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Regulation of Coagulation
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Positive Feedbacks of Coagulation
Their Role in Threshold Regulation

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Abstract—Tissue factor (TF), the initiator of coagulation, continuously circulates in the plasma, and the clotting system "idles," generating very low levels of active clotting enzymes, clotting products, and by-products. Given the enormous amplification potential of the clotting cascade, rigorous control is required to ensure that such low-level stimulation does not cause massive system amplification and response. We propose that among the various mechanisms of regulation, activation thresholds may play a major role. These arise when positive-feedback reactions, of which there are several in the clotting system, are regulated by inhibitors. Such thresholds act like switches, so that small stimuli and/or nonproductive local conditions will generate no response, whereas larger stimuli or the existence of local prothrombotic conditions will produce a full, explosive response. We review here the evidence for system idling, the structures of the various feedback mechanisms of clotting, the mechanisms by which they can produce threshold behavior, and the possible role of thresholds in system regulation. **(*Arterioscler Thromb Vasc Biol*. 2005;25:2463-2469.)**

Key Words: blood coagulation ■ positive feedback ■ threshold ■ protease ■ inhibitor

In his proposal of clotting pathways as a cascade system, Macfarlane[1] suggested that such cascades function as extremely potent biologic amplifiers. His ideas were soon confirmed in a mathematical analysis by Levine.[2] However, the cascade hypothesis initially included no regulatory controls, among the key ones being the presence of inhibitors of the clotting proteases and positive-feedback steps, which regulate earlier steps in the cascade. Khanin and Semenov[3] first proposed the idea that inhibitors and feedback steps together may lead to another type of control: activation thresholds.

Positive Feedbacks

In positive feedback, a later enzyme in the clotting cascade either enables or greatly accelerates an earlier step. In terms of system structure, these reactions are very complex, and it seems likely that they could have evolved by natural selection only if they provided a substantial benefit. Let us consider the cofactor, factor VIII (FVIII), as an example. FVIII is required for the action of FIXa on FX, but FVIII circulates in an inactive or near-inactive state, and during clotting, it must be activated by thrombin to form the active cofactor FVIIIa. The fundamental question is what benefit is afforded by such a mechanism? Would it not be to an animal's advantage to synthesize FVIIIa in an active state, ready to contribute immediately on the appearance of FIXa? There would then be no time lag in FX activation, and it is difficult to see how that could be a disadvantage. On the other hand, it has been sometimes said that the most remarkable thing about the clotting system is not that blood can clot so rapidly and so effectively but that it does not clot all the time. We believe that the inhibitory control of positive feedbacks plays a major role in ensuring that this does not happen.

The "Idling" Clotting System

Evidence for Idling: Thrombin and FXa

It has been more than 30 years since Nossel and colleagues[4] detected low, steady-state levels of fibrinopeptides in normal plasma. These have a short half-life, on the order of 3 to 5

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minutes,[4] and to maintain a steady-state level, they must be continuously generated. This in turn means that continuous, low levels of thrombin must always be present. And because thrombin also has a short half-life, being constantly subject to inhibition,[5] it must also be continuously generated. We can take this argument still farther up the clotting cascade to the previous step. The enzyme that generates thrombin is FXa, and just like thrombin, it is continuously subject to inhibition, though at somewhat lower rates.[6] Thus, to maintain a steady-state level, these enzymes must be continuously generated. These conclusions have been confirmed experimentally by Bauer and colleagues.[7,8]

Steady States

The steady-state maintenance of an idling level depends on the balance between formation rate and inactivation rate. The simplest case is seen when an enzyme's formation rate is constant, or zero-order, and its inactivation is first-order. Consider an enzyme E that is generated at a constant rate, $d[E]/dt = C$ (eg, nmol·L^{-1}·min^{-1}). If at the same time it is subject to first-order inactivation, $d[E]/dt = -k[E]$ (eg, units of k are min^{-1}), then a steady state will be attained when the enzyme concentration is such that $C = k[E]$. Thus, the steady-state concentration is $[E]_{ss} = C/k$ (nmol/L). Although this description is written in solution-phase terms, it is also valid for the generation of enzymes on cell surfaces or other membranes, with a solution-phase inhibitor like antithrombin III (ATIII) providing inactivation. Although this form of steady-state regulation is true for thrombin and FXa, things get more complicated in the initial reactions that generate FXa.

Regulation of FIX and FVIIa

The enzymes that form FXa are (1) FIXa, in company with its regulatory cofactor FVIIIa, and (2) FVIIa, in company with its cofactor TF. In both cases, the free enzymes continuously circulate at low levels, but neither is subject to much regulation in the absence of the active cofactor.[9–11] Their function is governed by their cofactors.

FVIII requires the feedback action of thrombin to allow formation of the active IXa:VIIIa complex that converts FX to FXa. This complex then loses activity by two mechanisms. First, FVIIIa is spontaneously unstable, decaying at a substantial rate,[12,13] although it (particularly the porcine protein) is more stable in the presence of FIXa and phospholipid.[13,14] Second, it is subject to later inactivation by the protein C pathway. The details of FVIIa regulation are very different, but still it is the cofactor TF that controls everything, being absolutely required for the inactivation of FVIIa by TF pathway inhibitor (ie, TF pathology inhibitor [TFPI]).[15] For both FIXa and FVIIa then, there is no significant inhibitory capacity in the plasma, and in the absence of their cofactors, they last a remarkably long time.[9–11] It is only through their cofactors that they are subject to regulatory control.

We therefore have an overall picture wherein regulation of the upper enzymes—FVIIa and FIXa—is dominated by their regulatory cofactors, and the lower enzymes—FXa and thrombin—are regulated by a permanent, first-order, inhibitory capacity in the form of ATIII. (There is a wrinkle in this broad picture, in the form of additional regulation of FXa by its cofactor FV, which we will address later.)

Sources of Idling

Although alternative means of system activation may contribute, eg, activation by monocyte cathepsin G,[16] the major source of the clotting system's idling is now quite clear, with the discovery of and substantial confirmation that low levels of TF continuously circulate in the plasma.[17–20] More important in regard to hemostasis mechanisms, this material can be recruited to aggregated platelets and into thrombi under flow conditions.[21,22] There are different forms of circulating TF. A major portion likely occurs in the form of microparticles derived from monocytes and polymorphonuclear leukocytes, which bind to platelets via P-selectin.[23–25] However, another plasma form of TF, formed by alternative splicing, has recently been described. This variant lacks the carboxy-terminal transmembrane domain of normal TF and is soluble, but it is still weakly procoagulant and can also be incorporated into thrombi under flow conditions.[26] Although the plasma TF level is certainly very low,[27,28] it rises significantly in numerous situations, including myocardial infarction and other cardiovascular disease, sepsis, disseminated intravascular coagulation, and diabetes.[18,20,27,29–31]

The presence of procoagulant TF in the normal circulation immediately raises the question of how, given the high efficiency of the clotting enzymes, the system avoids continuous substantial activation. It might be thought that the requirement of many of these reactions for anionic phospholipid (see later sections) fully answers the question, but actually none of the relevant enzymes—FVIIa, FIXa, and FXa—is completely inactive in the absence of its cofactor(s). To answer the question in part, we link two features that we think are central: the regulation of positive feedback steps by inhibition of feedback enzymes and the concept of activation thresholds. The bulk of the evidence for the latter comes from mathematical analysis of both small and large parts of the system.

The Positive-Feedback Steps of Clotting

Figure 1 shows a biased view of the thrombin-generating pathways of clotting, with the emphasis on positive feedback steps (bold triple arrows). Although other feedback steps have been described in the laboratory, at this point we identify four that are of certain importance, and all are catalyzed by FXa or thrombin.

The activation of TF:VII by FXa is the major initiating feedback loop of clotting. When TF is available to the plasma, the former binds with very high affinity to FVII or FVIIa. Most of the available TF surely binds to the inactive zymogen FVII, thereby forming the TF:FVII complex. However, plasma also contains low levels of FVIIa, typically ≈0.5% of the level of FVII,[32] and we expect that a proportionate amount of TF will be bound to it. This would be sufficient to start the FXa-catalyzed feedback that leads to bulk conversion of TF:VII to TF:VIIa.

The activation of FVIII by thrombin is another important step. As mentioned before, FVIII is the regulatory cofactor for FIXa, but the former circulates as the inactive or almost

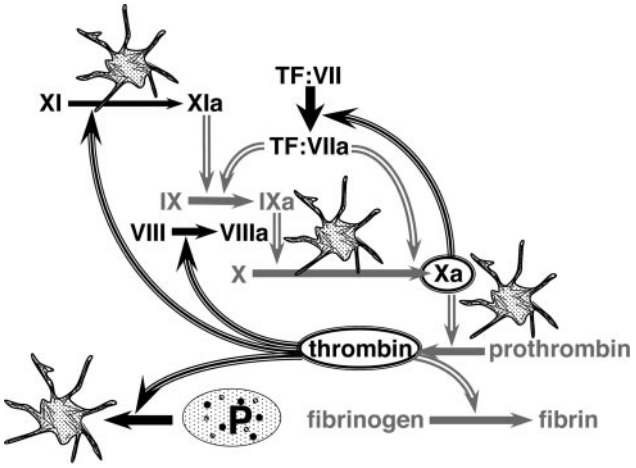

Figure 1. The major positive feedback loops of clotting. Four significant feedback loops are highlighted by bold triple arrows (see text). Solid arrows denote a reaction; open (and bold) arrows denote the action of an enzyme in catalyzing a reaction. P indicates platelet; other abbreviations are as defined in text.

inactive precofactor. Thus, FIXa generation will not propagate through the cascade until FVIII is activated. Although FXa is capable of activating FVIII,[33] it is thrombin that undoubtedly plays the major role.[13,34]

FXI is activated in a very-long-range feedback loop by thrombin[35] and especially so in the presence of activated platelets.[36,37] Although the reaction is not particularly efficient, it seems capable of explaining the partial requirement for FXI in normal hemostasis.

Thrombin is a major activator of platelets, and activated platelets are required for numerous reactions of the central core of the clotting pathways, providing anionic phospholipid for the reactions of vitamin K–dependent proteins and a number of receptors involved in hemostasis. Although platelet-released agonists are important, the action of thrombin also qualifies as a major positive feedback step. Platelet activation is also the major source of functional FVa in normal hemostasis, which is released from α-granules to the platelet membrane in an already active state. In contrast, plasma FV appears to have little or no function.[38,39] This is an important point, because it relegates plasma FV and its feedback activation by thrombin to a minor role.

The Inhibition of Feedback Enzymes

That FXa and thrombin are the enzymes that catalyze these feedback loops is central to our hypothesis because these two enzymes are regulated by ATIII. Because this inhibitor is present in plasma in substantial molar excess over even the precursors of its targets, it provides a permanent, first-order inhibitory capacity. First-order kinetics are expressed in terms of a first-order rate constant or a half-life. In human plasma in the absence of heparin, the half-life of thrombin is $\approx$15 seconds, and that of FXa is $\approx$1 minute.[5,6] It is likely that in the vasculature, which is lined with heparinlike material on the endothelial surface,[40–43] inhibition is faster than this; nevertheless, first-order kinetics still hold.

Thresholds

We now come to the crux of our discussion. If a feedback enzyme is inhibited, the result is a threshold system, with the threshold level of stimulus being controlled by the kinetic balance of formation and inhibition of the feedback enzyme. Below the threshold, the system will not "fire," but above the threshold, explosive propagation will ensue. However, the thresholds are not fixed: they will shift according to changes in the kinetics of either formation or inhibition. For instance, the catalytic rates of FXa and thrombin formation are vastly increased in the presence of anionic phospholipid, which will lower the threshold proportionately and enable a full response, even at very low stimulus levels. Conversely, on the inhibition side, inhibition by ATIII can be greatly accelerated by heparin, which will raise the threshold. We discuss this in more detail next.

Experimental Evidence

Experimental proof for a threshold of the sort proposed herein is very difficult because it requires the measurement of zymogen activation in the presence of enzyme inhibitors and—critically—proof of stability (ie, an experimental nonevent) at low stimulus levels. In passing, we note that plasma studies at very low levels of system activation by TF are especially difficult. Not only may the levels of FXa and/or thrombin generation be very small, but also effective and complete inhibition of contact activation is required, generally with corn (maize) trypsin inhibitor. A related requirement is proof of nonlinearity and especially demonstration that the stimulus-response curve does not pass through the origin; ie, there is no response until a finite threshold is reached. For instance, a simple saturating hyperbola relating a response (R) to a stimulus (X), $R = {}_{max}[X]/(K+[X])$, is nonlinear: it provides for saturating behavior at high [X] and a nearly linear reduction in response at low [X]. Nonetheless, it passes through the origin. A small number of apparent thresholds have been experimentally demonstrated in large systems, 1 of the more convincing being that of Ataullakhanov et al,[44] who measured the plasma clotting response to contact activation as a function of Ca^{2+} ion concentration and observed a very sharp cutoff at low Ca^{2+} concentration (0.25 to 0.5 mmol/L). van't Veer and Mann[45] have similarly reported a sharp change in the response of a complex pure system to changes in a number of variables, in particular, inhibition mechanisms. However, in neither case was complete stability observed below the "threshold," and the specific role of feedback steps was not considered. We ourselves have recently demonstrated an activation threshold in a quite artificial feedback system—the regulation of FXII autoactivation by FXIIa inhibition—but the conditions of this study were not relevant to physiologic hemostasis.[46]

Models

In the absence of experimental evidence, we turn to mathematical models. There are two general types of mathematical model, each with advantages and disadvantages. The more common technique is to specify a system in as much detail as known and required at the level of individual concentrations, rate constants, binding equilibria, flow rates, viscosities, diffusion coefficients, etc. Simpler models, with all reactants in the solution phase, may be specified entirely as ordinary differential equations; models incorporating transport pro-

cesses (flow and diffusion) and membrane phases will usually incorporate partial differential equations; and yet others may use stochastic particle-tracking techniques. These models are solved computationally. They generate an accurate picture of what happens under any specific set of conditions, kinetic parameters, initial concentrations, etc. Although they can produce strikingly "real" simulations, they can be very large, sometimes requiring that >100 quantitative parameters be specified for a single simulation. However, enough is now known of the kinetics of clotting to make the exercise meaningful, particularly when only a few variables (eg, concentrations, kinetic parameters) are of interest. On the other hand, describing the more general properties of a large system over a large range of conditions is difficult.

Analytical Models

An alternative approach is to reduce a system, such as a feedback loop or a collection of them, to its minimal structure and examine it mathematically to determine the general system properties. Although such models eliminate all of the details, they do generate fundamental not condition-specific conclusions. Levine[2] was the first to do this, by making a small model of the initial cascade of clotting that convincingly demonstrated its amplification potential. We have used the same approach to investigate some general properties of positive feedback.[47,48]

Mathematical Analysis: an Example

Although some readers may prefer to skip this section, we think it useful to illustrate the principles of the analytical approach by analyzing the simplest positive-feedback system (real examples are mentioned below), wherein an enzyme E catalyzes its own formation from a zymogen precursor Z in the presence of an inhibitor INH, everything being in the solution phase:

$$Z \xrightarrow{E} E \xrightarrow{INH} \text{inactivated E}$$

An initial trace of E must be provided to enable the feedback loop to run. By Michaelis and Menten, the rate (or velocity V) of enzyme generation at any instant is

$$V_{gen}=k_{cat}[E][Z]/(K_m+[Z])$$

We can simplify this by concentrating on the *initial* rate of E formation, which will be sufficient to tell whether or not the feedback loop fires. At this stage, [Z] is essentially constant, and so we can define a first-order constant, $k_f=k_{cat}[Z]/(K_m+[Z])$, and enzyme generation can be written as $V_{gen}=k_f[E]$. The kinetics of enzyme inhibition or decay are assumed also to be first order with a rate constant k_i, so that the rate of enzyme inhibition is $V_{inh}=-k_i[E]$. The *net* rate of generation of E at any instant is therefore

$$V_{net}=V_{gen}-V_{inh}=k_f[E]-k_i[E]=(k_f-k_i)[E]$$

It is clear that the enzyme can only be generated, ie, the system can only fire, if $k_f>k_i$. Below this threshold, $k_f<k_i$, the initial trace of E present decays exponentially, and the system remains permanently stable. Above the threshold, $k_f>k_i$, explosive exponential generation of E occurs. Of critical importance, because neither k_f nor k_i includes an enzyme-concentration term, the position of the threshold depends on [Z], k_{cat}, K_m, and k_i and is independent of the initial trace level of enzyme present in the system, so long as some finite amount of E is initially present.

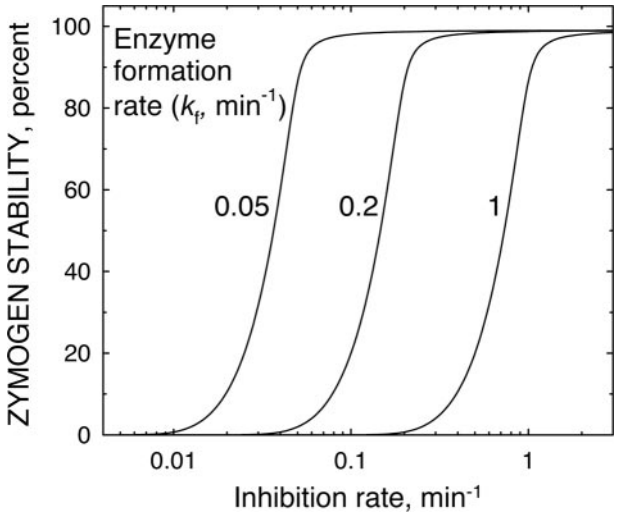

Figure 2. For an autocatalytic feedback loop (see text), the amount of zymogen remaining at infinite time was calculated as a function of (1) the rate of enzyme inhibition (varying on the abscissa) and (2) varying efficiencies of the autocatalytic activation (k_f, labeled).

Figure 2 shows simulated thresholds in an autocatalytic system, with the stability of the zymogen (at infinite time) plotted as a function of the inhibition rate of the feedback enzyme. The three curves were obtained at different efficiencies of autoactivation, ie, k_f. The controlling balance between feedback activation rate and inhibition rate is clear.

Lag Times

The initial trace level of E, $[E]_0$, does not determine whether a feedback system will fire, but when the threshold is exceeded and the system does fire, $[E]_0$ controls the lag time, or the time required to generate the full response. It is relevant that the clotting system idles, so that there are always traces of all enzymes available, and there is no need to consider how one gets the "egg" in the absence of the "chicken," or vice versa. For one particular feedback loop (see Figure 3), we have examined in detail the relation of lag time to the stimulus size and the initial trace level of enzyme.[47]

More Complex Feedback Loops

Simple autocatalytic feedback exists in the clotting cascade at 2 points: (1) in contact activation, the initial activation of

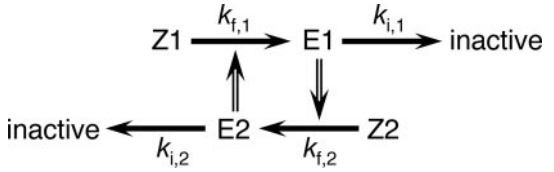

Figure 3. A 2-zymogen, 2-enzyme feedback loop. Kinetic constants are as follows: (1) catalytic efficiency, $k_f=k_{cat}[Z]/(K_m+[Z])$ and (2) inhibition rate, k_i. Constants are first order, with units of time^{-1}. Abbreviations are as defined in text.

FXII to FXIIa is autocatalytic[49] and (2) in the TF pathway, the TF:FVIIa complex is capable of autoactivating TF:FVII.[50–52] Because of low efficiencies, however, we think it unlikely that either has a major functional role in the normal course of hemostasis. The more significant real feedback steps of clotting listed earlier are more complex. We have examined some that are amenable to analysis and have shown that the overall threshold properties are maintained.[48] However, because of the complexities of real clotting reactions, eg, feedback loops on cofactors; more complex inhibition mechanisms, etc, it is not possible to formulate analytic models that are both accurate representations and mathematically soluble. Nonetheless, two key properties of longer-range feedback are clear. In Figure 3, we show a model of a 2-zymogen, 2-enzyme feedback, with both enzymes subject to inactivation. This model is close to the situation for the feedback activation of TF:FVII by FXa, but in the real world, the inhibition of TF:FVIIa is much more complex than just first-order decay. It is nevertheless instructive to note that the threshold in this system is regulated by the products of the kinetic parameters for enzyme generation and enzyme inhibition: $k_{f,1}k_{f,2}>k_{i,1}k_{i,2}$. For instance, if the inhibition rates of both enzymes are increased 5-fold, a 25-fold shift in the threshold is predicted (see the later section on anticoagulation). And importantly, this multiplier effect is retained in longer-range feedback.[48]

Numerical Models

As we have already mentioned, detailed models that are solved numerically are the more common approach, and this has the advantage that the conditions simulated can be much closer to the physiologic system. Earlier models focused on smaller parts,[3,12,53,54] whereas later ones included enzyme inhibitors,[55] a phospholipid membrane, and the protein C system.[56,57] Although none has focused on feedback loops or thresholds, two have produced striking evidence for excitation thresholds.[56,57] These reports also happen to include detailed consideration of the role of the (anionic) phospholipid membrane in clotting. This can be modeled in a manner similar to that for protein molecules in the solution phase, in which small vesicles are simulated to provide an equally distributed concentration of membrane. More complex is simulating the role of a true membrane surface, such as the one that activated platelets might provide, for then one needs to incorporate the complications of the boundary layer and the rates of diffusion between the membrane and solution phases.

Flow and Membrane Surfaces

Finally, there is the enormous complication of flow, which transports reactants along a vessel, and diffusion, which is largely responsible for transport of reactants to a membrane and of products away from it. This is a complex subject and not within the scope of this review. For interested readers, however, references 57 through 60 provide an introduction. In addition to their studies of a comprehensive ordinary differential equation model,[57] Gentry et al[58] have used stochastic particle-tracking methods to model a membrane-bound reaction under flow at its most detailed (FX activation by TF:FVIIa) and have demonstrated the critical role of the transport of product away from the membrane. In a rather less complex situation, we have examined a model of autolytic feedback (cf Figure 2) occurring on a restricted area of stationary membrane, or "patch," in the presence of flow. In addition to the kinetic balance of enzyme generation and inhibition, this threshold is regulated by both the flow rate and the size of the active membrane patch on which the feedback is localized.[59] Loss of feedback enzyme activity thus occurs by two mechanisms: (1) inhibition of the enzyme on the patch, essentially as we have previously considered for solution systems (see earlier sections), and (2) dissociation, diffusion, and flow away from the patch. Thus, depending on the other conditions already discussed (kinetic parameters, etc), small procoagulant patches may not support a feedback loop and excitation, whereas larger patches may. Although the simplification inherent in such a model far removes it from reality, such as a patch actually representing a clump of activated platelets, the possible additional regulation of thresholds by the physical dimensions of a procoagulant area are of interest.

Controlling the Threshold

We mentioned that TF continuously circulates in the blood. On the basis of the previous discussion, we return to the questions of (1) how, in the absence of vascular damage, the system initiates no response and (2) how, in appropriate situations, it can generate a maximal response. Our fundamental hypothesis concerns the balance of enzyme-generation kinetics and enzyme-inhibition kinetics in regulating thresholds. This predicts that one should be able to prevent or enable the clotting response by changing either, and it is here that we look for evidence. Although experimental confirmation is lacking, we think that supporting clinical evidence exists in the two major classes of anticoagulant therapy: those directed at increasing enzyme-inhibition rates and those directed at reducing enzyme-generation rates.

Varying Inhibition Kinetics

Evidence has existed for >50 years that increasing inhibition rates raises the system threshold during heparin therapy. Patients generally survive typical heparin doses without significant bleeding, and this implies that even under anticoagulation conditions, the system can still respond to hemostatic stimuli. Thus, whereas the more rapid inhibition raises the threshold, larger stimuli will still be able to make the system fire. On the other hand, if the inhibition rate is too high, patients do bleed. This can occur not only with aggressive heparin therapy but also with very-high-affinity enzyme inhibitors. Examples include the early hirudin-based, direct thrombin inhibitors, which (we would conclude) inhibited thrombin so fast as to raise the excitation threshold of the system to unsustainable levels.

It has generally been tacitly assumed that heparin therapy or indeed, any antienzyme therapy simply reduces the overall response to any stimulus; ie, for any particular degree of vascular insult, a response can still be generated, but the size of the response is reduced along a more-or-less continuum, depending on the heparin dose or the enzyme inhibition rate.

We propose that this is not the case and that an important factor in such therapy is not so much the amount of thrombin formed at all sites of damage but the activation threshold. In other words, when inhibition rates are increased, it takes a larger stimulus, ie, a larger vascular insult, to exceed the threshold; but when the threshold is exceeded, the system is still capable of a nearly full response and adequate clot formation.

Although most of the evidence concerning inhibition kinetics involves increasing inhibition rates and raising a threshold, there is also clinical evidence of the impact of lowering rates. This occurs in heterozygous ATIII deficiency, wherein the inhibition rates of FXa and thrombin are roughly half normal. Moreover, both enzymes are involved in multistep feedback loops, for which our hypothesis would predict a multiplier effect (see earlier sections). In a simple two-step feedback loop, for example (Figure 3), a reduction in the inhibition rates of both enzymes of 50% would reduce the threshold by 75%. Although the details are certainly not this simple, we consider the general conclusions to be relevant to the question of how relatively small reductions in inhibitory efficiency might lead to a substantially increased risk of thrombosis.

Varying Enzyme-Generation Kinetics

The other class of anticoagulants is the coumarins, which cause the synthesis of clotting factors that are kinetically defective: in binding Ca^{2+} ions and because of this, in their activation on anionic phospholipid membranes. Activated platelets, aggregating at a site of vascular injury, are the primary source of such lipid (and FVa), and in kinetic terms, they enormously increase the rate constants for formation of the vitamin K–dependent enzymes (or k_f, in the analytic discussion detailed earlier). They thus enable feedback loops to fire when, in the absence of activated platelets, they would not. In coumarin therapy, however, catalytic efficiencies, k_f, are deliberately reduced. In threshold terms, this is exactly complementary to increasing the inhibition rates, and this fundamentally may be expected to similarly regulate activation thresholds.

Summary

Much of our thesis about the importance of positive feedback loops and threshold control is as yet unsupported by experimental results, and the hemostatic system is a great deal more complicated than the "bare-bones" systems that we have examined. Nevertheless, the fact remains that under normal conditions, the system is capable of surviving, without any significant response, a continuous, low-level stimulus. We propose that this largely results from the system's positive feedback loops' acting as stimulus-sensitive and condition-sensitive switches.

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