Caution in the interpretation of continuous thrombin generation assays: a rebuttal

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See also Butenas S, Mann KC. Caution in the interpretation of continuous thrombin generation assays This issue, pp 1084-5

We share the concern of Butenas and Mann [1] about the influence of the signaling substrate in continuous measurement of thrombin generation. Any substrate necessarily binds to thrombin and, moreover, is liable to bind to other clotting proteases. It therefore always inhibits thrombin and is potentially an inhibitor of the clotting mechanism at other sites as well. Given the importance of thrombin-driven feedback reactions, inhibition of thrombin will, theoretically, always influence prothrombin conversion. Whether and under what conditions this is of practical importance remains to be answered for each single substrate. In the articles in which we introduced continuous thrombin generation measurement with chromogenic [2] or with fluorogenic [3] substrates, we have shown that prothrombin conversion is not significantly affected by the proposed substrates but thrombin decay is slowed down in a predictable manner because plasmatic antithrombins cannot interact with thrombin that is occupied by the substrate. It might suffice to refer to these articles but in view of its accruing clinical relevance we readdress the subject for the currently most-used substrate: Z-Gly-Gly-Arg-aminomethylcoumarine (AMC).

From Table I it can be seen that Z-Gly-Gly-Arg-AMC indeed prolongs the initiation phase (lag time) of thrombin generation at low tissue factor (TF) concentrations, be it less in our experiments than in those of Butenas and Mann. We agree that this is likely to be due to inhibition of thrombin-driven feedback reactions. The effect on the lag time in TF-induced thrombin generation in plasma is weaker than that on the reconstituted 'coagulosome', which suggests that caution is in its place when extrapolating from reconstituted systems to the 'isolated organ' plasma. The information contained in the lag time is essentially the same as that in conventional clotting times. It is a key question whether the substrate interferes with the information that is unique for the thrombogram, i.e. that on the amount of prothrombin converted and on the time course of the thrombin production.

Plasma contains the natural antithrombin α2macroglobulin (α2M), the 'bait region' of which is split by thrombin after which thrombin remains bound to the α2 M molecule and, although biologically inactive, retains its activity towards the signal substrate. The residual amidolytic activity, after
Table I Effect of substrate on feedback phenomena

<table>
<thead>
<tr>
<th>Substrate (μM)</th>
<th>0</th>
<th>104</th>
<th>208</th>
<th>416</th>
<th>832</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lag time TF: 5 pm (s)</td>
<td>16</td>
<td>105 ± 6</td>
<td>104 ± 6</td>
<td>107 ± 8</td>
<td>127 ± 7</td>
</tr>
<tr>
<td>Lag time TF: 1 pm (s)</td>
<td>16</td>
<td>225 ± 13</td>
<td>226 ± 13</td>
<td>234 ± 11</td>
<td>253 ± 12</td>
</tr>
<tr>
<td>a2 M–IIa end-level 5 pm (nm)</td>
<td>8</td>
<td>92.2 ± 5.7</td>
<td>90.6 ± 6.5</td>
<td>93.9 ± 6.4</td>
<td>96.8 ± 5.9</td>
</tr>
<tr>
<td>a2 M–IIa end-level 1 pm (nm)</td>
<td>8</td>
<td>74.7 ± 5.4</td>
<td>70.8 ± 6.3</td>
<td>79.8 ± 5.0</td>
<td>77.2 ± 4.9</td>
</tr>
<tr>
<td>Inhib. by 10 nm TM TF 5 pm (%)</td>
<td>8</td>
<td>68 ± 5</td>
<td>69 ± 4</td>
<td>67 ± 5</td>
<td>62 ± 4</td>
</tr>
<tr>
<td>Inhib. by 4 nm TM TF 1 pm (%)</td>
<td>8</td>
<td>81 ± 6</td>
<td>83 ± 7</td>
<td>78 ± 6</td>
<td>77 ± 5</td>
</tr>
</tbody>
</table>

The lag time is defined as the moment that the thrombin concentration curve crosses the thrombin concentration value of 10 nm. The inhibition by thrombomodulin (TM) gives the α2macroglobulin–(a2 M–) thrombin end-level with TM as a percentage of the end-level without TM. Inhib., inhibition; TF, tissue factor.

Fig. 1. Influence of substrate concentration on thrombin activity. (A) Amidolytic activity, i.e. thrombin and α2macroglobulin–thrombin activity; (B) thrombin activity only; (C) thrombin activity compensated for binding to substrate. Defibrinated pooled normal plasma. Reaction conditions as in [2]. Five pm tissue factor. Substrate concentrations from top to bottom: 832 μM (black), 416 μM (red), 208 μM (black), 104 μM (red).

thrombin generation is over, is due to this complex. The total amount of complex formed is a measure of the amount of thrombin molecules that have been available for reaction with a2 M (see further [4]), whether they have been reversibly bound to the substrate or not. In Fig. 1A, we see that thrombin generation curves that are determined at different substrate concentrations (104–832 μM, 5 pm TF) eventually yield identical amounts of a2M–thrombin activity. Also, at 1 pM of TF, a concentration at which thrombin generation is dependent upon antithrombic factors [5], the level is independent of the substrate concentration (Table I). Identical (low) end-levels of a2 M–thrombin were also found when hemophilic plasmas were tested at different substrate concentrations (results not shown). We conclude that the presence of substrate did not alter the extent of prothrombin conversion.

As expected, the end-level of a2 M–thrombin drops when a natural inhibitor of prothrombin activation is induced through addition of thrombomodulin (TM). This decrease is more important when no substrate is present than it is at high substrate concentrations (Table I). So the thrombin-driven negative feedback through the protein C system is affected by the presence of substrate, be it that at the usual substrate concentration (416 μM) the effect is barely significant.

Peaks of amidolytic activity increase significantly with the substrate concentration (Fig. 1A). This is not due to the higher reaction velocity at higher substrate concentration, because the reaction velocities are converted into enzyme concentrations by comparison with a fixed amount of thrombin activity measured at the relevant substrate concentration. The increase must be ascribed to the fact that thrombin is protected from antithrombins by the substrate. This is especially evident when the activity due to a2 M–thrombin is subtracted (see [4] for the method employed) (Fig. 1B).

In the reaction mixture, thrombin is partitioned between the free form and that bound by substrate. Antithrombin acts on the free form only, i.e. on a fraction that is K_m/(K_m + S) of the total enzyme. Multiplication of measured thrombin concentrations by K_m/(K_m + S) should therefore compensate for the slowing down of thrombin decay by substrate. In accordance with this hypothesis, this multiplication produces curves that in good approximation are identical and independent of substrate concentration (Fig. 1C, see also [2]).

Effects on the course of prothrombin conversion

In Fig. 1C, the effect of substrate binding on thrombin breakdown has been abolished. The resulting curves represent thrombin generation as if antithrombin action would have been normal. The curves are so similar as to be indistinguishable, except for the effect on the lag phase. We conclude that not only the amount of prothrombin converted is not influenced by the substrate but also the time course of prothrombin conversion is hardly or not influenced.

In conclusion, to probe a system means disturbing it, so when proposing a new technique one has to investigate what this disturbance amounts to. This we did in our original publications and here we show additional evidence that the extent and the time course of prothrombin conversion are minimally disturbed by the presence of Z-Gly-Gly-Arg-AMC at 416 μM. Thrombin inactivation is slowed down to an exactly predictable extent. The necessary effect on thrombin-driven feedback, in plasma, does not cause an important increase in the lag time but does diminish the action of added thrombomodulin by about 10%.
These conclusions pertain to Z-Gly-Gly-Arg-AMC at the usual concentration, i.e. at around twice its $K_m$. They should not be extrapolated to other substrates or to higher concentrations. They certainly should not be interpreted as a laissez passer for any other substrate. Thrombin generation experiments with high-affinity substrates, e.g. those performed in [6], are very likely to reflect a coagulation mechanism in which feedback mechanisms are severely disturbed.

Disclosure of Conflict of Interests
The authors state that they have no conflicts of interest.

References