thromboxane A_2 analog, U46619, and ADP evoked transient rises in cytosolic $[Ca^{2+}]_i$, which finally returned to basal levels. Under some conditions, e.g. rapid stirring at 1000 rpm or with micromolar concentrations of U46619, $[Ca^{2+}]_i$ did not completely return to this basal level. In these cases, measurements of the release of lactate dehydrogenase indicated lysis of 5–10% of the platelets. Since under most conditions, however, platelet agonists evoked essentially terminating Ca^{2+} signals, it was possible to determine the accumulative rises in $[Ca^{2+}]_i$ during the response, i.e. the time integral $\int\Delta[Ca^{2+}]_{i,t}dt$. The CMP of a certain agonist was subsequently defined as the maximal value reached by this integral at the end of the response. It should be noted that calculation of the CMP required correction for leakage of fura-2 dye out of the platelets and, if applicable, for lysis of platelets (see Experimental Procedures section).

Table 1. CMP of saturating doses of platelet agonists in the presence and absence of extracellular $CaCl_2$. Fura-2-loaded platelets (2×10^8/2 ml) were activated with α-thrombin (40 nM), SFLLRN (100 μM), ADP (20 μM) or U46619 (1 μM) in the presence of 1 mM EGTA or 1 mM $CaCl_2$. Incubations were carried out with aspirin-treated platelets in the presence of apyrase (0.1 U ADPase/ml), where indicated (ASA/APY). Areas under the recorded $[Ca^{2+}]_i$/time curves were determined to obtain CMP values. Data are mean values ± SE (*n*) of 3–5 experiments with platelets from different blood donors. n.d., not determined.

Agonist	CMP with $CaCl_2$	CMP with EGTA
	nM · s	
α-Thrombin	109300 ± 13200 (5)	15100 ± 300 (5)
α-Thrombin + ASA/APY	94000 ± 5200 (5)	n.d.
SFLLRN	30000 ± 300 (5)	7200 ± 1000 (5)
ADP	9000 ± 200 (3)	4100 ± 400 (3)
U46619	8300 ± 1000 (3)	4500 ± 600 (3)

We first studied several factors determining the CMP of α-thrombin and SFLLRN. In the presence of extracellular $CaCl_2$, the increase in $[Ca^{2+}]_i$ evoked by 10 nM α-thrombin was much higher and more prolonged than with extracellular EGTA (Fig. 1A). Measurements of the $[Ca^{2+}]_i$ time integrals gave CMP values that were about seven-times higher when external $CaCl_2$ was present (Fig. 1B). Determination of the CMP at various α-thrombin concentrations resulted in dose/effect plots that saturated at about 40 nM α-thrombin both in the presence and absence of $CaCl_2$ (Fig. 1C). The peptide SFLLRN elicited Ca^{2+} responses of much shorter duration than those with α-thrombin (Fig. 1D). Accordingly, this resulted in relatively low CMP values even at a saturating concentration of 100 μM SFLLRN (Fig. 1E–F). In platelets from five different donors, the maximal CMP with high α-thrombin was on average 109000 nM · s and 15100 nM · s in the presence of external $CaCl_2$ and EGTA, respectively, while that with high SFLLRN was 30000 nM · s and 7000 nM · s (Table 1). Typically, especially with α-thrombin, considerable differences were found in the CMP of platelets from various individuals.

To investigate the contribution of endogenously released ADP and thromboxane A_2 to the high and prolonged Ca^{2+} signal with α-thrombin/$CaCl_2$, we measured the CMP in the presence of ADP-degrading apyrase with platelets treated with the cyclooxygenase inhibitor, aspirin. This resulted in a small reduction of the CMP of about 15% (Table 1). Thus, the high CMP of α-thrombin was not primarily caused by Ca^{2+} signals evoked by released ADP or thromboxane. This was confirmed by the observation that even saturating doses of U46619 (1 μM) or ADP (20 μM) elicited short $[Ca^{2+}]_i$ transients with low CMP values (Table 1).

To determine whether the sustained Ca^{2+} influx-driven response of α-thrombin is detectable in individual platelets, we measured the Ca^{2+} responses of single platelets immobilized on fibinogen in the presence of RGDS and apyrase to prevent spontaneous (i.e. fibrinogen adhesion-dependent) activation [23, 29]. In the absence of agonist, 97% of the bound platelets remained low in $[Ca^{2+}]_i$, and many responded to 0.5 nM α-thrombin (71%) or 50 μM SFLLRN (85%) by repetitive spiking in $[Ca^{2+}]_i$, when external $CaCl_2$ was present. The duration of the spiking signal was in these cells much longer with α-thrombin than with SFLLRN (Fig. 2), which is in agreement with the cell suspension experiments.

The CMP at varying concentrations of α-thrombin or SFLLRN. There is good evidence that the continuous presence of α-thrombin is required to obtain a full Ca^{2+} response in platelets [18]. In agreement with this, we observed with platelets in suspension that α-thrombin-evoked Ca^{2+} signals were immediately abrogated upon addition of the active-site inhibitors antithrombin III (1 μM) or FRP chloromethylketone (0.2 μM). When added at 1 min after α-thrombin, these inhibitors reduced the remaining CMP by 90.6 ± 2.6% and 88.3 ± 2.0%, respectively (mean ± SE, $n = 3$).

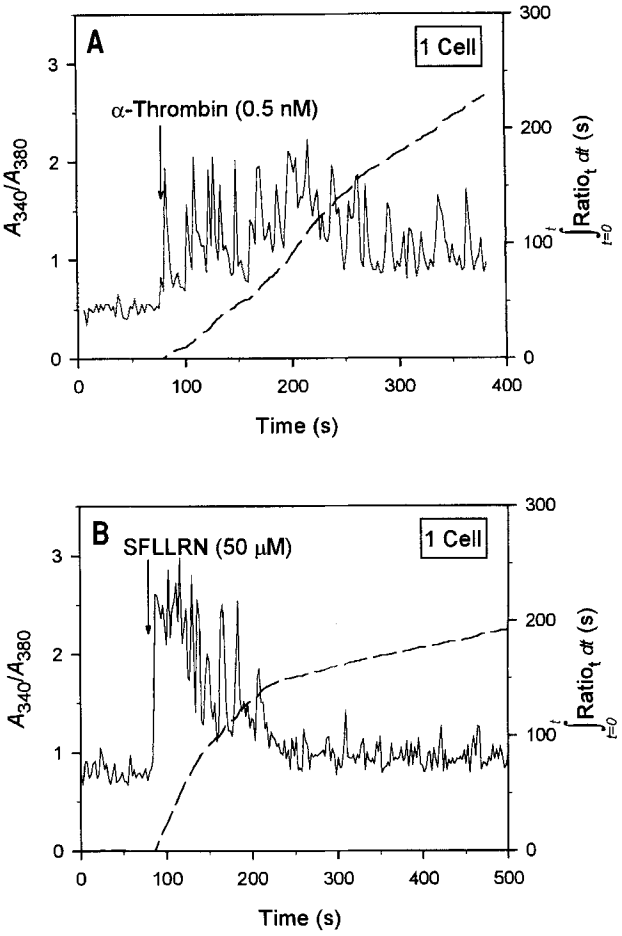

Fig. 2. Duration of Ca^{2+} response evoked by α-thrombin or SFLLRN in single platelets. Fura-2-loaded platelets were immobilized and activated with 0.5 nM α-thrombin or 50 μM SFLLRN in the presence of 1 mM $CaCl_2$, as indicated. Changes in the 340 nm/380 nm fluorescence ratio (———) are given, indicative of changes in $[Ca^{2+}]_i$, of one platelet representative of > 50 platelets. Time integrals of accumulative increases in fluorescence ratio (- - -) are also shown.

We then questioned whether platelets may also respond to *increasing* concentrations of α-thrombin or SFLLRN. In a first set of experiments, the platelets were stimulated with a low dose of 1 nM α-thrombin that was followed by a higher dose of 10 nM (Fig. 3A). This resulted in CMP values that were a factor of 8.3 ± 0.4 (mean ± SE, $n = 3$) higher with the high concentration. Similar results were obtained upon sequential addition of 2.5 μM and 25 μM SFLLRN (Fig. 3B). In other experiments, α-thrombin was gradually added to the platelets using a motor-driven perfusion pump. α-Thrombin infusion at a low rate of 1.5 nM/min resulted in a lower but more prolonged increase in $[Ca^{2+}]_i$ than infusion at 4.0 nM/min (Fig. 3C and D). Nevertheless, CMP values at the end of the experiment were almost identical under either condition, i.e. 101000 ± 4600 nM · s and 108100 ± 7300 nM · s, respectively (mean ± SE, $n = 3$ individuals). Within each experiment with platelets from the same do-

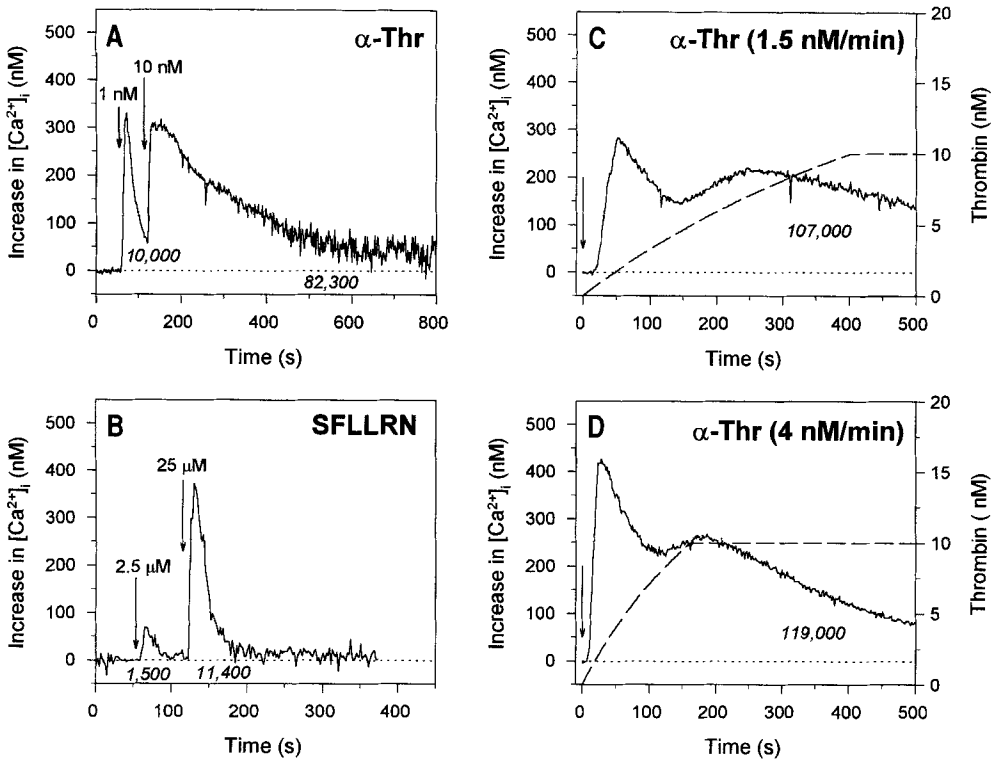

Fig. 3. Effect of increasing concentrations of α-thrombin or SFLLRN on Ca^{2+} responses in platelets in suspension. (A, B) Suspensions of fura-2-loaded platelets (2×10^8/2 ml) were activated in the presence of 1 mM $CaCl_2$ by rapid mixing with successively 1 nM and 10 nM α-thrombin (α-Thr), or with 2.5 μM and 25 mM SFLLRN, as indicated. (C, D) Platelet suspensions containing 1 mM $CaCl_2$ were mixed with α-thrombin that was gradually added at a rate of 1.5 nM/min (C) or 4 nM/min (D). Continuous lines give changes in cytosolic $[Ca^{2+}]_i$, after correction for the leakage of fura-2 dye out of the platelets. Discontinuous lines represent changes in α-thrombin concentration. Numbers in italic are CMP values at the end of the Ca^{2+} response (in nM · s). Data are representative for 3–5 independent experiments.

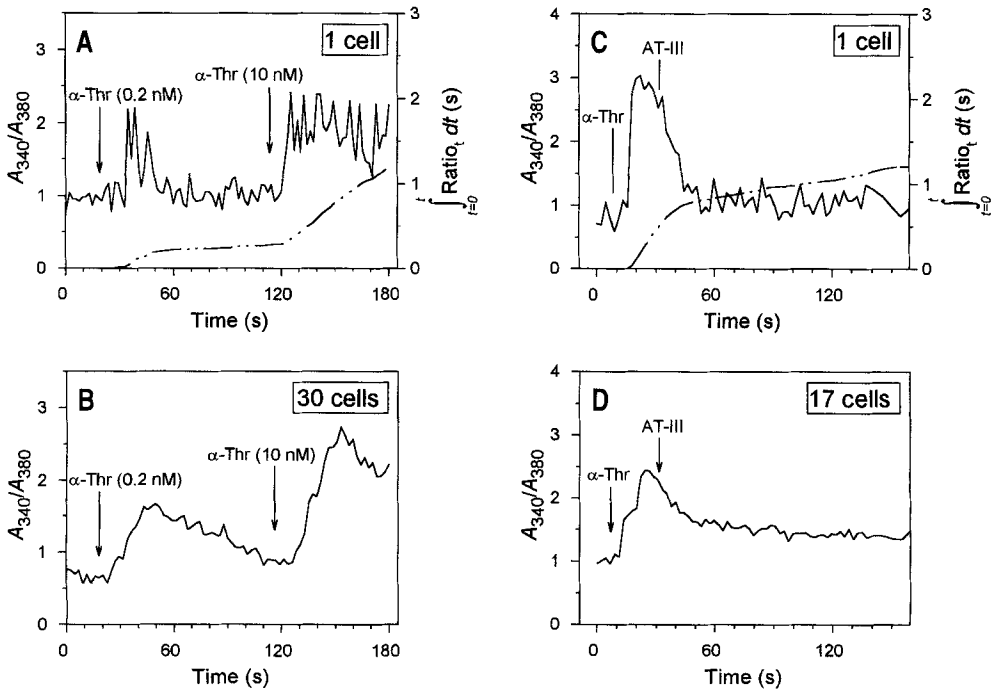

Fig. 4. Effect of increasing or decreasing concentration of α-thrombin on Ca^{2+} responses in single platelets. (A, B) Fura-2-loaded platelets immobilized on fibrinogen were activated with 0.2 nM and 10 nM α-thrombin (α-Thr) in the presence of 1 mM $CaCl_2$, as indicated. (C, D) Immobilized platelets were stimulated with 10 nM α-thrombin in the presence of 1 mM $CaCl_2$, after which 1 μM antithrombin III (AT-III) was added. Similar results were obtained when 1 μM hirudin was used instead of antithrombin III. Traces represent changes in the 340 nm/380 nm fluorescence ratio (——) or time integrals of accumulative increases in fluorescence ratio (– · · –) of individual cells (A, C), or are averaged data from 17 to 30 cells (B, D). Data are representative for three or more experiments.

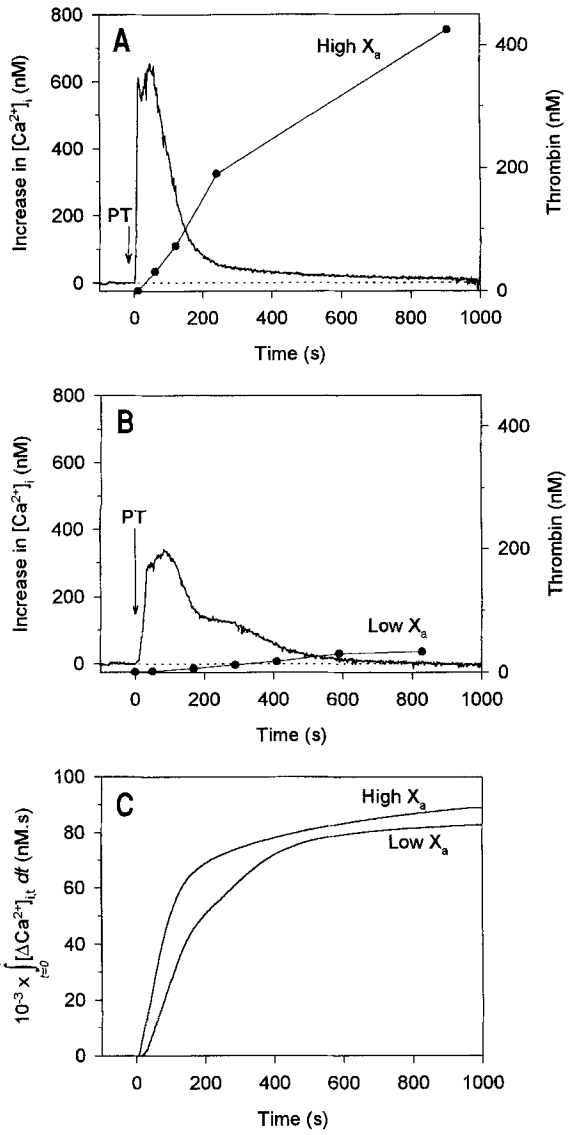

Fig. 5. Effects of *in-situ*-generated α-thrombin on Ca^{2+} responses in platelets in suspension. Fura-2-loaded platelets (2×10^8/2 ml) were incubated with factor Va (1.35 nM) and $CaCl_2$ (2 mM) under stirring at 37°C. Factor Xa was added at a concentration of 0.45 nM (A) or 0.01 nM (B). Prothrombinase activation was started 2 min later by addition of 0.5 μM prothrombin (PT). (A, B) Changes in $[Ca^{2+}]_i$ were recorded and corrected for leakage of fura-2. Samples were taken at regular times to determine the α-thrombin concentration (●). (C) Time plots are given of areas under $[Ca^{2+}]_i$/time curves as determined by the integral, $\int \Delta[Ca^{2+}]_{i,t}dt$. Maximal values reached by this integral represent the CMP of α-thrombin at the end of the experiment. Data are representative for three independent experiments.

nor, the difference in CMP values between slow and more rapid infusion was only 7 ± 3%. Infusion of SFLLRN at rates of 4 μM/min or 10 μM/min (with final concentrations of 25 μM) also resulted in comparable CMP values of 12500 ± 1000 nM · s and 18500 ± 1200 nM · s, respectively (mean ± SE, n = 3 individuals). These results thus suggest that α-thrombin- and SFLLRN-induced Ca^{2+} signaling is only slightly influenced by previous responses to lower concentration of agonist.

The capability to respond to varying α-thrombin levels may or may not be a property of single platelets in the population. To investigate this, platelets on fibrinogen were incubated with low and then higher α-thrombin concentrations. Most cells (78%) responded to the higher α-thrombin concentration by additional trains of $[Ca^{2+}]_i$ spikes of high frequency (Fig. 4A and B). It also appeared that the spiking was dependent on the continuous presence of active α-thrombin, since in 42 out of 60 analyzed cells, it was rapidly abolished with antithrombin III or hirudin (Fig. 4C and D).

The CMP of α-thrombin generated *in situ* by activation of prothrombinase. Another way to expose platelets to gradually increasing α-thrombin concentrations is by incubation with the coagulation factors Va and Xa and prothrombin. It has been shown that suspensions of washed platelets contain sufficient procoagulant membrane surface to support activation of the prothrombinase complex and, thus, to generate α-thrombin *in situ* [27]. Platelet incubation with 2 mM $CaCl_2$, 1.35 nM factor Va, 0.45 nM factor Xa, and 0.5 μM prothrombin resulted in a rapid formation of α-thrombin with an initial rate of 1 nM/s and reaching a concentration of 420 nM α-thrombin after 15 min (Fig. 5A). The formation of α-thrombin was accompanied by a potent increase in $[Ca^{2+}]_i$, after which $[Ca^{2+}]_i$ returned to almost basal level after 5 min. When this experiment was performed with a lower concentration of 0.01 nM factor Xa, this resulted in a 20-times slower rate of α-thrombin formation (0.05 nM/s) reaching an end level of 33 nM after 15 min, and in a lower but more prolonged rise in $[Ca^{2+}]_i$ (Fig. 5B). The integral, $\int \Delta[Ca^{2+}]_{i,t}dt$, thus reached its maximum at a later time, when factor Xa was low (Fig. 5C). Nevertheless, final CMP values were similar under conditions of high and low factor Xa concentration, i.e. 98000 ± 6100 nM · s and 89700 ± 14600 nM · s, respectively (mean ± SE, n = 3 individuals). Within each experiment using platelets from one donor, these values differed with only 11 ± 6%. Control experiments showed that omission of one of the components of the prothrombinase complex (factor Va or Xa or prothrombin) prevented α-thrombin formation, while $[Ca^{2+}]_i$ remained low. In the presence of high factor Xa, α-thrombin formation was reduced by the addition of 1 μM antithrombin III to 23.9 ± 2.3% of the control value, while the CMP was reduced to 49.1 ± 7.8% (mean ± SE, n = 3).

Effects of *in-situ*-generated α-thrombin were also studied on single platelets by activating prothrombinase with procoagulant phospholipid vesicles, factors Va and Xa, and prothrombin. In a typical experiment, 51 of 66 analyzed platelets started to show $[Ca^{2+}]_i$ spikes soon after the first appearance of α-thrombin, and many of these cells showed progressively increasing Ca^{2+} signals while α-thrombin was accumulating (Fig. 6A and B). Upon addition of antithrombin III, spiking in $[Ca^{2+}]_i$ terminated in most (74%) of the responding platelets (Fig. 6C and D), indicating that the spikes were secondary to α-thrombin formation.

Effects of α-thrombin concentration and time on the CMP. The data described so far suggest that the CMP of α-thrombin is determined by two factors: the agonist concentration (Figs 1, 3 and 4) and the time it is present (Figs 4 and 6). For experiments where the α-thrombin level was varied, we evaluated the combined effects of α-thrombin concentration and time by constructing plots of the time-integral of the Ca^{2+} response as a function of the time-integral of the α-thrombin concentration. Regardless of whether α-thrombin was added by infusion (see Fig. 3) or whether it was formed *in situ* (see Fig. 5), these plots were independent of the rate of α-thrombin infusion or formation (Fig. 7).

DISCUSSION

α-Thrombin has a high CMP because it evokes a prolonged Ca^{2+} signal. To quantify the Ca^{2+} concentration and time charac-

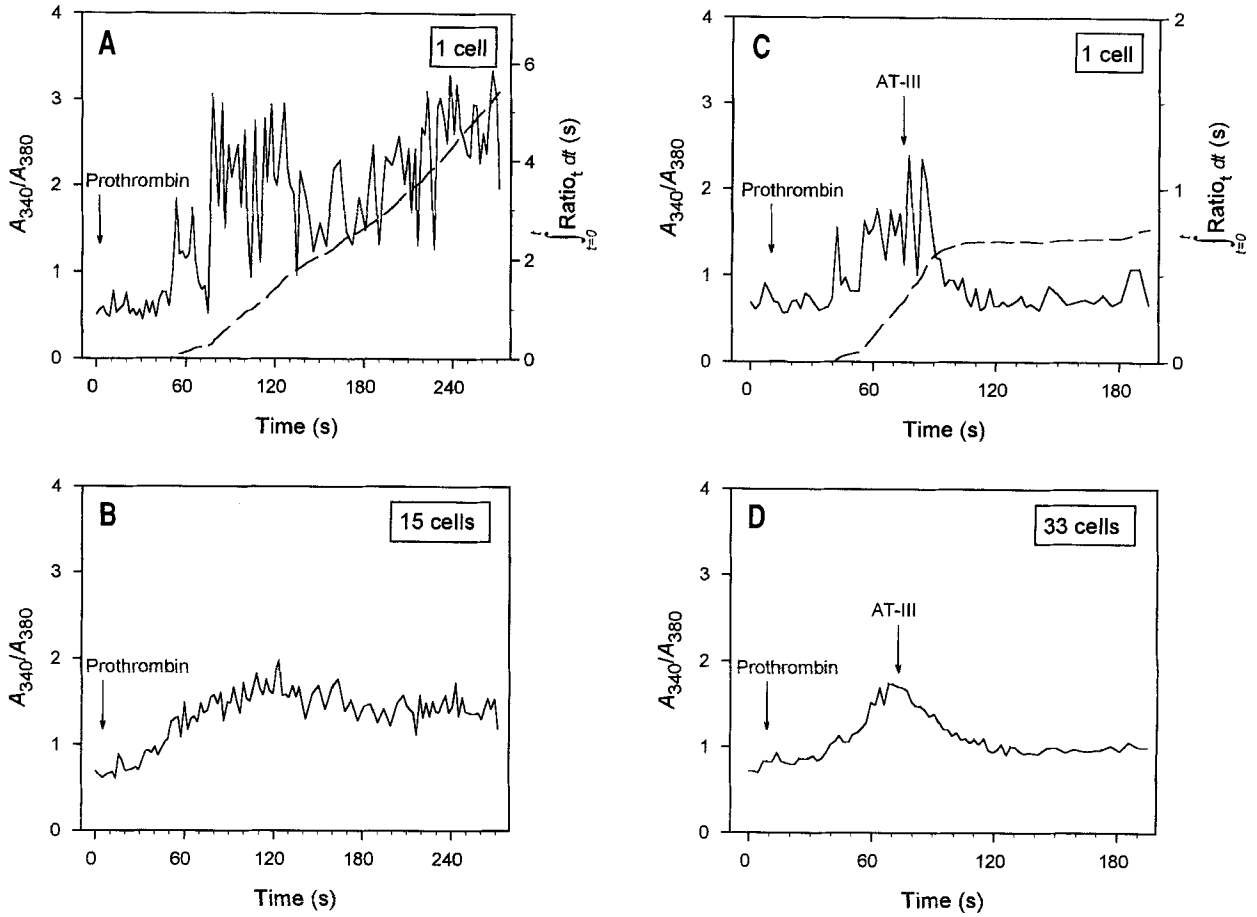

Fig. 6. Effect of *in-situ*-generated α-thrombin on Ca^{2+} responses in single platelets. Fura-2-loaded platelets were immobilized, and incubated with phospholipid vesicles (2 μM), factor Va (0.24 nM), factor Xa (0.1 nM), and $CaCl_2$ (2 mM). Activation was started by the addition of prothrombin (0.5 μM). Where indicated, 1 μM antithrombin III (AT-III) was given after the addition of prothrombin. Traces represent changes in 340 nm/380 nm fluorescence ratio (——) or time integrals of accumulative increases in fluorescence ratio (– – –). Responses are shown of individual cells (A, C), or of averages from 15 or 33 cells (B, D). Data are representative for three experiments (>200 platelets).

teristics of Ca^{2+} responses evoked by α-thrombin and other platelet agonists, we determined time integrals of the increases in $[Ca^{2+}]_i$, $\int \Delta[Ca^{2+}]_i dt$. The CMP of an agonist was defined as the maximal value reached by this integral at the end of the response. With α-thrombin, SFLLRN and compounds activating purinergic or thromboxane receptors, the rises in $[Ca^{2+}]_i$ were completely transient, suggesting that the Ca^{2+}-ATPases present in the plasma membrane and endomembranes are fully active in antagonizing these Ca^{2+} signals. The assumption can then be made that the $[Ca^{2+}]_i$-time integral primarily reflects the number of mobilized Ca^{2+} ions, and not the rate by which these ions are pumped out of the cytosol by Ca^{2+}-ATPases. In other words, the half-life time of every mobilized Ca^{2+} ion is likely to be equal. Since these agonists evoke repetitive spiking in $[Ca^{2+}]_i$ in single platelets, where rapid increases in $[Ca^{2+}]_i$ are always followed by rapid decreases [23], this assumption seems to be valid for the conditions of study.

With saturating doses of proteolytically active α-thrombin, much higher CMP values are obtained than with high SFLLRN, ADP, or U46619. This is true for incubations carried out in the presence of EGTA, where the Ca^{2+} signal is exclusively caused by mobilization of Ca^{2+} from intracellular stores (Table 1). However, this difference is most obvious (a factor 4–10) in the presence of extracellular $CaCl_2$, when influx of external Ca^{2+} also contributes to the Ca^{2+} signal. With $CaCl_2$, α-thrombin typically evokes a high Ca^{2+} response of quite long duration (Fig. 1A), although this is subjected to considerable donor-to-donor variation (Table 1). Accordingly, the high CMP of α-thrombin is largely due to a prolonged Ca^{2+} influx-driven component of the Ca^{2+} signal. It is probably not a consequence of the release of ADP or thromboxane from activated platelets, because treatment of platelets with aspirin or apyrase gives only slightly decreased CMP values. This is in agreement with the rather low CMP values measured after stimulation of the purinergic or thromboxane receptors with ADP or U46619, respectively (Table 1).

The α-thrombin-evoked Ca^{2+} signal is determined by the instantaneous α-thrombin level. The high and prolonged Ca^{2+} signal of α-thrombin is highly sensitive to changes in α-thrombin concentration during the course of the Ca^{2+} response. For instance, increases in $[Ca^{2+}]_i$ change with the α-thrombin concentration, when increasing amounts of α-thrombin are added to platelets by either sequential addition (Fig. 3A) or continuous infusion (Fig. 3C and D). Conversely, the CMP of α-thrombin is reduced with thrombin active-site inhibitors, such as antithrombin III or FPR chloromethylketone (see text). In single, immobilized platelets, sequential addition of increasing amounts of α-thrombin results in an increased frequency of spiking in $[Ca^{2+}]_i$ (Fig. 4A and B), whereas active-site inhibitors terminate the spiking with α-thrombin (Fig. 4C and D). Similar results are obtained when α-thrombin is continuously increased by reconstituted prothrombinase, using purified factor Xa, factor Va, and prothrombin. Individual platelets respond to the accumulation of

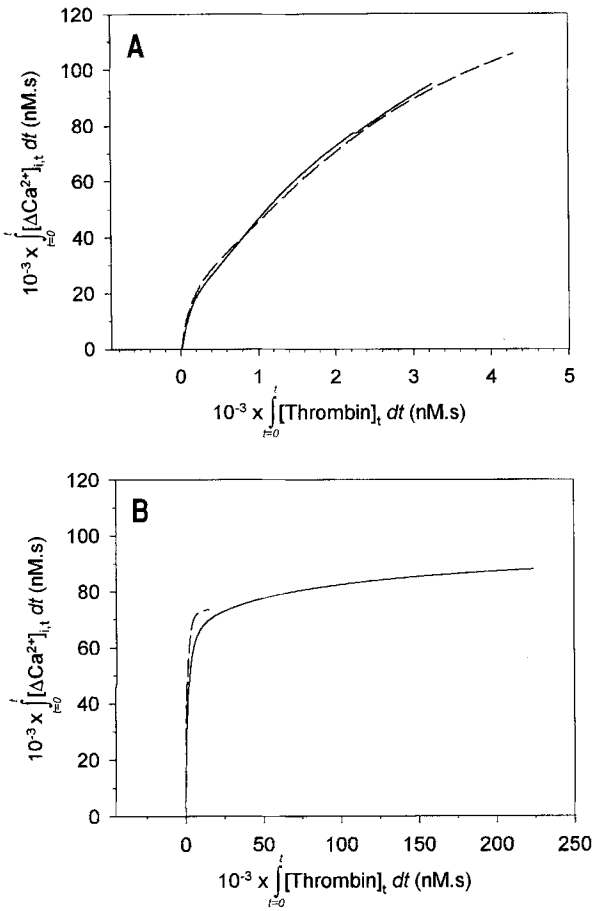

Fig. 7. Effects of concentration and time of α-thrombin on Ca^{2+} responses in platelets in suspension. Data from the experiments shown in Fig. 3C and D and Fig. 5A and B were used to construct plots of $[Ca^{2+}]_i$/time integrals, $\int \Delta[Ca^{2+}]_{i,t}dt$, as a function of the time integrals of the α-thrombin concentration, $\int[\text{thrombin}]_t dt$. (A) Platelets were activated by addition of α-thrombin at a rate of 1.5 nM/min (——) or 4 nM/min (– – –), as described for Fig. 3. (B) Platelets were activated by *in-situ*-generated α-thrombin in the presence of 0.01 nM (——) or 0.45 nM (– – –) factor Xa, as described for Fig. 5. For each condition, nearly overlapping curves were obtained in at least two other experiments with platelets from various donors, although the maximal CMP values could differ by 25% from one experiment to another.

in-situ-generated α-thrombin by accelerated spiking in $[Ca^{2+}]_i$, and spiking stops when antithrombin III is added (Fig. 6).

The observation that (single) platelets continuously respond to increasing and decreasing concentrations of α-thrombin leads to the conclusion that α-thrombin has an instantaneous effect on the generation of the Ca^{2+} signal, implying that its continuous proteolytic activity is required to maintain the Ca^{2+} response. For instance, antithrombin III, hirudin, and FRP chloromethylketone all have immediate, reducing effects on the Ca^{2+} signal of (single) platelets (Figs 4 and 6). However, we have also found that the maximal CMP with saturating α-thrombin (> 10 nM) is essentially insensitive to the rate of α-thrombin infusion or formation *in situ* (Fig. 3C and D and Fig. 5C). This suggests that the (prolonged) Ca^{2+} signal of α-thrombin is not or only slightly subjected to homologous desensitization due to previous α-thrombin activity. Since CMP values of SFLLRN are also independent of the infusion rate, this lack of desensitization may be a property of the concerned protease-activated receptors: apparently, the Ca^{2+} signal of newly activated receptor molecules is not influenced by that of previously activated receptors.

There is good evidence for the existence of SFLLRN-insensitive α-thrombin receptors on platelets [7–11, 13]. Although glycoprotein Ib complexes [10, 11] and a second protease-activated receptor [13] have been put forward, the identity of the alternative receptors is still unclear. Our data also advocate the presence of more than one receptor type. Saturating doses of α-thrombin have a much higher maximal CMP than SFLLRN because of a higher and more sustained influx of external Ca^{2+}. While this confirms the idea that part of the α-thrombin-evoked Ca^{2+} signal is not mediated by type 1 protease-activated receptors [9–11], the results also suggest that the signaling is completely dependent on proteolytic activity. This supports the proposal of Hayes et al. [12], that α-thrombin activates platelets solely because of its enzymatic nature. Although glycoprotein Ib is highly sensitive to proteolysis [11], it remains to be investigated whether/how cleavage of this glycoprotein may be responsible for part of the α-thrombin-evoked Ca^{2+} signal.

The cumulative Ca^{2+} response with α-thrombin is determined by the product of α-thrombin concentration and time. If α-thrombin activates platelets mainly by a proteolytic mechanism, its activating effect should be a function of both the α-thrombin concentration and the time it is present. We measured that the cumulative Ca^{2+} response of platelets is a function of the time integral of the α-thrombin concentration, independent of the rate at which α-thrombin accumulates (Fig. 7). In other words, the product of α-thrombin concentration and time defines the integrated Ca^{2+} signal over that time. From these considerations, it is tempting to conclude that the time integral of the α-thrombin concentration defines the number of receptor cleavages leading to Ca^{2+} mobilization. This closely resembles the earlier reported effects of α-thrombin on fibroblasts transfected with mutated thrombin receptors, where it was found that cumulative receptor cleavage by α-thrombin correlates with cumulative phospholipase C activity and it was concluded that every cleaved receptor produces a fixed quantum of second messengers [5, 21]. Our data similarly suggest that cleavage of the natural thrombin receptors on platelets results in fixed quanta of Ca^{2+} mobilization and Ca^{2+} influx. Thus, the α-thrombin concentration at a certain time may determine the rate of receptor cleavage and thereby the number of Ca^{2+} quanta mobilized at that moment, whereas the time during which α-thrombin is active may determine the total number of cleaved receptors and thus the total number of Ca^{2+} quanta (the integrated Ca^{2+} signal).

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