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Gating of Thrombin in Platelet Aggregates by pO_2-Linked Lowering of Extracellular Ca^{2+} Concentration†

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ABSTRACT: Platelet accretion at sites of vascular injury yields a neo-tissue comprising packed platelets and having an interstitial space not supplied with blood. Within growing thrombi platelet masses become anoxic and depolarize to yield interstitial cation concentrations characteristic of the more voluminous platelet cytosol, with extracellular $[Ca^{2+}]$ falling below that adequate to support the plasma clotting system. The platelet-associated clotting system reactivates during disaggregation of the thrombi *in vitro*, which proceeds with high yield of apparently basal, functional platelets when specific anticoagulants are included in the disaggregating media. The capacity of regulatory demand to lower extracellular $[Ca^{2+}]$ in the microenvironment of platelet aggregates provides a physiological basis for evolution of the highly cooperative calcium interactions of the hemostasis system.

Cytosolic Ca^{2+} is a cornerstone of intracellular signal transduction. In contrast, extracellular reactions in fluids or on cells have not been regarded as acutely ion-regulated, inasmuch as modest (50%) falls in extracellular $[Ca^{2+}]$, rises in $[K^+]$, or change in $[H^+]$ are lethal. Some intracellular processes have been found sensitive (in cell culture) to extracellular Ca^{2+} (Nemeth & Carafoli, 1990), but outside the context of calcium homeostasis no mechanism has been established that imparts extracellular fluctuations of adequate amplitude for acute intracellular or extracellular signaling.

The hemostasis system responds cooperatively to $[Ca^{2+}]$ *in vitro* but is near-saturated *in vivo* at physiological $[Ca^{2+}]$ in plasma and other extracellular fluids (Tucker et al., 1983). Analyzed most thoroughly for prothrombin activation (Tucker et al., 1983; Prendergast & Mann, 1977), exceptional Ca^{2+} cooperativity arises from post-ribosomal γ-carboxylation of the first 9−11 glutamic acid residues (gla) of the vitamin-K-dependent zymogens to yield a microdomain that folds around and buries five conjugated calcium ions (Soriano-Garcia et al., 1989), thereby to acquire the configuration needed for assembly with other subunits on platelet, endothelial, or leukocyte membranes. In spite of the evolution of the gla-domain in at least five hemostasis zymogens and its conservation in vertebrates (Doolittle et al., 1962), no regulatory role for this unique and exceptionally complex structure has been elucidated. Nonetheless, the co-evolution in vertebrates of a family of zymogens bearing post-ribosomal carboxylations with stringent sequence and vitamin (K) requirements, all to impart cooperative responses to a metal ion, would be an unprecedented extravagance for a system having no regulatory demand for that metal ion. Yet, owing to stringency of $[Ca^{2+}]_{free}$ in blood plasma, the calcium bound to gla has been regarded as structural; regulation of hemostasis has been attributed rather to inhibitors, feed-back proteolysis, and platelet or vascular receptor activities (Esmon, 1993). Regulation of hemostasis with extracellular calcium further appears counterintuitive in light of the

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Table 1: Arterial Thrombi Formed *in Vivo* Yield Single Platelets and Thrombin *in Vitro*[a]

medium for disaggregation *in vitro*	single platelets (% total)	thrombin molecules per released platelet	ATP released per platelet (% of blood)
Hanks' salts	19 ± 2	na	18 ± 3
+hirudin	46 ± 3[b]	35000 ● 8000[c]	60 ± 13[b]
+TAP/hirudin[d]	44 ± 7[b]	5100 ± 1700[c]	76 ± 19[b]
+TAP	38 ± 4[b]	na	41 ± 10[b]

[a] Porcine carotid arteries were crush-injured and allowed to accrete platelets for 30 min. The injured segments then were ligated, dissected free, assayed for ^{111}In content, opened, rinsed gently with Hanks' solution, bisected longitudinally, and then incubated (1 h, 37 °C in T-150 culture flasks swirled at 50 rpm) in Hanks' salts (50 mL) plus glucose (1 g/L) and bovine albumin (3 mg/mL), buffered (pH 7.4, no HCO_3) with NaOH-Hepes (20 mM) and containing, as indicated, hirudin, tick anti-prothrombinase, or both (1 μM each). Released platelets were counted both with a Coulter counter (single platelet window) and by ^{111}In radioactivity, which were congruent. Thrombin was measured as thrombin−hirudin complex. [b] $p < 0.01$(unpaired *t*-test relative to Hanks'). [c] $p < 0.04$ (paired *t*-test). [d] Abbreviation: TAP, tick anti-prothrombinase.

capacity of activated platelets to secrete calcium (Murer, 1969), which would presume to raise an already saturating concentration. Here we report, however, that the extracellular enzyme system prothrombinase and associated platelet cohesive reactions are tightly coupled to fluctuations in interstitial [Ca^{2+}] in the microenvironment of platelet aggregates. During platelet thrombosis *in vivo* and during disaggregation *in vitro* of platelet thrombi harvested from porcine carotid arteries, the process of platelet disaggregation *per se* generates thrombin from interstitial prothrombin resident within the platelet mass *in vivo* but which, until the onset of disaggregation, is restrained from activation by pO_2-coupled, microenvironmental sequestration of extracellular [Ca^{2+}].

EXPERIMENTAL PROCEDURES

Materials. Recombinant desulfatohirudin variant 1 (Revasc) was provided by Ciba Pharmaceuticals, Horsham, U.K. (Wallis, 1993), and recombinant tick anti-prothrombinase (Waxman et al., 1990) was provided by Robert Johnson, Merck Research Laboratories, West Point, PA. Hanks' salts powder (without $NaHCO_3$) obtained from Gibco, Inc. was formulated with 1 g/L D-glucose, 3 mg/mL bovine albumin (fatty acid free), and 20 mM NaOH-HEPES, pH 7.4; Hanks' solution contains 1.3 mM Ca^{2+}.

Platelet Thrombi. Pigs, 20−30 kg (protocol approved by the Mayo Foundation Animal Care and Use Committee), were anesthetized with ketamine/xylazine. Carotid arteries were dissected free of surrounding tissue and then crushed over 1 cm of length with a hemostat. This procedure provides a strong stimulus that reproducibly yields within 30 min thrombi (2 × 8 mm, D × l) comprising mostly platelets (McBane et al., 1995). Platelet content of thrombi was measured as ^{111}In radioactivity of autologous platelets labeled and readministered the previous day (Dewanjee et al., 1981). Platelet counts in samples of peripheral blood and culture media were measured with a Coulter counter with the window set for single platelets.

Assay Procedures. Bioluminescence coupled to ATP secretion by platelets was measured with an SLM/OLIS spectrofluorimeter modified to accommodate a thermostated (30 °C) sample compartment attached directly to the photomultiplier housing. Assay samples (50 μL) of platelets (500−1000/μL) in Hanks' solution were mixed in the cuvette with 50 μL of the luminescence reagent (Hanks' solution containing 0.1 mg/mL luciferin and 0.03 mg/mL luciferase); luciferin and luciferase were obtained from Sigma Chemical Co., St. Louis MO.

Thrombin was measured as thrombin−hirudin complex (84% thrombin, 16% hirudin) by ELISA (Bichler et al., 1991), where thrombin−hirudin was captured by affinity-purified immobilized rabbit anti-thrombin Ig and then detected with a double-antibody reagent set comprising chicken anti-hirudin Ig and alkaline-phosphatase-conjugated rabbit anti-chicken Ig.

Interstitial pO_2, pH, [K^+], and [Ca^{2+}] were measured within growing thrombi with a fiber-optic fluorimeter operated in photon counting mode (Gehrich et al., 1986); fibers for pO_2 and pH were fashioned as described. For K^+ and Ca^{2+}, fibers were tipped with the ion-permeable copolymer of polyvinylalcohol-co-vinyltrifluoracetate doped with PBFI for K^+ or with magnesium green for Ca^{2+} (Molecular Probes, Inc.). In aqueous electrolyte solutions the PBFI fiber was sensitive to $1 < [K^+] < 100$ mM, while the magnesium green fiber responded to $0.2 < [Ca^{2+}] < 2$ mM and was not perturbed by Mg^{2+} in the same range of concentrations. In plasma after addition of EDTA or EGTA (10 mM) the magnesium green fiber yielded a fluorimeter response of 320 photon cpm, equivalent to that of 0.3 mM aqueous $CaCl_2$ solution; aqueous 1 mM $CaCl_2$ yielded 780 cpm.

RESULTS

Platelet thrombi in porcine carotid arteries removed and bisected 30 min after traumatic injury yielded 19% of their platelets as single corpuscles when stirred in Hanks' balanced salt solution (Table 1). Incorporation of hirudin or tick anti-prothrombinase into the medium increased the platelet yield to near half. With hirudin alone an average of 35 000 molecules of thrombin per disaggregated platelet appeared in the medium as thrombin−hirudin complex; addition of tick anti-prothrombinase with the hirudin reduced recovery of thrombin−hirudin by 85%.

Transmission electron micrographs of intact thrombi show retention within the platelets of dense bodies and α-granules, both sensitive to release by thrombin (Figure 1A). Of the platelets from thrombi disaggregated in Hanks' solution (Figure 1B), 148/392 (38%) on electron micrographs from two thrombi retained at least one secretory granule, and 31/392 (8%) were counted as discoid (one axis > 2× the other). Addition of hirudin (Figure 1C) or tick anti-prothrombinase (Figure 1D) increased the prevalence of corpuscles retaining their organelles [443/517 (77%); $X^2 = 63$, $p < 0.001$] and counted as discoid [177/517 (34%); $X^2 = 20$, $p < 0.001$].

In keeping with ultrastructure, platelets disaggregated *in vitro* in Hanks' solution with hirudin and tick anti-prothrombinase and then stimulated with thrombin secreted ATP with kinetics and yields near those of platelets obtained from peripheral blood (Figure 2). Platelets recovered in medium without the anticoagulants released much less ATP.

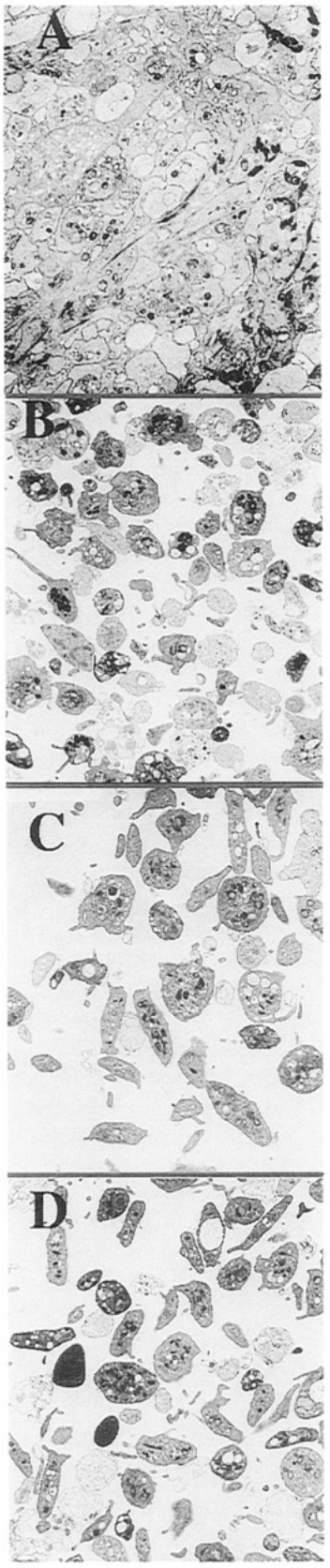

FIGURE 1: Ultrastructure of platelets recovered from arterial thrombi. Arterial segments bearing thrombi (30 min) were fixed (A) or bisected longitudinally, stirred (cf. Table 1) in Hanks' solution (B) plus hirudin (C) or tick anti-prothrombinase (D), and then fixed by addition of Trump's solution to the medium. Disaggregated fixed platelets were concentrated by centrifugation into 1% agar, post-fixed (OsO_4), and stained (UO_2Ac_2).

The paradox raised by the data in Table 1, that thrombin generation is required to maintain cohesion of platelets in a near-basal state while platelet disaggregation is required for thrombin generation, can be resolved by considering the thrombus a tissue. Platelets buried under an accretion zone are separated from blood, the source of glucose and O_2. Ischemic platelets would lose metabolic (but not storage pool) ATP and with it their capacity to maintain chemiosmotic gradients. Within the thrombus the interstitial volume is insignificant relative to that of the neo-tissue (Figure 1A). Were ion pumps lost, membranes would depolarize and the interstitial space would equilibrate with the much more voluminous cytosol, which contains 5 mM Na^+ (100 mM in plasma), 100 mM K^+ (5 mM in plasma), pH 7.0 (7.4 in plasma), and <0.001 mM Ca^{2+} (1.5−2 mM in plasma). Induction by thrombin of membrane Ca^{2+} channels (Zschauer et al., 1988) might also contribute to a fall in interstitial $[Ca^{2+}]$. The $[Ca^{2+}]$ need fall but to 0.2 mM to arrest prothrombinase (Tucker et al., 1983). This hypothetical control mechanism was tested *in vivo* by inserting fluorimeter-coupled optical fibers (0.2 mm diameter), tipped with ion-specific dyes, into arteries undergoing thrombosis. As platelets aggregated around the fibers, pO_2 and cation concentrations in the interface changed in the predicted directions: pO_2 approached zero, pH fell, and $[K^+]$ rose to cytosolic concentrations and $[Ca^{2+}]$ fell below detection limits of the dye (Figure 3). When the animal was given intravenous hirudin, disaggregation of the thrombus was reflected by return of the O_2 and cation signals to those of pre-thrombosis flowing blood. Inspection of the arteries after the experiments revealed no macroscopic thrombus residue, whereas fibers removed from animals not given hirudin were encrusted with white (platelet) thrombus. Whole blood clots induced *in vitro* had no impact on fluorimeter cation or pO_2 signals.

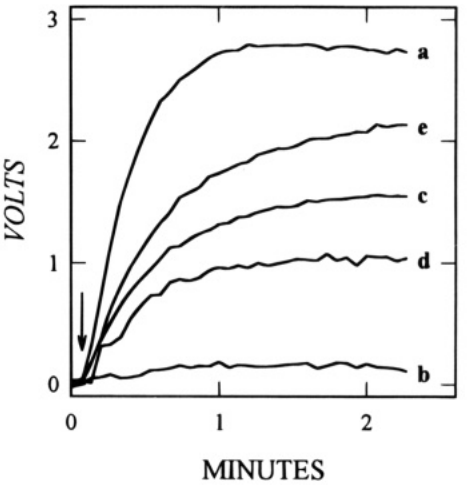

FIGURE 2: Secretion of ATP *in vitro* by platelets recovered from thrombi. Arterial segments bearing thrombi (30 min) were quartered and then platelets were disaggregated (cf. Table 1) in Hanks' solution (b) plus hirudin (c), tick anti-prothrombinase (d), or both (e). The suspensions were diluted to platelet concentrations (Coulter counter) of that in a sample of blood obtained without anticoagulant from the same pig and diluted immediately 1:500 in Hanks' solution (a). Samples (50 μL) of the dilute platelet cultures were added to 50 μL of luciferase reagent in the luminometer cuvette. To initiate the response, 50 μL of thrombin (100 nM in Hanks' solution) was added through a septum (arrow).

DISCUSSION

With the first example of an extracellular enzyme system under acute control by calcium ion fluctuations our findings

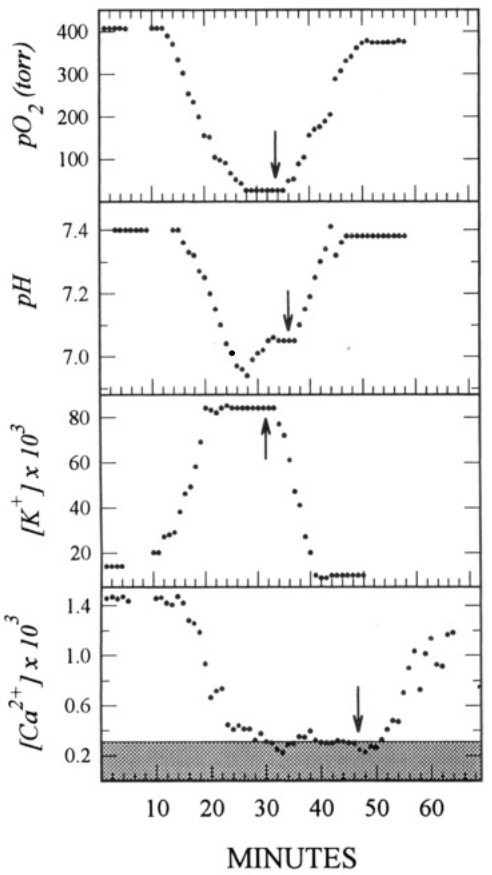

FIGURE 3: Anoxia and extracellular cation redistribution within platelet thrombi *in vivo*. Partial pressure of O_2 and cation activities were measured by fluorescent signals acquired through optical fibers having indicator dyes bonded to the fiber tip. For each measurement a fiber was inserted via catheter into a carotid artery of an anesthetized pig, a baseline was collected, the fiber was withdrawn 2 cm (data gap preceding 10 min), and the artery was crushed with a hemostat. The fiber was then redeployed, data were collected until the signal plateaued, and then hirudin (1 mg/kg body weight) was administered intravenously (arrow) to initiate dethrombosis. Each graph shows data from a different animal. The lower response threshold of the Ca^{2+} fibers is indicated by the cross-hatched area.

provide a premise for cooperative regulation of hemostasis with calcium and a basis for evolution of the gla-domain. Although the ultimate coupling of the process to hypoxia is inferential, the ion fluxes all are predictable consequences of loss of oxidative phosphorylation. Arrest of thrombin generation by decreases in $[Ca^{2+}]$ requires that the process be cooperative; if Ca^{2+} binding were simply hyperbolic, prothrombin activation could only be moderated. The magnitude of prothrombinase suppression is underscored by the sensitivity of platelets to thrombin. The change from discoid to spherical shape and desensitization of the platelets to further stimulation with thrombin occur within minutes when platelets are treated with as little as 0.5 nM thrombin (O'Brien, 1965; Reimers et al., 1973; Smith and W. G. Owen, unpublished results); the electron micrographs and ATP secretion responses (Figures 1 and 2) were obtained from vessels undergoing thrombosis for 30 min and then stirred for an additional hour in media *in vitro*. The suppressed ATP secretion by platelets recovered without anticoagulants may reflect both the reductions in granule content and refractoriness to thrombin.

Prothrombin is likewise sensitive to inhibiting proteolysis by thrombin, particularly when $[Ca^{2+}]$ is low (Jackson et al., 1974). Because platelets and prothrombin are so sensitive to thrombin and the action on both is proteolytic (Owen et al., 1974; Vu et al., 1991), the minor amount of old thrombin in the thrombus, *i.e.*, that recovered by hirudin during disaggregation with tick anti-prothrombinase in the medium (Table 1), might be expected to have had more impact on platelet form or function and to have degraded prothrombin. However, thrombin has some properties that might provide for microenvironmental suppression of proteolytic activity. First, the clotting activity of thrombin shows a Na^+ dependence that is amplified by decreasing pH in the physiological range (Mathur et al., 1993), so anoxia-coupled platelet depolarization would serve to suppress thrombin activity directly. The approach of extracellular $[K^+]$ to cytosolic levels (Figure 3) provides a measure of the segregation of the interstitial fluid from plasma ions, from which reciprocal changes in $[Na^+]$ may be inferred. More profound thrombin inhibition is provided hypothetically by glycoprotein Ib-IX, which reversibly inhibits the clotting activity of thrombin (De Marco et al., 1994) and has been speculated to act as a moderator of thrombin activity (Leong et al., 1992). The interstitial space of the thrombus, bounded by membranes 100 nm apart (Figure 1A), has ultrafiltration dimensions (Loike et al., 1993). Platelets have 500−1000 thrombin binding sites attributable to glycoprotein Ib-IX (Harmon & Jamieson, 1986), so in the interstitial environment [glycoprotein Ib-IX] is of the order of 0.1 mM, or 10^5 times its dissociation constant for thrombin; consequently, any thrombin in this environment would be thoroughly sequestered until the platelets dilute their surface area by disaggregation.

A simple interplay of geometry and mass action, by linking thrombin-generating enzyme assemblies to anoxia-coupled passive ion fluxes in the interstitial microenvironment, provides a regulatory basis for evolution of the gla domain. Not addressed directly by our findings, the platelet integrin $\alpha_{IIb}\beta_3$ requires cytosolic ATP (Kloprogge et al., 1986) and extracellular Ca^{2+} (Phillips & Baughan, 1983) to support platelet cohesion, and so might be likewise controlled. For platelets, the system can be envisaged as a cohesion servo, but our findings invite the possibility of cation-regulated systems in interstitial spaces of any tissue experiencing ischemia and incidentally support the idea (Mustard et al., 1966) that platelet thrombi formed *in vivo* are intrinsically reversible.

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