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Efflux of Cyclic GMP from Activated Human Platelets

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SUMMARY

Activation of human platelets is associated with an increased level of cGMP, when total cGMP in individual samples is measured. However, by discriminating between intracellular and extracellular cGMP we were able to demonstrate that cGMP accumulates in the extracellular space only, whereas the level of intraplatelet cGMP actually decreases. Therefore, during the first minutes of platelet aggregation cGMP is released from the cell, and it thereby escapes hydrolysis by intracellular phosphodiesterases. In contrast, during direct activation of soluble guanylyl cyclase by nitrovasodilators, such as sodium nitroprusside, the newly synthezised cGMP remains mainly inside the cells. Elevation of intracellular calcium and activation of protein kinase C are likely to be involved in promoting cGMP efflux. Our results are discussed in contrast to the general hypothesis that the cGMP increase associated with platelet aggregation may represent a feedback mechanism designed to terminate early events of activating signal transduction. According to our data the apparent cGMP increase results from cGMP release from thrombocytes, rather than soluble guanylyl cyclase activation. This cGMP efflux provides a mechanism of decreasing the intracellular cGMP level upon stimulation with platelet agonists and thus favors platelet activation.

cGMP, an important second messenger, is produced by various guanylyl cyclase (GTP pyrophosphate-lyase; EC 4.6.1.2) isoenzymes and degraded by different cGMP phosphodiesterases (1-3). It is generally believed that cGMP exerts most of its biological actions by interacting with specific protein kinases, causing a distinct pattern of protein phosphorylation (4). In the case of platelets a 50-kDa protein has been reported to be phosphorylated in a cGMP-dependent fashion; its function, however, remains unknown (5). Smooth muscle vasorelaxants activate soluble guanylyl cyclase and cause marked elevations of cGMP, in this way counteracting the effects of vasoconstrictor hormones (6, 7). The physiological vasorelaxant endothelium-derived relaxing factor has been identified as NO (for reviews, see Refs. 8 and 9). NO directly activates soluble guanylyl cyclase by binding to its ferroheme group (10, 11).

Using human platelets it was reported that aggregatory agents, such as ADP, thrombin, epinephrine, collagen, prostaglandin endoperoxides, and the calcium ionophore A23187, increase cGMP levels (12-14). Thus, a proaggregatory second messenger role of cGMP was initially suggested (15). However, it was soon determined that several NO-generating vasodilators also elevate intraplatelet cGMP levels but markedly inhibit platelet aggregation (16, 17). By analogy with smooth muscle cells, several authors now propose that the rise in cGMP levels observed during platelet aggregation functions in feedback inhibition of early activating events, rather than being a positive signal itself (17, 18). Endogenous NO production by platelets was suggested as a trigger for stimulation of soluble guanylyl cyclase in activated platelets. A calcium/calmodulin-dependent NO synthase was described that, associated with a cytosolic calcium increase, becomes active during early platelet activation (19). Although this would provide a logical explanation for the observed cGMP increase during platelet activation, the existence of a platelet NO synthase is still controversial (20).

Recently we focused on the inhibitory activity of 12-HPETE in platelet activation (21). We demonstrated potent antiaggregatory activity of several fatty acid hydroperoxides that was produced by activation of soluble guanylyl cyclase and a concomitant cGMP increase. Furthermore, we investigated activation of soluble guanylyl cyclase by oxidizing agents in combination with SNP, using human platelets as a model system (22). In the course of platelet activation 12-HPETE is produced from free arachidonic acid (23). The function of this lipoxygenase pathway in the regulation of platelets is still unknown. We were interested in determining to what extent these mechanisms, NO synthesis and 12-HPETE production, contribute to the rise in cGMP associated with platelet activation. However, simple experiments revealed surprising evidence that during platelet activation the apparent cGMP increase is in fact due to cGMP release from the cell, which is actually associated with a moderate decrease in the intraplatelet cGMP level.

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ABBREVIATIONS: NO, nitric oxide; 12-HPETE, 12-hydroperoxyeicosatetraenoic acid; SNP, sodium nitroprusside; LDH, lactate dehydrogenase; PMA, phorbol myristate acetate.

Experimental Procedures

Materials

cGMP was bought from Boehringer Mannheim (Mannheim, Germany), cGMP-2′-*O*-succinyl-[^{125}I]iodotyrosine methyl ester was purchased from Amersham (Braunschweig, Germany), and anion channel blockers and succinyl-cGMP were obtained from Sigma Chemical Co. (Deisenhofen, Germany). Collagen A was purchased from Biochrom KG (Berlin, Germany). SNP was obtained from Aldrich Chemical Co. (Steinheim, Germany). All other reagents were of the highest grade of purity available and were obtained from local commercial sources.

Methods

Preparation of washed platelets. Blood was obtained from healthy volunteers who had not taken any drugs for at least 2 weeks. Preparation of washed platelets was carried out as outlined previously (24). To remove any erythrocytes, after the first centrifugation step platelets were resuspended in lysis buffer (0.61%, w/v, NH_4Cl, 7.5 mM $KHCO_3$, 0.1 mM EDTA) for 10 min at room temperature. A 10-fold excess of washing buffer was then added, following the procedure outlined in Ref. 24.

Measurement of cGMP. For measurement of extracellular cGMP, 400 μl of washed human platelets (stirred platelet suspension; 800 rpm) were incubated at 37° with the compounds indicated. After incubations, samples were centrifuged at 10,000 × *g* for 20 sec. The supernatant was removed for extracellular cGMP measurement.

For measurement of intracellular cGMP, the resulting pellet (see above) was resuspended and lysed by addition of 800 μl of ice-cold 10% trichloroacetic acid. After samples had been kept on ice at 4° for 1 hr, the lysate was centrifuged again at 10,000 × *g* for 10 min and 600 μl of the supernatant were neutralized by addition of 400 μl of a 1:1 (v/v) mixture of 1,1,2-trichlorotrifluoroethane and tri-*n*-octylamine (25), followed by vigorous mixing. After centrifugation (1 min at 10,000 × *g*) three phases were obtained. The upper phase was the neutralized sample containing all water-soluble components. A 500-μl portion of the upper phase was removed for measurement of intracellular cGMP.

The determination of intra- and extracellar as well as total cGMP was carried out by radioimmunoassay as described (26). The preparation of polyclonal antibodies against cGMP was outlined in Ref. 22. Total cGMP is the sum of intra- and extracellular cGMP. Counting and calculation of the calibration curve were done automatically with a Philips PW 4800 γ counter with a PW 4801 integrated single-board computer.

Protein determination. Protein concentrations of platelet samples were determined according to the method of Lowry *et al.* (27).

Measurement of LDH release. LDH release into the medium was taken as an indicator of cell rupture during aggregation. After each incubation platelets were centrifuged as described above and LDH activity in the supernatant was determined according to the method of Vassault (28).

Statistical methods. Results are expressed as mean values ± standard deviations of a number of determinations in individual experiments with samples from different blood donors. Student's unpaired *t* test was used to determine the significance of differences between means, and a *p* value of <0.05 was taken as significant.

Results

Thrombin, a commonly used receptor agonist, induces rapid and irreversible platelet aggregation. Activation was followed by increased light transmission of a stirred washed platelet suspension (Fig. 1). Platelet response was fast during the first 2 min after agonist addition, reaching maximal values after 4–6 min of incubation. According to previously reported data (12–14), platelet aggregation should be accompanied by a cGMP increase in response to various agonists. Measuring cGMP, as outlined in Experimental Procedures, in the whole sample, i.e., neutralized supernatant of the trichloroacetic acid precipitate, we indeed observed a cGMP increase after thrombin addition.

The increase in cGMP was rapid during the initial phase of aggregation (first 2 min) and slowly progressed up to 5 min, after which no additional significant cGMP increase was detectable. Interestingly, discrimination between intracellular cGMP and cGMP that had been released from the cells revealed a completely different picture. After thrombin addition, cGMP inside platelets decreased slightly, with a tendency to stabilize at a lower intracellular level after roughly 2 min. The opposite effect was seen concerning the level of extracellular cGMP, which dramatically increased during the onset of platelet activation, followed by a minor increase with longer incubation times. Similar distributions of intracellular versus extracellular cGMP were seen after collagen A- or PMA-induced platelet activation (data not shown). Under the same experimental conditions no LDH leakage from platelets was measured (data not shown). The separation of intracellular and extracellular cGMP requires approximately 20 sec. During this time intracellular cGMP phosphodiesterases would efficiently hydrolyze cGMP; intracellular levels of cGMP thus represent a steady state resulting from cGMP formation and destruction. This potential interference has to be considered in all experiments; however, the overall significance of our results is unaffected because centrifugation does not interfere with any of these processes.

After establishing that during platelet activation only the extracellular component is responsible for the rise in total cGMP, we measured the distribution of cGMP when soluble guanylyl cyclase was directly activated, i.e., by the nitrovasodilator SNP, which is known to release NO spontaneously. Incubating washed platelets with increasing concentrations of SNP revealed a marked cGMP increase up to roughly 20 μM SNP, followed by a plateau phase where higher concentrations of the NO donor did not cause an additional cGMP increase (Fig. 2).

Discriminating between intracellular and extracellular cGMP, SNP clearly causes an intraplatelet cGMP increase without any substantial efflux from the cells. Released cGMP did not exceed 10% of the total cGMP content, measured over the entire concentration range of SNP. It must be taken into account that the intracellular concentration of cGMP after addition of SNP is severalfold higher, compared with the cGMP concentration after addition of thrombin (compare Figs. 1 and 2).

Previously we reported that fatty acid hydroperoxides caused an inhibition of platelet activation by increasing the level of cGMP (21). Incubating washed human platelets with 10 μM 12-HPETE increased the cGMP content significantly. Investigation of the distribution of cGMP under these conditions revealed that the cGMP increase was mainly intracellular (data not shown). Generally, intracellular cGMP elevation due to guanylyl cyclase activation is not necessarily associated with cGMP release from platelets.

To examine further the cGMP release during platelet activation, together with direct soluble guanylyl cyclase activation, we studied the effects of thrombin and SNP applied alone or added in combination (Table 1). When platelets were stimulated first with thrombin, followed by SNP, a significantly higher cGMP efflux was detected, compared with the SNP control. In the converse experiment, when SNP was added

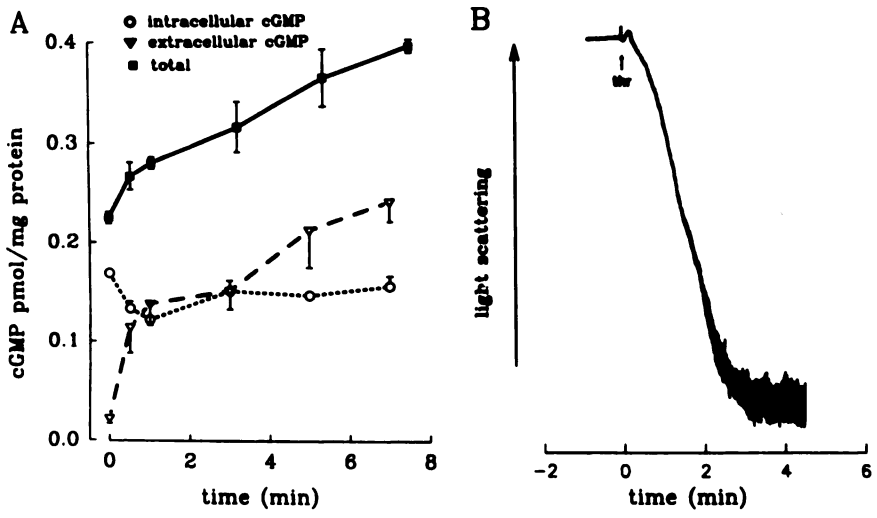

Fig. 1. Thrombin causes platelet aggregation and cGMP efflux. A, Time-dependent cGMP efflux after addition of thrombin. Washed platelets were incubated with 250 mU/ml thrombin. At the indicated times, intra- and extracellular cGMP levels were separately determined, as described in Experimental Procedures. Results are given as the mean ± standard deviation of three different experiments. B, Typical aggregation curve produced with thrombin (250 mU/ml). Increase of light transmission versus time was recorded.

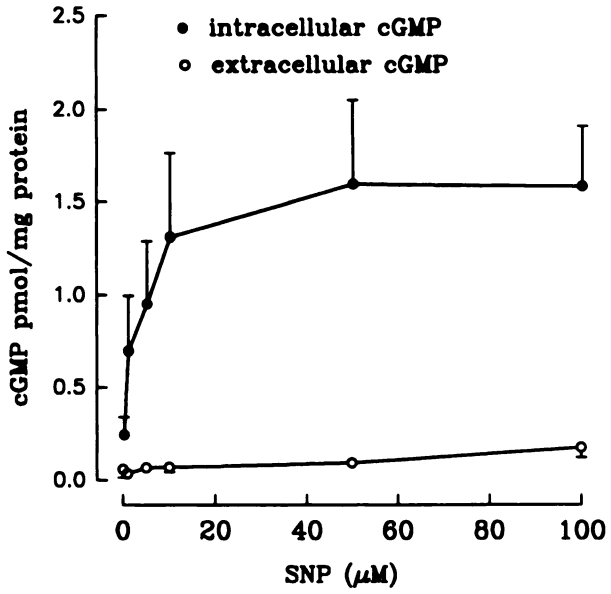

Fig. 2. cGMP increase after the addition of SNP. Washed human platelets were incubated with increasing concentrations of SNP for 3 min, followed by determination of intra- and extracellular cGMP as outlined in Experimental Procedures. Results are given as the mean ± standard deviation of three different experiments.

TABLE 1

Platelet aggregation promotes cGMP efflux

The table demonstrates changes in intracellular and extracellular cGMP concentrations induced by the indicated treatments in washed platelet suspensions. The values for extracellular cGMP were directly measured by radioimmunoassay in the cell-free supernatant. The intracellular cGMP concentration was calculated from the cGMP content in the neutralized trichloroacetic acid precipitate of the cell pellet, assuming an average platelet volume of 5 fl and a platelet number of 5×10^8 ml^{-1}. Values represent means ± standard deviations of individually calculated results from three experiments. Platelet aggregation is also indicated.

	cGMP		Aggregation
	Intracellular	Extracellular	
	nM		
Control	180 ± 6	0.01 ± 0.03	–
Thrombin (250 mU/ml) (3 min)	166 ± 11	0.44 ± 0.01	+
SNP (10 μM) (3 min)	1314 ± 265	0.29 ± 0.12	–
Thrombin (2 min) + SNP (3 min)	1218 ± 444	0.71 ± 0.10	+
SNP (1 min) + thrombin (2 min)	1360 ± 462	0.23 ± 0.06	–

before thrombin cGMP efflux again was as low as in the SNP control. The extracellular level of cGMP after SNP treatment was roughly the same as that after thrombin addition. In thrombin-treated cells no increase in intracellular cGMP was obvious and cGMP release was fed solely by basal turnover. In contrast, the 10-fold higher concentration gradient across the plasma membrane after SNP addition may cause some nonspecific release. However, the release was considerably enhanced after cell activation by thrombin. When SNP was added alone or before thrombin, platelets did not aggregate. Because platelet aggregation was initiated by thrombin added before SNP, it seems that platelet aggregation allows cGMP to escape.

Elevation of intracellular cGMP before stimulation with a receptor agonist blocks receptor-mediated activating signal transduction pathways in platelets. To gain further insight into the underlying mechanisms of cGMP efflux from activated platelets, we used thapsigargin to increase intracellular free calcium and phorbol esters to stimulate protein kinase C under conditions of guanylyl cyclase activation. During these studies we again simultaneously recorded platelet aggregation, to establish cGMP efflux without aggregation. The following experiments, presented in Fig. 3, were performed in the presence of SNP to inhibit platelet aggregation and to cause an intraplatelet cGMP increase.

Thapsigargin, a commonly recognized, selective inhibitor of endoplasmic reticulum calcium ATPases, is known to cause an intracellular calcium increase and platelet activation (29). However, in the presence of SNP thapsigargin neither induces platelet aggregation nor promotes significant cGMP extrusion. By using PMA, a known activator of protein kinase C, only a marginally significant cGMP extrusion was produced, although the aggregatory response during the first 3 min after agonist addition was inhibited. More importantly, the combination of thapsigargin and PMA promoted substantial cGMP efflux in response to SNP without inducing significant platelet aggregation during the first 3 min after agonist addition. Therefore, cGMP escape from platelets is facilitated by the two critical events in the activation of platelet signaling, namely increases in calcium concentration and activation of protein kinase C.

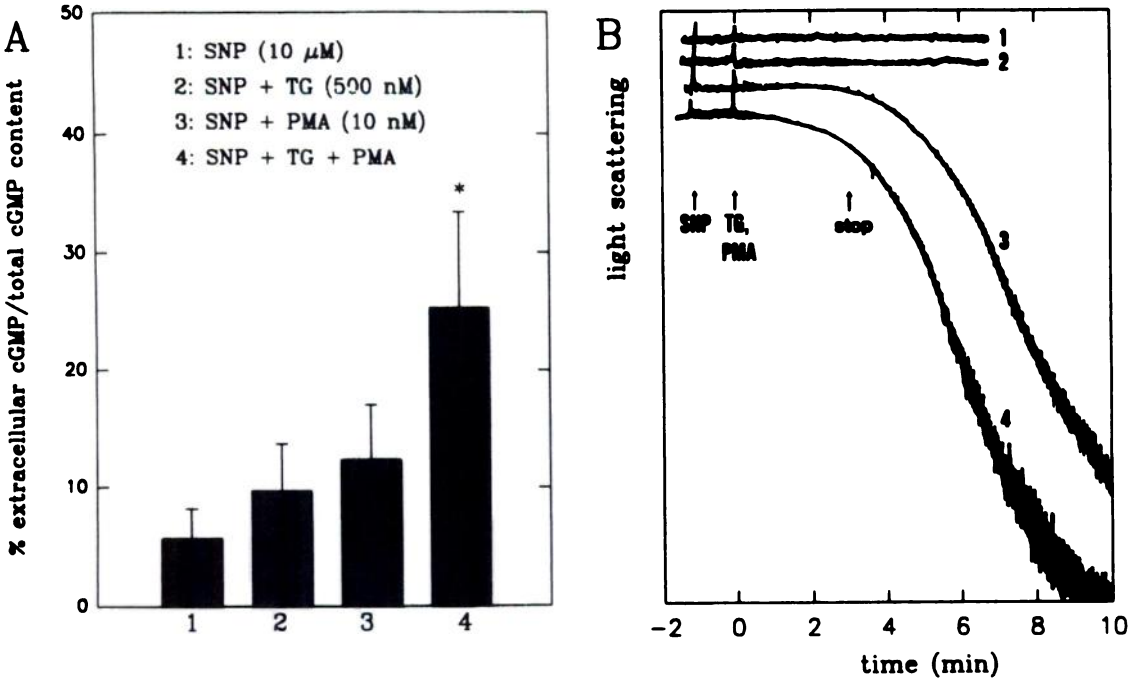

Fig. 3. Thapsigargin and PMA promote cGMP release. A, Washed platelets were preincubated with 10 μM SNP for 1 min, followed by a 3-min incubation with the substances indicated (*TG*, thapsigargin). Intra- and extracellular cGMP levels were determined. Further details are as in Fig. 1. B, Aggregation traces corresponding to different experimental conditions as described in A. As indicated (*arrows*), samples were withdrawn for cGMP determination after a total of 4 min of incubation.

Discussion

The observations documented in this report confirmed the results of earlier investigations (12–14), that platelet aggregation is accompanied by an increase in total cGMP. However, our results extend these findings, pointing to a different distribution of cGMP. Discriminating between intracellular and extracellular cGMP, our experiments indicate that after thrombin stimulation a cGMP increase is evident only in the extracellular compartment. In contrast, the level of regulatively effective intracellular cGMP is decreased. The observed apparent cGMP increase reflects escape of the cyclic nucleotide rather than soluble guanylyl cyclase activation by a yet unidentified second messenger. Basal turnover of intracellular cGMP is under the control of formation and degradation. In contrast, extracellular cGMP is no longer hydrolyzed by cytosolic phophodiesterases and therefore accumulates after having escaped from platelets. A different picture emerges when soluble guanylyl cyclase is activated by SNP directly or, alternatively, by the action of fatty acid hydroperoxides. Under these conditions cGMP dramatically increases inside the cell. Although there is some minor release of cGMP in this instance, it is not related to the intracellular level and may be simply triggered by the 10-fold higher concentration gradient across the membrane. One way to explain the appearance of extracellular cGMP after cell activation and stimulation of soluble guanylyl cyclase is via a saturable or rate-limiting extrusion step of low capacity.

However, the release of cGMP from the cells was enhanced by stimulation of platelets with thrombin before soluble guanylyl cyclase activation. cGMP efflux was also considerably increased when thapsigargin and phorbol ester were used to increase intracellular free calcium and to activate protein kinase C, respectively. Thus, both signals seem to be involved in the regulation of cGMP efflux. Furthermore, these experiments prove that cGMP release is not due to a nonspecific membrane pertubation or simply a result of cell damage.

cGMP efflux from various cells has already been reported, for example in the case of hepatocytes (30, 31), cerebellar slices (32), and pancreatic lobules (33). In those reports, an elevated intracellular cGMP level was associated with extrusion of the cyclic nucleotide. In contrast to our experiments, in those cases cGMP efflux occurred after hours, as a result of induction of NO synthase (30). For hepatocytes and cerebellar slices, it was demonstrated that anion channel blockers like probenecid reduced cGMP efflux. Unfortunately, anion channel blockers are potent but nonspecific antiaggregatory compounds (34). In our hands probenecid and 4,4-diisothiocyanatostilbene-2,2′-disulfonic acid inhibited cGMP extrusion, but we were not able to determine whether this was due to anion channel-blocking activity or nonspecific inhibition of platelet activation. Thus, a potential role of anion channels in cGMP release from human platelets remains to be established. Interestingly, in addition to cGMP, intracellular cAMP escapes from several cell types by an energy-dependent mechanism that shares many properties with active transport (35, 36). The observation that both cyclic nucleotides escape from cells could provide a useful comparison aiming at pharmacological manipulation of the efflux systems (37).

Our study questions the hypothesis that cGMP acts as a negative feedback regulator of platelet activation. cGMP increases only extracellularly and this does not allow a specific antiaggregatory activity, because extracellular cGMP cannot interact with cGMP-specific kinases. Considering the antiaggregatory potential of cGMP, a mechanism actually rapidly decreasing its intracellular level would be a mechanism favoring platelet aggregation. This is likely to be even more relevant *in vivo*, where circulating platelets are subjected to a certain

intravascular tone of NO released from vascular endothelium, which probably up-regulates intracellular cGMP in platelets and thus applies a steady constraint on platelet activation. In physiological or pathopysiological situations requiring platelet aggregation, efficient activation of the cells can be achieved by decreased synthesis of antiaggregatory compounds in combination with a platelet mechanism to accelerate their own activation by rapidly removing the intracellular counteracting signal cGMP. The presence of a cGMP efflux system in various cell types, including platelets, indicates that the level of cGMP is efficiently regulated in three ways, (i) cGMP production by activation of soluble guanylyl cyclase, (ii) removal of cGMP by phosphodiesterase-catalyzed hydrolysis, and (iii) cGMP efflux from the cell. A set of several phosphodiesterases with different K_m values for cGMP may serve to balance the intracellular cGMP level according to the activation state of guanylyl cyclase, whereas cGMP efflux provides a means of rapid removal of cGMP from the intracellular compartment, thus giving way to activation of otherwise inhibited cell functions.

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