Synergistic Disaggregation of Platelets by Tissue-Type Plasminogen Activator, Prostaglandin E₁, and Nitroglycerin

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Endothelial cells produce at least three substances that can attenuate the platelet aggregation response: tissue-type plasminogen activator; the platelet inhibitory prostaglandins I₂ and E₁; and endothelium-derived relaxing factor, one form of which exhibits properties of nitric oxide. Since platelet aggregates formed in vivo are involved in the initiation of many clinically important occlusive vascular syndromes, we tested the hypothesis that these endothelial products act synergistically to disperse platelet aggregates. Our data reveal that tissue-type plasminogen activator, prostaglandin E₁, and nitroglycerin (an organic nitrate activator of guanylate cyclase analogous to endothelium-derived relaxing factor) act synergistically to disaggregate platelets and do so in part by modulation of platelet cyclic nucleotides. These data suggest a potential mechanism by which the endothelium protects against the formation of platelet aggregates in vivo and offer a potential strategy for improving the efficacy of thrombolytic therapy. (Circulation Research 1989;65:796–804)

The evidence that platelets play a role in ischemic vascular events is conclusive and has resulted in the emergence of antiplatelet therapy for vasculo-obstructive disorders.¹² Platelets contribute to the clinical expression of atherothrombotic disorders³⁴ by their activation and the subsequent formation of circulating aggregates. Several endogenous molecules that maintain blood fluidity, as well as some pharmacologically useful antiplatelet agents, protect against thrombotic events by inhibiting platelet adhesion and aggregation and by dispersing formed platelet aggregates. The limitations of aspirin therapy in thrombosis may thus in part be explained by its inability to disaggregate platelets.⁵

In vivo, platelet activity is modulated by the endothelium. Three endothelial products have now been demonstrated to attenuate platelet function by distinct biochemical mechanisms. Prostaglandins I₂ and E₁ (PGE₁) inhibit aggregation and induce disaggregation by increasing platelet cyclic AMP (cAMP)³⁰⁻⁴⁹; tissue-type plasminogen activator (tPA) disperses platelet aggregates by selective proteolysis of platelet-bound fibrinogen¹⁰; and endothelium-derived relaxing factor (EDRF) inhibits platelet aggregation by a cyclic GMP (cGMP)-dependent mechanism.¹¹⁺¹² While specific roles for these substances may exist individually in vivo, it is also possible that they act in concert to disaggregate platelets. However, there is little available data on the additive or synergistic effects of these endothelial products or their analogues on platelet function.¹³⁻¹⁵

In this study, we examine the interactions among tPA, PGE₁, and nitroglycerin (NTG) on platelet disaggregation and investigate their mutual effects on platelet cyclic nucleotides. NTG is used here as an organic nitrate activator of guanylate cyclase analogous to EDRF. Our data reveal that tPA, PGE₁, and NTG can act synergistically to disaggregate platelets in plasma and suggest a potential mechanism by which the endothelium can protect against thrombus formation in vivo. In addition, these studies offer a potential strategy by which the efficacy of tPA as a thrombolytic agent may be enhanced.

Materials and Methods

Materials
Adenosine 5'-diphosphate (ADP) and NTG were purchased from Sigma (St. Louis, Missouri). N-
acetylcysteine was purchased from Mead Johnson (Evansville, Illinois). PGE, was provided by the Upjohn Company (Kalamazoo, Michigan). Recombinant, predominantly single-chain tPA was provided by Genentech (San Francisco, California). Radioimmunoassay kits for the determination of cGMP and cAMP were purchased from New England Nuclear (Boston, Massachusetts).

Platelet aggregation

Venous blood was obtained from healthy volunteers who had not ingested aspirin (or any other platelet active drug) for at least 10 days and was anticoagulated with 13 mM sodium citrate. Platelet-rich plasma (PRP) was prepared by centrifugation at 150g for 10 minutes at 25° C, and platelet-poor plasma was prepared by centrifugation at 800g for 10 minutes. Platelet counts were determined with a Coulter Counter (model Z, Coulter Electronics, Hialeah, Florida) and adjusted to 1.5 X 10^8 platelets/ml by the addition of the platelet-poor plasma.

Platelet aggregation was monitored using a standard nephelometric technique in which 0.4-ml aliquots of PRP were incubated at 37° C and stirred at 900 rpm in a dual channel aggregometer (Payton Associates, Buffalo, New York). In experiments with NTG, 5.9 mM N-acetylcysteine (a reduced thiol used to replenish platelet thiol stores depleted with storage after phlebotomy) was preincubated with PRP for 4 minutes at 37° C before aggregation. In all experiments, aggregation was induced with a Coulter Counter (model Z, Coulter Electronics, Hialeah, Florida) and adjusted to 1.5 X 10^8 platelets/ml by the addition of the platelet-poor plasma.

Platelet Aggregation

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Platelet Disaggregation

Disaggregation of platelets in PRP was induced by the addition of tPA, PGE, and NTG, or combinations of these agents exactly 1 minute after aggregation had been induced by ADP, by which time maximal aggregation had occurred (corresponding to 60% maximal light transmittance). Disaggregation was quantitated by measuring the maximal rate of decrease in light transmittance after addition of disaggregating agents.

Cyclic Nucleotide Assays

Measurements of cAMP and cGMP were performed using standard methods by radioimmunoassay. Ten percent trichloroacetic acid was used to terminate reactions at different time points before and after the addition of disaggregating agents. Simultaneous measurements of cAMP and cGMP were made from ether-extracted supernatants of samples. Acetylation of samples with acetic anhydride was used to increase the sensitivity of the cGMP assay. Basal values of cGMP and cAMP were found to be 0.26±0.08 pmol/10^8 platelets and 51±2.5 pmol/10^8 platelets, respectively. The sensitivity of the cGMP assay was approximately 0.0025 pmol, whereas that of the cAMP assay was approximately 0.02 pmol. The reproducibility between assays was 6.8% for cAMP and approximately 10% for cGMP, whereas the reproducibility within assays was less than 5%.

Statistics

Paired samples were compared by Student's t test. Dose-effect data were also evaluated by two-way analysis of variance (ANOVA). Values of p<0.05 were considered significant.

Results

Disaggregation to Individual Agents

tPA, PGE, and NTG all induced concentration-dependent disaggregation of platelets (Figure 1). PGE, was the most potent of these agents with disaggregation observed in the 100 nM concentration range. tPA and NTG were less potent as disaggregating agents when used alone.

Although threshold disaggregating concentrations of tPA varied little from subject to subject with demonstrable disaggregation in response to tPA (1.9-3.6 μM), not all individuals tested disagreed to tPA. The reason for relative individual platelet refractoriness to tPA is unclear, but on retesting, refractoriness was reproducible in the same (2/14) subjects.

Disaggregation to Combinations of Two Agents

Figure 2 shows concentration-effect curves for interactions between tPA and NTG (panel A), PGE, and NTG (panel B), and tPA and PGE, (panel C). According to the isobole method (hyperplane theorem), assuming mutual exclusivity, criteria for synergistic interaction are met 1) for 1.9 μM tPA and 3.6 nM-3.6 μM NTG and for 0.80 μM tPA and 3.6 μM NTG; 2) for 250 nM PGE, and 36 nM-36 μM NTG, for 2.5-25 nM PGE, and 3.6-36 μM NTG, and for 25-250 pM PGE, and 36 μM NTG; and 3) for 0.42 μM-1.9 μM tPA and 250 nM-2.5 μM PGE, and for 1.9 μM tPA and 25 nM PGE,. Subthreshold concentrations of these agents used alone led to disaggregation when used in combination with one another over the ranges listed indicating synergy. At some of the higher concentrations of individual agents used in this study, the effects of combinations of agents cannot be shown to meet the criteria for synergy without extending the concentration-effect curves of individual agents to technically unfeasible concentrations. This technical limitation becomes important when the concentration-effect curve does not saturate (see Appendix); thus, synergy between agents at the highest (nonsaturating) concentrations cannot be confirmed.

Responses to combinations of agents are also displayed in Figure 3 in a three-dimensional plot in which the additive response of a pair of agents is superimposed on the observed response at any given combi-
FIGURE 1. Plots showing platelet disaggregation dose-response to single agents. Panel A: Disaggregation dose-response to prostaglandin E, (PGE,). Panel B: Disaggregation dose-response to nitroglycerin (NTG). Panel C: Disaggregation dose-response to tissue-type plasminogen activator (tPA). Each point represents the mean±SEM of five experiments.

Disaggregation to Combinations of Three Agents

To test the interaction among tPA, PGE, and NTG, maximal subthreshold (nondisaggregating) combinations of two agents were added to subthreshold and greater concentrations of the third agent (Figure 4). For all of these agents, the individual threshold disaggregating concentration of any single agent was lowered further in the simultaneous presence of the other two agents (Figure 5). As determined by application of the hyperplane theorem to the individual concentration-effect relation of individual agents (Figure 1), synergy among all three agents is demonstrated, under the following conditions: 1) when tPA is added to subthreshold concentration of NTG and PGE, between 0.78 and 1.8 µM tPA; 2) when NTG is added to subthreshold concentrations of PGE, and tPA, between 3.6 nM and 3.6 µM NTG; and 3) when PGE, is added to subthreshold concentrations of tPA and NTG, between 250 pM and 25 nM PGE,.

Cyclic Nucleotide Measurements and Synergism

To test the effect of synergistic interactions among agents on cyclic nucleotide levels, measurements were made in the presence of maximal subthreshold (nondisaggregating) concentrations of individual agents that, in combination, induced (synergistic) disaggregation. At the disaggregating concentrations used in this study, aggregate dispersal was observed 4–16 seconds after the addition of disaggregating agent. Measurements of cyclic nucleotides were therefore made before the addition of disaggregating agents and compared with measurements 4 seconds after the addition of agents but before any observed disaggregation (t=4 sec) and 64 seconds after the addition of disaggregating agents (t=64 sec), by which time any disaggregation that might occur was manifest (Figure 6).

Individual agents. The addition of subthreshold concentrations of NTG induced small elevations (0.43±0.14 pmol/10^8 platelets, or a 39±5% increase) in cGMP by 4 seconds that remained significantly elevated at 64 seconds (0.39±0.15 pmol/10^8 platelets, or a 28±6% increase); cAMP levels were not affected by NTG (Figure 6A). In contrast, subthreshold concentrations of PGE, induced small elevations in cAMP by 4 seconds (68±8 pmol/10^8 platelets, or a 10±2% increase) that remained elevated at 64 seconds (76±7 pmol/10^8 platelets, or a 24±3% increase); PGE, had no significant effect on cGMP levels (Figure 6B). Subthreshold concentrations of tPA had little measurable effect on either cyclic nucleotide (Figure 6C).

Cyclic nucleotide levels in synergistic combinations. Experiments were performed as described above in which subthreshold concentrations of individual agents were used, which in all combinations met the criteria for synergy in their ability to induce disaggregation. Synergistic disaggregation by tPA and PGE, was preceded by a small (58±15 pmol/10^8 platelets, or a 10±2% increase) elevation in cAMP that persisted at 64 seconds (59±18 pmol/10^8 platelets, or a 33±4% increase) (Figure 6D) and was not significantly different from that induced by PGE, alone. At the same time, this combination had little effect on cGMP. Synergistic disaggregation by tPA and NTG was preceded by an elevation in cGMP
Synergy in Platelet Disaggregation

FIGURE 2. Plots showing platelet disaggregation dose-response to combinations of two agents. Panel A: Tissue-type plasminogen activator (tPA) and nitroglycerin (NTG). Disaggregation dose-responses to NTG are shown in the presence of increasing concentrations of tPA. Open circles represent 3.4 μM tPA, closed circles represent 1.9 μM tPA, open triangles represent 0.8 μM tPA, and closed triangles represent 0.41 μM tPA. The upper three curves in panel A (3.4 μM, 1.9 μM, and 0.8 μM tPA) were significantly different from the lower curve (0.41 μM tPA) to p<0.05 by two-way analysis of variance (ANOVA). In addition, the upper two curves in panel A (3.4 μM tPA and 1.9 μM tPA) were significantly different from the corresponding portion of the curve in Figure 1B to p<0.05 by two-way ANOVA. Panel B: Prostaglandin E₁ (PGE₁) and NTG. Disaggregation dose-responses to NTG are shown in the presence of increasing concentrations of PGE₁. Open circles represent 2.5 μM PGE₁, closed circles represent 250 nM PGE₁, open triangles represent 25 nM PGE₁, closed squares represent 250 pM PGE₁, and closed squares represent 25 pM PGE₁. The uppermost curve in panel B (2.5 μM PGE₁) was significantly different from the lowermost curve (25 pM PGE₁) to p<0.01 by two-way ANOVA, whereas the middle two curves (250 nM and 25 nM PGE₁) were different to p<0.05. The lowest two curves in panel B (25 μM PGE₁ and 250 pM PGE₁) were not significantly different from one another. The upper three curves in panel B (2.5 μM, 250 nM, and 25 nM PGE₁) were significantly different from the corresponding portion of the curve of Figure 1B to p<0.05 by two-way ANOVA. Panel C: tPA and PGE₁. Disaggregation dose-responses to PGE₁ are shown in the presence of increasing concentrations of tPA. Open circles represent 3.5 μM tPA, closed circles represent 1.9 μM tPA, open triangles represent 0.82 μM tPA, closed triangles represent 0.42 μM tPA, and open squares represent the absence of tPA. Only the uppermost curve (3.5 μM tPA) in panel C was significantly different from the lowermost curve (0.42 μM tPA) and from the corresponding portion of the curve of Figure 1A to p<0.05 by two-way ANOVA. Each point represents the mean of four experiments. Error bars were deleted for the purpose of clarity.

(0.36±0.07 pmol/10⁸ platelets, or a 19±2% increase) that persisted at 64 seconds (0.47±0.33 pmol/10⁸ platelets, or a 55±22% increase) (Figure 6E) and was not significantly different from that induced by NTG alone. At the same time, cAMP levels were not enhanced by this combination. Synergistic disaggregation by NTG and PGE₁ induced small but significant elevations in both cyclic nucleotides (Figure 6F).

Similar elevations in both cAMP (72±12 pmol/10⁸ platelets, or a 38±4% increase) and cGMP (0.45±0.18 pmol/10⁸ platelets, or a 42±12% increase) preceded disaggregation when tPA was added to the combination of NTG and PGE₁, and these remained elevated throughout the first minute of disaggregation (Figure 6G). Elevations in cAMP induced by the combination NTG, PGE₁, and tPA were not significantly different from those induced by PGE₁ alone, and elevations in cGMP were not significantly different from those induced by NTG alone. Thus, in all experimental combinations of two or three agents, elevations in cGMP appeared to reflect the presence of NTG alone (although a very small effect induced by PGE₁ could not be excluded), and elevations in cAMP appeared to reflect the presence of PGE₁. Accordingly, no interaction is apparent between these agents on intracellular platelet cyclic nucleotides, as defined by Berenbaum.¹⁸

Discussion

The endothelium can inhibit platelet function and modulate the local hemostatic milieu by the synthesis and secretion of several substances. Prostacyclin and PGE₁ are powerful endothelial inhibitors of platelet aggregation; they also dissociate platelet aggregates.⁵⁻⁹ Inhibition of platelet aggregation by prostacyclin and PGE₁ is mediated by cyclic AMP, and disaggregation by prostacyclin has also been associated with a rise in this cyclic nucleotide.⁵⁻⁹
FIGURE 3. Three-dimensional plot demonstrating synergy among pairs of agents that disaggregate platelets. Predicted (additive) dose-response planes (hatched) are plotted based on individual dose-response relations and are compared with the observed (open) dose-response relation for tissue-type plasminogen activator (TPA) and nitroglycerin (NTG) (panel A), TPA and prostaglandin E₁ (PGE₁) (panel B), and NTG and PGE₁ (panel C). Note that the individual dose-response relations for individual agents comprise the edge of the plane on the frontolateral faces of the cube.

Our demonstration of an elevation in cAMP that precedes disaggregation to PGE₁ further substantiates the relation between elevations in cAMP and disaggregation by antiplatelet prostaglandins.

A platelet inhibitory effect of EDRF that is mediated by cGMP is readily explained by the recent evidence that the chemical and pharmacological properties of one important form of EDRF are identical to those of nitric oxide.20,21 The inhibition of platelet aggregation and platelet disaggregation by nitric oxide are both cGMP-dependent events, as first demonstrated by Mellie and colleagues.8 NTG is denitrified to its active nitric oxide metabolite (via an S-nitrosothiol intermediate22) and was thus chosen as an EDRF analogue in the present study. The antiaggregatory actions of NTG are well described,23 and we now demonstrate for the first time its ability to disperse platelet aggregates by a cGMP-dependent pathway.

TPA-induced disaggregation of platelets was recently described and attributed to the selective proteolysis of cohesive, platelet-bound fibrinogen10; however, cyclic nucleotide measurements were not included in that study.10 Schäfer and colleagues13 were unable to detect an effect of plasmin on platelet cAMP but did not test directly the effect of TPA on cAMP levels. At the subthreshold concentrations used in this study, TPA had no important effect on either nucleotide when used alone or in combination with NTG or PGE₁ yet, TPA potentiated platelet disaggregation by NTG and PGE₁, providing further support for a cyclic nucleotide-independent mechanism by which TPA facilitates platelet disaggregation.

There is little published data on the interactions among endothelial products or their analogues and effects upon platelet function. Inhibition of platelet aggregation by prostacyclin is known to be potentiated by plasmin, and an argument for synergy between these two agents in the inhibitory process has been made.13 Levin and colleagues15 demonstrated “synergistic” inhibition of platelet aggregation by nitroprusside (a nitric oxide-containing compound) and endothelial cell supernatants, and MacDonald and colleagues14 suggested that synergy exists between EDRF and prostacyclin as inhibitors of platelet aggregation. Synergism, as correctly defined, is an effect of a combination of agents that is greater than expected from their individual concentration-effect relations.18,19 The effect of a combination of agents tested at two isolated concentrations provides little useful information about synergy since the concentration-effect relation necessary to examine synergy remains unknown. Prior reports that attempt to establish synergy among endothelial-derived inhibitors of platelet function are faulty in this respect.13-15 In the present study the concentration-effect relations among TPA, NTG, and PGE₁ were determined over broad concentration ranges, alone and in combination, and pharmacological criteria for synergy between and among agents as defined by the isobole theorem are met.18,19 Therefore, this study represents the first true demonstration of synergy among endothelial products or their analogues on platelet disaggregation. It is
FIGURE 4. Platelet disaggregation in response to combinations of nitroglycerin (NTG), prostaglandin E\(_1\) (PGE\(_1\)), and tissue-type plasminogen activator (tPA). Panel A: A representative experiment in which subthreshold (nondisaggregating) concentrations of 0.78 \(\mu\)M tPA (1), 25 nM PGE\(_1\), and 3.6 \(\mu\)M NTG (2), or all three agents at the same concentrations (3) were added 1 minute after induction of aggregation with ADP. Panel B: Similar experiment in which 3.6 \(\mu\)M NTG (1) is compared with 0.78 \(\mu\)M tPA and 25 nM PGE\(_1\) (2), and all three agents (3). Panel C: Similar experiment in which 25 nM PGE\(_1\) (1) is compared with 0.78 \(\mu\)M tPA and 3.6 \(\mu\)M NTG (2), and all three agents (3).

important to point out, however, that because of limitations in the maximal concentrations of certain agents (such as tPA) that could be used, we were not able to establish that synergism exists over the complete range of concentrations of all three agents.

While the presence of synergy is clearly established from the present study, the biochemical mechanism for the effect remains to be determined. We have attempted to investigate the biochemical mechanism of synergy among tPA, NTG, and PGE\(_1\), first by analysis of cyclic nucleotide levels. This approach was chosen for the following reasons: 1) Cyclic nucleotides play a central role in the regulation of platelet function by NTG and PGE\(_1\), and the effects of tPA on platelet cyclic nucleotides are not known. 2) The effects of (these) agents in combination on platelet cyclic nucleotides and on the platelet regulatory function of both cyclic nucleotides in the simultaneous presence of their respective enhancers is not well understood. 3) An interaction between platelet cyclic nucleotides by mutual regulation of phosphodiesterase activity exists as a potential mechanism for synergy between agents that modulate cyclic nucleotide levels. Cooperative and inhibitory interactions between cAMP on cGMP phosphodiesterase have been described although in platelets these regulatory mechanisms are not well understood. A cGMP-binding phosphodiesterase modulated by cAMP, a cAMP specific phosphodiesterase, and a nonspecific cyclic nucleotide phosphodiesterase have been described in platelets. Cyclic nucleotides might effect interactions between agents that regulate their levels by acting as competitive inhibitors of phosphodiesterase by allosteric interactions with these enzymes or possibly by mediating enzyme phosphorylation.

The important observation of simultaneous elevations in cAMP and cGMP in platelets preceding synergistic disaggregation contradicts the notion that these cyclic nucleotides may act in opposite fashion in relation to one another in platelets. Furthermore, the controversy over the regulatory role of cGMP in platelets notwithstanding in the present study cGMP rose before NTG-induced disaggregation and remained elevated above resting levels while platelets were dispersed. This observation at least suggests a causal role and is in keeping with the observed kinetics of cGMP elevation in smooth muscle in response to EDRF or nitrates.

The documented synergy among tPA, NTG, and PGE\(_1\), did not translate into similar synergy in cyclic nucleotide elevations. These data suggest that synergistic disaggregation by the agents studied here occurs by different, mutually exclusive mechanisms. Synergy in this study reflects a greater relative potency of individual platelet inhibitory mechanisms when acting in concert with other mutually exclusive mechanisms than when acting alone. Lack of a quantitative and temporal correlation between changes in cyclic nucleotides and platelet function hinders the present analysis. The technical inability to detect very small or nearly instantaneous changes in cyclic nucleotides in response to the lowest concentrations of agents studied prevents a complete description of the interactions among these agents. Alternative mechanisms that might be invoked by which synergistic interactions may occur in this system include effects...
FIGURE 5. Plots showing platelet disaggregation dose-response to the combination of nitroglycerin (NTG), prostaglandin E₁ (PGE₁), and tissue-type plasminogen activator (tPA; TPA in figure). Panel A: tPA was added to maximal subthreshold (nondisaggregating) concentrations of NTG (1.0±1.7 μM) and PGE₁ (81±113 nM) (open circles). A dose-response to tPA alone was performed on paired samples for comparison (closed circles) (p<0.02 by two-way analysis of variance [ANOVA]). Panel B: NTG is added to maximal subthreshold concentrations of tPA (0.56±0.06 μM) and PGE₁ (8.8±5.1 nM) (open circles). A disaggregation dose-response to NTG alone was performed on paired samples for comparison (closed circles) (p<0.01 by two-way ANOVA). Panel C: PGE₁ is added to maximal subthreshold concentrations of NTG (1.9±0.8 μM) and tPA (0.56±0.06 μM) (open circles). A disaggregation dose-response to PGE₁ alone was performed on paired samples for comparison (closed circles) (p<0.05 by two-way ANOVA). Each point represents the mean±SEM of four experiments.

In summary the data presented here show that 1) disaggregation of platelets by tPA, NTG, and PGE₁ is the result of synergistic interaction among these agents, 2) disaggregation by PGE₁ is preceded by elevations in cAMP and disaggregation by NTG is preceded by elevations in cGMP, 3) simultaneous elevations in cAMP and cGMP occur in platelets in association with synergistic disaggregation by NTG and PGE₁, in the presence of tPA, 4) biochemically different, noninteractive mechanisms act in concert in the synergistic disaggregation of platelets by tPA, NTG, and PGE₁, and 5) synergy occurs at concentrations of these drugs that are pharmacologically achievable in vivo.

These data provide new insights into the potential mechanism by which the endothelium protects against the formation of platelet aggregates and maintains blood fluidity. With regard to the potential relevance of this work in improving thrombolytic efficacy in patients, it is important to recognize the inherent limitations of an in vitro clot system and the discrepancies between the effectiveness of interactive combinations of thrombolytic agents in vitro and in vivo. Interestingly, we observed a relatively narrow dose range over which synergy was demonstrated among agents in this study. A similar “window of synergy” has been observed between tPA and single-chain urokinase-type plasminogen activator in patients. Because recent studies have demonstrated the importance of antplatelet agents in accelerating clot lysis and improving thrombolytic efficacy, the work presented here may also have therapeutic implications.

Appendix

Interactions Between Two Agents: The Special Case of Sigmoidal Concentration-Effect Relations

The isobole (hyperplane) theorem has been constructed for the general case of interactions among two or more agents, regardless of their concentration-effect relations. To identify synergistic interactions between two or more agents each with sigmoidal concentration-effect relations, more direct expressions must be used and characterized in the limits of the concentration-effect relation (i.e., at the plateau); clearly, the importance of this fact rests on the inability to achieve an “equieffective” concentration of a single agent when that single agent’s response saturates well below that observed in the combination.

Consider a system in which two mutually exclusive ligands, A and B, bind to receptors R₁ and R₂, respectively, to produce response R. The interaction between the ligands and their respective receptors can be generalized by the following equations:

\[ K_A = \frac{AR_1}{(R_1A^o)} = \frac{AR_1}{[(A_1-AR_1)(R_1-A_1)]} \]

and

\[ K_B = \frac{BR_2}{(R_2B^o)} = \frac{BR_2}{[(B_1-AR_1)(B_1-B_2)]} \]
FIGURE 6. Bar charts of cyclic nucleotides that were measured and compared with basal values in platelets at 4 seconds (open bars) and 64 seconds (closed bars) after the addition of maximal subthreshold (nondisaggregating) concentrations of individual agents alone and in combinations resulting in synergistic disaggregation. Each point represents the mean ± SEM of three experiments each performed in duplicate except for the measurement of cyclic GMP (cGMP) in bar D where n = 2, performed in duplicate. A, nitroglycerin (NTG); B, prostaglandin E$_1$ (PGE$_1$); C, tissue-type plasminogen activator (tPA); D, tPA + PGE$_1$; E, tPA + NTG; F, NTG + PGE$_1$; G, tPA + NTG + PGE$_1$. Each time point was compared with its paired t = 0 point (100%). *p < 0.01; **p < 0.02; ***p < 0.05.

where $K_A$ and $K_B$ represent the association constants for A and B and their respective receptors, $AR_1$ and $BR_2$ represent the ligand-receptor complexes, n and m represent the degree of cooperativity of ligand binding for A and B, respectively (i.e., define the “steepness” of the concentration-effect switch), and the subscript, T, refers to the total concentration of a given ligand or receptor in the system under consideration.

When $A_T >> AR_1$ and $B_T >> BR_2$, these equations can be rearranged as:

$$AR_1 = \frac{(K_A A_T^n)}{(1 + K_A A_T^n)}$$

and

$$BR_2 = \frac{(K_B B_T^m)}{(1 + K_B B_T^m)}$$

and the observed response, $R$, can be defined as the sum of these two expressions, which we will normalize to unit response with specific activity coefficients, $\alpha$ and $\beta$, as follows:

$$R = \alpha AR_1 + \beta BR_2 = 1$$

The isobole, $I$, by which interactions between A and B can be considered, is defined in the general case as:

$$I = (\alpha AR_i + \beta BR_j)$$

where $AR_i$ and $BR_j$ represent the “equieffective” concentrations of $AR_1$ and $BR_2$, respectively, that is, those that alone produce the same effect as $AR_1$ and $BR_2$ in combination.

Two specific cases can be considered in sigmoidal concentration-effect relations. In the first case, consider the midportion of the concentration-effect curve in which an effective linear concentration-effect relation exists. Under these circumstances, $A_T^n = K_A^{-1}$ and $B_T^m = K_B^{-1}$; thus $K_A A_T^n - 1$ and $K_B B_T^m - 1$. Since, over the linear concentration-effect range, $AR_1 = BR_2 = \alpha AR_1 + \beta BR_2$, substituting these values in Equation 3 leads to the simple expression, $I = 1$, which is consistent with the general case of Berenbaum. Under these conditions, synergy is said to exist if $I < 1$.

In the second case, consider the uppermost portion of the concentration-effect curve, that is, at saturation. Under these circumstances, $K_A A_T^n >> 1$ and $K_B B_T^m >> 1$. Thus, substituting these values into Equations 1 and 2 and simplifying the expressions for substitution into Equation 3, we find that $AR_1 = AR_1^e = BR_2 = BR_2^e$, or $\alpha = \beta = 1$; thus, $I = 2$. Under these circumstances, synergy exists if $I < 2$. The implications of this derivation are that when one wishes to examine a system for synergistic interactions at concentrations of agents that individually produce saturating response characteristics, any response noted with the combination greater than the response at saturating concentrations of individual agents implies synergy. Thus, the concentration-effect curve does not need to be extended to excessive or prohibitive concentrations of individual agents in an effort to establish an “equieffective” dose of the single agent; in fact, there is no
such concentration. Such was the case for PGE1 and NTG, for example, in the present study.

Acknowledgments
The authors wish to thank Drs. John P. Cooke and Michael Mendelsohn for their helpful comments, Ms. Patricia Amarante and Ms. Deborah Smick for excellent technical support, and Ms. Nancy Beatty for superb secretarial assistance.

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Key Words: platelets • tissue-type plasminogen activator • prostaglandin E1 • nitroglycerin
Synergistic disaggregation of platelets by tissue-type plasminogen activator, prostaglandin E1, and nitroglycerin.

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Circ Res. 1989;65:796-804
doi: 10.1161/01.RES.65.3.796

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