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Molecular Basis of the Synergistic Inhibition of Platelet Function by Nitrovasodilators and Activators of Adenylate Cyclase: Inhibition of Cyclic AMP Breakdown by Cyclic GMP

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SUMMARY

We investigated the roles of cyclic GMP and cyclic AMP in the inhibition of rabbit platelet aggregation and degranulation by two nitrovasodilators, sodium nitroprusside (SNP) and 3-morpholinosydnonimine (SIN-1; the active metabolite of molsidomine), with particular reference to the synergistic interaction of these drugs with prostaglandin E_1 (PGE_1). Changes in platelet cyclic [^{3}H]GMP and cyclic [^{3}H]AMP were measured by rapid and sensitive prelabeling techniques, the validity of which were confirmed by radioimmunoassays. Incubation of the platelets with 0.1 to 10 μM SNP alone for 0.5 min caused progressively greater inhibitions of platelet function associated with large dose-dependent increases in cyclic [^{3}H]GMP and 1.4- to 3.0-fold increases in cyclic [^{3}H]AMP. However, addition of SNP with the adenylate cyclase activator, PGE_1, at a concentration of the latter that had little effect alone, caused much larger increases in cyclic [^{3}H]AMP and greatly enhanced the inhibition of platelet aggregation. SIN-1 had effects similar to those of SNP, although it was less active. The adenylate cyclase inhibitor 2′,5′-dideoxyadenosine (DDA) diminished the increases in cyclic [^{3}H]AMP caused by SNP or SIN-1 in both the presence and absence of PGE_1 but reduced the inhibition of platelet function caused by the nitrovasodilators only in the presence of PGE_1. These results suggest that, although cyclic GMP may mediate the inhibition of rabbit platelet function by high concentrations of nitrovasodilators added alone, the synergistic interaction of lower concentrations with PGE_1 depends on an enhanced accumulation of cyclic AMP. Synergistic effects on cyclic [^{3}H]AMP accumulation were also observed on incubation of platelets with SNP and adenosine, another activator of adenylate cyclase. Hemoglobin, which binds nitric oxide, blocked or reversed the increases in *both* cyclic [^{3}H]GMP and cyclic [^{3}H]AMP in platelets caused by the nitrovasodilators added either alone or with PGE_1. Cilostamide, a selective inhibitor of platelet low K_m cyclic AMP phosphodiesterase, had effects on platelet cyclic [^{3}H]AMP accumulation identical to those of SNP, suggesting that the action of the latter depends on inhibition of the same enzyme. M&B 22,948, a selective inhibitor of cyclic GMP phosphodiesterase, potentiated the increases in *both* cyclic [^{3}H]GMP and cyclic [^{3}H]AMP caused by SNP. A hyperbolic relationship was found between the increases in cyclic [^{3}H]GMP and cyclic [^{3}H]AMP caused by different concentrations of SNP; this relationship was not affected by addition of M&B 22,948. The results strongly suggest that the increases in platelet cyclic [^{3}H]AMP caused by nitrovasodilators in the presence or absence of activators of adenylate cyclase are mediated by the inhibition by cyclic GMP of cyclic AMP breakdown. This study provides the first evidence that cyclic GMP exerts important functional effects through a cyclic GMP-inhibited low K_m cyclic AMP phosphodiesterase, an enzyme known to be present in many cells including platelets [*Adv. Second Messenger Phosphoprotein Res.* **22:** 1–38 (1988)].

Certain nitrovasodilators, in particular SNP (1, 2) and SIN-1 (3), a metabolite of molsidomine, are potent inhibitors of platelet aggregation *in vitro*. Organic nitrates are much less effective but, at least in the case of nitroglycerin, can be activated by thiol compounds (4, 5). There is also evidence that some nitrovasodilators can suppress platelet reactions *in vivo*, suggesting that an antiplatelet action may supplement their effects on vascular smooth muscle in the treatment of cardiovascular disease (6–9). Nitrovasodilators are believed to exert their effects via the release of nitric oxide, which stimulates the formation of cyclic GMP by soluble guanylate cyclase (10). Consistent with this view, both SNP (11–13) and SIN-1 (3, 5) activate platelet guanylate cyclase and markedly increase cyclic GMP in intact platelets. Cyclic GMP has been assumed to mediate vascular relaxation by stimulating the phosphorylation of critical protein substrates by cyclic GMP-dependent protein kinase (10). A similar mechanism has been proposed for plate-

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ABBREVIATIONS: SNP, sodium nitroprusside; SIN-1, 3-morpholinosydnonimine; EDRF, endothelium-derived relaxing factor; PGI_2, prostacyclin; PGE_1, prostaglandin E_1; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; PIPES, piperazine-*N*,*N*′-bis-(2-ethanesulfonic acid); 5-HT, 5-hydroxytryptamine (serotonin); DDA, 2′,5′-dideoxyadenosine; PAF, synthetic platelet-activating factor (1-*O*-octadecyl-2-*O*-acetyl-*sn*-glycero-3-phosphocholine); M&B 22,948, 2-*o*-propoxyphenyl-8-azapurin-6-one; TLC, thin layer chromatography.

lets, in which the cyclic GMP-dependent phosphorylation of specific but as yet unidentified proteins has also been described (12, 14). However, studies in this laboratory (12) and at least two others (3, 15) have demonstrated small increases in cyclic AMP in human platelets incubated with either SNP or SIN-1, and preliminary studies have suggested that this cyclic AMP contributes to the inhibitory effects of SNP on platelet aggregation (16).

Recent evidence has indicated that nitric oxide released from the vascular endothelium may play a physiological role in the regulation of vascular tone and of platelet interaction with the vessel wall. Thus, EDRF, which is released from the endothelium under basal conditions as well as at an accelerated rate in the presence of many pharmacological stimuli, has been identified as nitric oxide (17–19). EDRF not only relaxes vascular smooth muscle but also inhibits platelet aggregation (20–22). In both cases, these effects are associated with the activation of guanylate cyclase (15, 19). The formation of nitric oxide, therefore, represents a mechanism by which the vascular endothelium can inhibit platelet and smooth muscle responses in addition to the formation of PGI_2, which exerts its effects through the receptor-mediated activation of adenylate cyclase (23).

In 1982, Levin *et al.* (24) reported that SNP and PGI_2 act synergistically to inhibit platelet aggregation. More recently, synergism between the actions on platelets of PGI_2 and EDRF (25, 26) or of PGI_2 and organic nitrates (27) has been demonstrated. Because PGI_2 and EDRF (nitric oxide) are likely to be generated simultaneously by stimulated vascular endothelium, a synergistic effect of these compounds on platelets could be of physiological importance (25). Moreover, the actions of exogenous nitrovasodilators on platelets could be enhanced by endogenous PGI_2 formation (27). The molecular mechanism responsible for these synergistic effects has not previously been determined. We demonstrate here that this synergism results from the ability of nitrovasodilators to enhance the accumulation of cyclic AMP in platelets, as a result of the inhibition of cyclic AMP breakdown by cyclic GMP. Our results provide the first demonstration that cyclic GMP exerts important functional effects through the cyclic GMP-inhibited cyclic AMP phosphodiesterase found in many cell types including platelets (28–30). Some of our findings have been reported in a preliminary form (31).

Experimental Procedures

Materials

[2,8-^{3}H]Adenine (34–38 Ci/mmol) from ICN (Irvine, CA) and [8-^{3}H]guanine (16 Ci/mmol) from Amersham (Oakville, Ontario, Canada) were adjusted to lower specific activities before use (see below). Cyclic AMP and cyclic GMP ^{125}I-radioimmunoassay kits, [U-^{14}C]ATP (538 mCi/mmol), [U-^{14}C]GTP (440 mCi/mmol), and cyclic [8-^{14}C]AMP (50 mCi/mmol) were obtained from NEN Canada (Lachine, Quebec, Canada). Cyclic [8-^{14}C]GMP (52 mCi/mmol), [*side chain*-2-^{14}C]serotonin, and ACS aqueous counting scintillant were supplied by Amersham (Oakville, Ontario, Canada). HP/b scintillant was from Beckman Instruments (Toronto, Ontario, Canada). Digitonin was obtained from Calbiochem-Behring (La Jolla, CA), charcoal (Darco G-60) from Fisher Scientific (Toronto, Ontario, Canada), and celite 535 from Johns Mansville (New York, NY). SNP, all nucleotides, PGE_1, HEPES, PIPES, bovine serum albumin (fraction V), rabbit hemoglobin, and neutral alumina (WN-3) were obtained from Sigma (St. Louis, MO). Dowex-50 resin (AG 50W-X8, 100–200 mesh, H^+ form) was obtained from Bio-Rad Laboratories (Mississauga, Ontario, Canada). Cellulose (MN 300 HR) was purchased from Brinkman Instruments (Toronto, Ontario, Canada). SIN-1 and molsidomine were gifts from Casella-Riedel (Frankfurt am Main, FRG) and M&B 22,948 from May & Baker Ltd. (Dagenham, UK). Cilostamide was generously provided by Dr. H. Hidaka of Nagoya University (Nagoya, Japan), DDA by Dr. R. A. Johnson of Stonybrook University (Stonybrook, NY), and PAF by Dr. H. R. Baumgartner of F. Hoffmann La Roche and Co. (Basel, Switzerland).

Methods

Preparation of washed platelets. Rabbit platelets were isolated by differential centrifugation and washed at room temperature in Ca^{2+}-free Tyrode's solution containing 0.35% bovine serum albumin, using a modification of the method of Ardlie *et al.* (32) in which the resuspension media were supplemented with 30–60 μg of apyrase/ml and 5.0 mM PIPES (buffered to pH 6.5 with NaOH). Washed platelets used for prelabeling assays of cyclic nucleotides were resuspended at 2.5×10^9/ml and then incubated at room temperature with 2 μM [^{3}H]guanine (7.4 Ci/mmol) to label the platelet metabolic GTP pool. Following incorporation of about 70% of the [^{3}H]guanine (45–60 min), 4 μM [^{3}H]adenine (4.0 Ci/mmol) was added to the suspension to label the metabolic ATP pool. The incubation was then allowed to continue for an additional 45–60 min, after which about 70% of the [^{3}H]adenine had been incorporated. It was necessary to add [^{3}H]adenine after [^{3}H]guanine because the former inhibited the uptake of the latter (but not vice versa). In parallel experiments in which platelet cyclic GMP and cyclic AMP were also determined by radioimmunoassay, platelet suspension was incubated with unlabeled 2 μM guanine followed by 4 μM adenine, to ensure that the platelets used were as similar as possible. When required to permit subsequent measurements of platelet degranulation, 1 μM [^{14}C]5-HT was added at the same time as [^{3}H]adenine to label the platelet dense granule contents. Uptake of [^{14}C]5-HT amounted to about 75%. After incubation with either labeled or unlabeled guanine and adenine, the platelets were again isolated by centrifugation and resuspended at $4.2–4.8 \times 10^8$/ml in Tyrode's solution (with Ca^{2+}) containing 3–6 μg of apyrase/ml and 5.0 mM HEPES, pH 7.35. This medium was warmed to 37° before resuspension of the platelets.

Incubations of platelets with drugs. Samples of platelet suspension were incubated at 37° with additions, giving a final volume of 0.5–1.2 ml. The final platelet concentration was always 4×10^8/ml. Additions were dissolved in 0.154 M NaCl where possible or in dimethyl sulfoxide (cilostamide and M&B 22,948). The final concentration of dimethyl sulfoxide (0.2% v/v) was the same in all samples in each experiment in which it was used. When cyclic [^{3}H]nucleotides were determined, incubations were terminated by mixing part or all of each sample with sufficient ice-cold 30% (w/v) trichloroacetic acid to give a final concentration of 5%. Cyclic [^{14}C]GMP and cyclic [^{14}C]AMP (700 dpm of each) were then added to each sample to permit measurement of the recovery of each cyclic nucleotide. In some experiments, 0.5 ml of each incubation mixture was also utilized for measurement of platelet aggregation or the release of [^{14}C]5-HT.

Determination of platelet cyclic [^{3}H]GMP and cyclic [^{3}H]AMP. Acidified samples containing recovery markers were mixed, allowed to stand overnight at 4°, and then centrifuged at $12,000 \times g$ for 2 min. The labeled cyclic nucleotides were isolated from the supernatants by chromatography, first on alumina and then on Dowex 50 resin, using a modification of the method of Jakobs *et al.* (33). The supernatants were applied to columns containing 1.5 g of alumina that had been washed with 5% (w/v) trichloracetic acid. The columns were then washed with an additional 12 ml of 5% trichloroacetic acid, 12 ml of water, and 2 ml of 0.2 M ammonium formate that had been adjusted to pH 6.0 with formic acid. The labeled cyclic nucleotides were then eluted with an additional 3 ml of the same ammonium formate solution. These eluates were acidified with 50 μl of 3.0 M HCl and applied to columns containing 5 ml (packed volume) of Dowex 50 resin (Bio-Rad AG 50W-

X8) that had been equilibrated with 0.05 M HCl. These columns were then washed with 6 ml of 0.05 M HCl and cyclic GMP was eluted with an additional 8 ml of 0.05 M HCl. After the columns were washed with two 4-ml aliquots of water, cyclic AMP was finally eluted in an additional two 4-ml portions of water. The eluates containing cyclic GMP (neutralized to pH 6 with HEPES and NaOH) and those containing cyclic AMP were lyophilized in scintillation vials and counted for ^3H and ^{14}C. The labeled cyclic AMP samples were counted in 0.5 ml of water and 8.0 ml of HP/b scintillant, whereas the labeled cyclic GMP samples were counted in a mixture of 0.5 ml of water and 8.0 ml of ACS scintillant, because of their high salt content. Following correction for background, quenching, ^{14}C crossover into the ^3H channel and recovery of ^{14}C-labeled cyclic nucleotides (about 50%), the ^3H-labeled cyclic nucleotides were expressed as percentages of the corresponding ^3H-labeled nucleoside triphosphates present in control platelets.

Measurement of [^{3}H]GTP and [^{3}H]ATP in platelets. Samples of control platelet suspension were mixed with 0.2 volumes of 30% (w/v) trichloroacetic acid. [^{14}C]ATP (about 30,000 dpm) was added to one sample and an equal amount of [^{14}C]GTP was added to another. Both samples were centrifuged at 12,000 × *g* for 2 min. Trichloroacetic acid was removed from the supernatants with 3 volumes of water-saturated diethyl ether. A sample of each aqueous extract (20 μl) was mixed with 0.1 μmol of unlabeled ATP and GTP, which were then separated by TLC in two dimensions on MN 300 HR cellulose plates. The first dimension solvent was *n*-butanol/acetone/acetic acid/14.8 M NH_3/H_2O (90:30:20:1:60 by volume) and the second dimension solvent was isobutyric acid/1 M NH_3/0.1 M EDTA (125:75:2 by volume). GTP and ATP were eluted from the cellulose with water and counted for ^3H and ^{14}C in HP/b scintillant. Values for platelet [^{3}H]ATP and [^{3}H]GTP were corrected in the same way as the ^3H-labeled cyclic nucleotides.

In some experiments, the specific radioactivities of the [^{3}H]GTP and [^{3}H]ATP in the platelet metabolic pool were determined in cytosol released by digitonin (34). Washed platelet suspension (10 ml; 4.2 × 10^9 platelets) was cooled to 0° and incubated for 10 min with 0.5 ml of 100 mM EDTA (adjusted to pH 8.0 with NaOH) and 0.28 ml of 1 mM digitonin in 0.154 M KCl. Following removal of the permeabilized platelets by centrifugation, the supernatant was extracted with trichloroacetic acid (final concentration 5%, w/v), recentrifuged, and applied, in a cold room, to a column containing a mixture of 40 mg of charcoal and 80 mg of celite. After the column was washed with water, nucleotides were eluted with 30 ml of solvent containing 19.2 ml of ethanol, 0.3 ml of 1 M NH_3, and water to volume. The eluate was dried by rotary evaporation, nucleotides were redissolved in 50 μl of H_2O, and the GTP and ATP were isolated by TLC in two dimensions, as described above. After elution from the cellulose, samples of GTP and ATP were counted for ^3H and the amounts present were determined spectrophotometrically, thus permitting calculation of their specific radioactivities and of the mass amounts of cyclic [^{3}H]GMP and cyclic [^{3}H]AMP formed from them.

Determination of platelet cyclic nucleotides by radioimmunoassay. Platelet cyclic GMP and cyclic AMP were separated, purified, and concentrated by a dual column procedure. Trichloroacetic acid extracts of unlabeled platelets (containing 6000 dpm of cyclic [^{3}H]GMP and of cyclic [^{3}H]AMP added to monitor cyclic nucleotide recoveries) were applied to columns containing 1 ml of Dowex 50 resin that had been equilibrated with 0.05 M HCl. Cyclic GMP was then eluted with 3 ml of 0.05 M HCl. After the columns were washed with 1 ml of H_2O, cyclic AMP was eluted with an additional 3 ml of H_2O. The eluates containing cyclic GMP or cyclic AMP were then applied to columns containing alumina (0.5 g) that had been washed with 4 ml of 0.2 M sodium acetate (pH 6.2) and 6 ml of 0.05 M HCl. After further washing of these columns with 2 ml of 0.05 M HCl, 4 ml of H_2O, and 0.75 ml of 0.2 M sodium acetate, the cyclic nucleotides were eluted with a final 1 ml of 0.2 M sodium acetate (recovery about 50%). Cyclic GMP and cyclic AMP were then acetylated and quantitated in separate radioimmunoassays (35).

Measurement of platelet aggregation and degranulation. These were studied at 37°C in a Payton aggregation module (Payton Associates, Scarborough, Ontario, Canada). Samples of the platelet incubation mixture (0.5 ml, containing 2 × 10^8 platelets) were stirred in siliconized aggregometer tubes with 10 nM PAF. The extent of aggregation was measured as the decrease in absorbance after 30 or 60 sec. When the release of granule [^{14}C]5-HT was determined, aggregation and degranulation were terminated by addition of ice-cold 0.154 M NaCl containing 1.5% formaldehyde and 5 mM EDTA. The samples were then centrifuged at 12,000 × *g* for 2 min and the [^{14}C]5-HT in the supernatant was determined by liquid scintillation counting in HP/b scintillant. The [^{14}C]5-HT released by PAF was expressed as a percentage of the total [^{14}C]5-HT in the platelets.

Statistics. In individual experiments, incubations were performed in triplicate or quadruplicate, as indicated, and all the results described were observed in at least three separate experiments. Values given are means ± standard errors. The significance of changes within experiments was evaluated by two-tailed unpaired *t* tests, whereas the significance of changes observed in multiple experiments was evaluated by two-tailed paired *t* tests after logarithmic transformation of the results.

Results

Prelabeling technique for simultaneous measurement of cyclic [^{3}H]AMP and cyclic [^{3}H]GMP: comparison with radioimmunoassays. Although the two-column procedure used in the present study (33) has been successfully used to measure changes in cyclic [^{3}H]AMP in rabbit platelets labeled with [^{3}H]adenine (36) or changes in cyclic [^{3}H]GMP in human platelets labeled with [^{3}H]guanine (37), these methods have not previously been combined or fully validated in rabbit platelets. Table 1 shows a direct comparison of the results obtained using prelabeling assays and radioimmunoassays to measure platelet cyclic AMP and cyclic GMP in parallel incubations of labeled and unlabeled platelets with SNP, PGE_1, or SNP and PGE_1. In this and two identical experiments, the specific activities of the [^{3}H]ATP and [^{3}H]GTP in the platelet metabolic (nongranular) pool were determined and the corresponding values for the pmol of cyclic [^{3}H]AMP and cyclic [^{3}H]GMP present were calculated. Under all experimental conditions used, these values were closely comparable to those obtained by radioimmunoassay, thus demonstrating the validity of the prelabeling assays for determining cyclic AMP and cyclic GMP in rabbit platelets (Table 1). In particular, these results eliminate the remote possibility that some of the changes in cyclic [^{3}H]AMP and cyclic [^{3}H]GMP caused by SNP or PGE_1 might reflect changes in the specific radioactivities of the precursor nucleoside triphosphates. Table 1 also shows some novel results. Thus, incubation of rabbit platelets with SNP not only increased platelet cyclic GMP but also caused small but significant increases in platelet cyclic AMP in the absence of PGE_1 and greatly potentiated the increases in cyclic AMP observed in the presence of PGE_1. In the three experiments in which both assays were performed, addition of 1 μM SNP without PGE_1 increased platelet cyclic AMP by 65 ± 7% and 93 ± 21%, as determined by the prelabeling assay and radioimmunoassay, respectively (mean values ± standard errors). These values are significantly different from the controls ($p < 0.05$) but not from each other. At the same time, addition of 1 μM SNP enhanced the increases in cyclic AMP caused by PGE_1 by 453 ± 44% and 693 ± 92% in these two assays; both values are highly significant ($p < 0.005$). The biological importance and mechanism of these effects are the subject of the experiments described in the remainder of this paper, in which the prelabeling assay alone

TABLE 1

Comparison of values obtained for platelet cyclic nucleotides using prelabeling assays and radioimmunoassays

Washed platelets were incubated with ^{3}H-labeled or unlabeled guanine and adenine. The former platelets were used to measure changes in cyclic [^{3}H]AMP and cyclic [^{3}H]GMP by the prelabeling technique and the latter, changes in cyclic AMP and cyclic GMP mass by radioimmunoassay (see Experimental Procedures). In both instances, samples of platelet suspension (4×10^8 platelets) were incubated for 0.5 min at 37° with the additions indicated (final volume, 1 ml). Incubations were terminated by addition of trichloroacetic acid; ^{14}C-labeled cyclic nucleotides were added to monitor the recoveries of cyclic [^{3}H]AMP and cyclic [^{3}H]GMP in the prelabeling assays and ^{3}H-labeled cyclic nucleotides were added to monitor the recoveries of cyclic AMP and cyclic GMP before radioimmunoassay. Cyclic nucleotides were then isolated and assayed as described in Experimental Procedures. Cyclic [^{3}H]AMP and cyclic [^{3}H]GMP determined in the prelabeling assays are expressed as percentages of the corresponding ^{3}H-labeled nucleoside triphosphate and as pmol/10^8 platelets, calculated from the specific activities of the [^{3}H]ATP and [^{3}H]GTP recovered from the platelet cytosol (see Experimental Procedures). Platelet cyclic AMP and cyclic GMP determined by radioimmunoassay are also expressed as pmol/10^8 platelets. All values are means ± standard errors from four identical incubation mixtures. Significant changes are shown.

Additions	Cyclic AMP			Cyclic GMP		
	Prelabeling assay		Radioimmunoassay	Prelabeling assay		Radioimmunoassay
	% of [^{3}H]ATP	pmol/10^8 platelets	pmol/10^8 platelets	% of [^{3}H]GTP	pmol/10^8 platelets	pmol/10^8 platelets
None	0.014 ± 0.000	7.2 ± 0.1	5.9 ± 0.7	0.011 ± 0.001	1.3 ± 0.2	0.3 ± 0.3
SNP (1.0 μM)	0.021 ± 0.000	10.9 ± 0.2*	11.0 ± 0.9*	0.056 ± 0.004	6.6 ± 0.5*	8.9 ± 1.1*
SNP (10 μM)	0.026 ± 0.000	14.0 ± 0.2*	11.7 ± 0.5*	0.293 ± 0.031	34.7 ± 3.7*	28.6 ± 1.4*
PGE_1 (0.04 μM)	0.053 ± 0.002	27.8 ± 1.2*	20.9 ± 1.4*	0.009 ± 0.001	1.0 ± 0.1	1.5 ± 0.6
PGE_1 (0.04 μM) + SNP (1.0 μM)	0.189 ± 0.003	99.7 ± 1.6*	103.3 ± 6.4*	0.047 ± 0.002	5.5 ± 0.2*	6.2 ± 1.3*

*$p < 0.01$.

was used. As shown in Table 1 and by the overall statistics given above, the standard errors observed in prelabeling assays were usually much smaller than those seen in the corresponding radioimmunoassays. The former assays were also much less expensive and time consuming.

In the prelabeling experiments on which this paper is based, labeling of [^{3}H]ATP and [^{3}H]GTP averaged 2.6×10^6 and 1.6×10^6 dpm, respectively, in 2×10^8 platelets (the number used in most individual incubation mixtures). No changes in platelet [^{3}H]ATP or [^{3}H]GTP occurred during the course of experiments. Basal values for platelet cyclic [^{3}H]AMP and cyclic [^{3}H]GMP averaged 0.017 ± 0.001% and 0.012 ± 0.001% of the corresponding [^{3}H]nucleoside triphosphates (means ± standard errors from 25 experiments). Whereas almost all the basal cyclic [^{3}H]AMP isolated by the two-column method described under Experimental Procedures co-chromatographed with authentic cyclic [^{14}C]AMP on subsequent TLC (first dimension solvent), the purity of the cyclic [^{3}H]GMP isolated under basal conditions varied from 40 to 100%, as determined by TLC. This probably reflects the fact that basal cyclic GMP levels in rabbit platelets are very low and indeed are barely measurable by radioimmunoassay (Table 1). However, all the cyclic [^{3}H]AMP isolated after incubation of the platelets with PGE_1 or SNP and all the cyclic [^{3}H]GMP formed in response to SNP co-chromatographed with the authentic ^{14}C-labeled cyclic nucleotides, when their purity was checked by an additional TLC step. These findings are consistent with the close correlation between the results obtained in the prelabeling assays and radioimmunoassays.

Effects of different concentrations of SNP. This compound caused dose-dependent increases in both cyclic [^{3}H]GMP and cyclic [^{3}H]AMP in rabbit platelets (Fig. 1). Relative to the basal values, the increases in cyclic [^{3}H]GMP were by far the greater, from 0.011 ± 0.003% of platelet [^{3}H]GTP to 0.028 ± 0.005%, 0.093 ± 0.017% and 0.707 ± 0.176% after 0.5-min incubations with 0.1, 1.0, and 10 μM SNP, respectively (mean values ± standard errors from 10 experiments). At the same time, cyclic [^{3}H]AMP increased from 0.021 ± 0.003% of platelet [^{3}H]ATP to 0.029 ± 0.004%, 0.044 ± 0.009%, and 0.062 ± 0.015% of the [^{3}H]ATP (mean values ± standard errors). Despite some variation in cyclic [^{3}H]nucleotide levels between experiments, these increases in cyclic [^{3}H]AMP were highly significant in each experiment (e.g., Table 1 and Fig. 1) or when all the results were analyzed by a paired t test ($p < 0.001$). Analysis of the time course of cyclic [^{3}H]nucleotide formation caused by SNP showed that the maximum increase in cyclic [^{3}H]GMP was reached earlier when higher concentrations were used and, with 10 μM SNP, declined rapidly after a 30-sec incubation (Fig. 1d). The highest concentration of SNP tested (100 μM; data not shown) caused even larger increases in cyclic [^{3}H]GMP in short incubations but little further increase in cyclic [^{3}H]AMP. The accumulation of cyclic [^{3}H]AMP tended to lag behind that of cyclic [^{3}H]GMP and, with 10 μM SNP, continued to increase after cyclic [^{3}H]GMP had begun to decline (Fig. 1d). These results suggested that the increases in cyclic GMP caused by SNP might stimulate the accumulation of cyclic AMP and that cyclic AMP might play a role in some of the inhibitory effects of SNP on platelet function. To test the latter possibility, we used DDA, an inhibitor of adenylate cyclase that attenuates the effects on platelets of compounds that increase platelet cyclic AMP (38). However, the inhibition of PAF-induced platelet aggregation and degranulation by 0.1–10 μM SNP was not significantly decreased by 200 μM DDA under the experimental conditions used in the present study (Figs. 2 and 3a), although DDA did cause significant inhibition of the accumulation of cyclic [^{3}H]AMP ($p < 0.05$, paired t test) which varied from 62% with 0.1 μM SNP to 38% with 10 μM SNP (Fig. 3b). DDA did not affect the increases in cyclic [^{3}H]GMP caused by SNP (Fig. 3c). These results suggest that cyclic GMP is likely to mediate most or all of the inhibition of platelet function caused by addition of SNP alone to rabbit platelets.

Synergistic effects of SNP and PGE_1. Although incubation of platelets with SNP alone caused dose-dependent inhibitions of PAF-induced platelet aggregation and degranulation, much more potent inhibitions were seen with 0.1–1.0 μM SNP in the presence of a concentration of PGE_1 chosen to have little effect alone (Figs. 2 and 3a). Thus, when SNP was added with 0.02 μM PGE_1, which inhibited platelet aggregation by only 6 ± 3%, the inhibition caused by 0.1 μM SNP was increased from 14 ± 3% to 62 ± 6% (mean values ± standard errors from five experiments). This synergism was less conspicuous with 10 μM SNP, which caused a potent inhibition of platelet aggregation

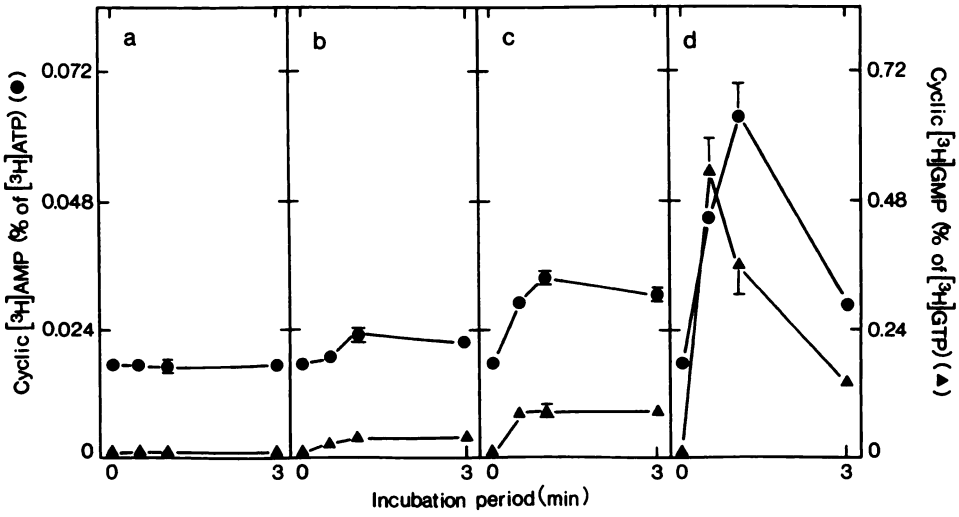

Fig. 1. Time course of the SNP-induced accumulation of cyclic [^{3}H]GMP and cyclic [^{3}H]AMP in platelets. Platelets were isolated and labeled with [^{3}H]guanine and [^{3}H]adenine (see Experimental Procedures). Samples of suspension containing 2×10^8 labeled platelets were incubated at 37° (final volume, 0.5 ml). Final concentrations of SNP were: a, none; b, 0.1 μM; c, 1.0 μM; and d, 10.0 μM. The incubations were terminated after 0, 0.5, 1.0, or 3.0 min by addition of trichloroacetic acid. Cyclic [^{3}H]GMP (▲) and cyclic [^{3}H]AMP (●) were isolated and expressed as percentages of the total platelet [^{3}H]GTP and [^{3}H]ATP, respectively; values are means ± standard errors from three identical incubation mixtures.

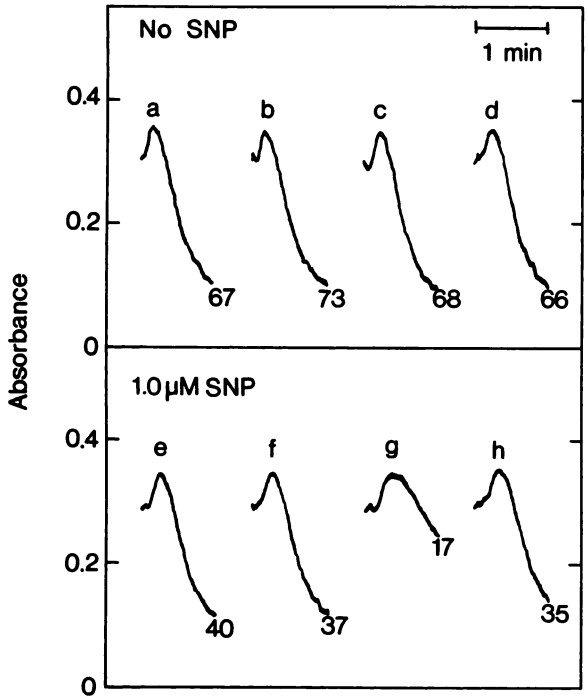

Fig. 2. Inhibition by SNP of PAF-induced platelet aggregation and [^{14}C] 5-HT release: synergism between SNP and PGE_1 and reversal of the effects of PGE_1 by DDA. Labeled platelets containing [^{14}C]5-HT (2×10^8 platelets in a final volume of 0.5 ml) were incubated at 37° without (a–d) or with 1.0 μM SNP (e–h) and the following other additions: a and e, none; b and f, 200 μM DDA; c and g, 0.02 μM PGE_1; d and h, 0.02 μM PGE_1 and 200 μM DDA. DDA was added 0.5 min before any SNP or PGE_1 and the incubations were continued for an additional 0.5 min. Platelet aggregation was then induced by the addition of 10 nM PAF, with stirring. Aggregation was recorded for 1 min, at which time the release of [^{14}C]5-HT was determined (see Experimental Procedures). Each incubation was performed in triplicate; representative aggregation traces and the corresponding values for release of [^{14}C]5-HT (as percentage of total platelet ^{14}C) are shown.

by itself (Fig. 3a). However, synergistic interactions between PGE_1 and SNP with respect to cyclic [^{3}H]AMP formation were observed with all SNP concentrations (Fig. 3b). The presence of PGE_1 did not, however, affect the increases in cyclic [^{3}H]GMP caused by SNP (Table 1 and Fig. 3c). The relationship between the increases in cyclic [^{3}H]AMP and cyclic [^{3}H]GMP found in platelets incubated with different SNP concentrations in the presence and absence of 0.02 μM PGE_1 is shown in Fig. 4. In both cases, apparent saturation effects were observed; half-maximal increases in cyclic [^{3}H]AMP were detected with increases in cyclic [^{3}H]GMP equivalent to 0.05–0.1% of platelet [^{3}H]GTP. This type of relationship is consistent with a role for cyclic GMP in inducing the accumulation of cyclic AMP.

The above results suggested that the synergistic inhibitory effects of SNP and PGE_1 on platelet responses to PAF were mediated by an increased accumulation of cyclic [^{3}H]AMP. In support of this hypothesis, addition of DDA was able to reduce the inhibition of aggregation caused by 0.1 μM SNP in the presence of 0.02 μM PGE_1 by about 50% (Fig. 3a). This effect was associated with a marked decrease in cyclic [^{3}H]AMP accumulation (Fig. 3b). Although DDA caused similar (about 65%) decreases in the cyclic [^{3}H]AMP that accumulated when higher concentrations of SNP were added with PGE_1, its ability to suppress the inhibitory effects of these drug combinations decreased progressively as the SNP concentration increased (compare Fig. 3, a and b). This may reflect a larger contribution of cyclic GMP to the inhibitory effects of SNP added with PGE_1 at the higher SNP concentrations studied.

Synergistic effects of SIN-1 and PGE_1. To determine whether the ability of SNP to enhance the accumulation of platelet cyclic [^{3}H]AMP is a general property of nitrovasodilators, parallel experiments were carried out with SIN-1. The results showed that when added alone, 10 μM SIN-1 had effects almost identical to those obtained with 1 μM SNP, causing a marked increase in platelet cyclic [^{3}H]GMP and a small but significant increase in cyclic [^{3}H]AMP. Together, SIN-1 and PGE_1 had a synergistic inhibitory effect on platelet aggregation (not shown) and degranulation (Fig. 5a), which was associated with a marked increase in the accumulation of cyclic [^{3}H]AMP. DDA attenuated both of these synergistic effects (Fig. 5). With 100 μM SIN-1, effects similar to those obtained with 10 μM SNP were observed (not shown).

Synergistic effects of SNP and adenosine. To establish whether the interactions between nitrovasodilators and PGE_1 described above were specific to PGE_1, cyclic [^{3}H]nucleotide accumulation was also measured in experiments in which adenosine, another activator of platelet adenylate cyclase (39), replaced PGE_1. Addition of 1 μM adenosine alone increased

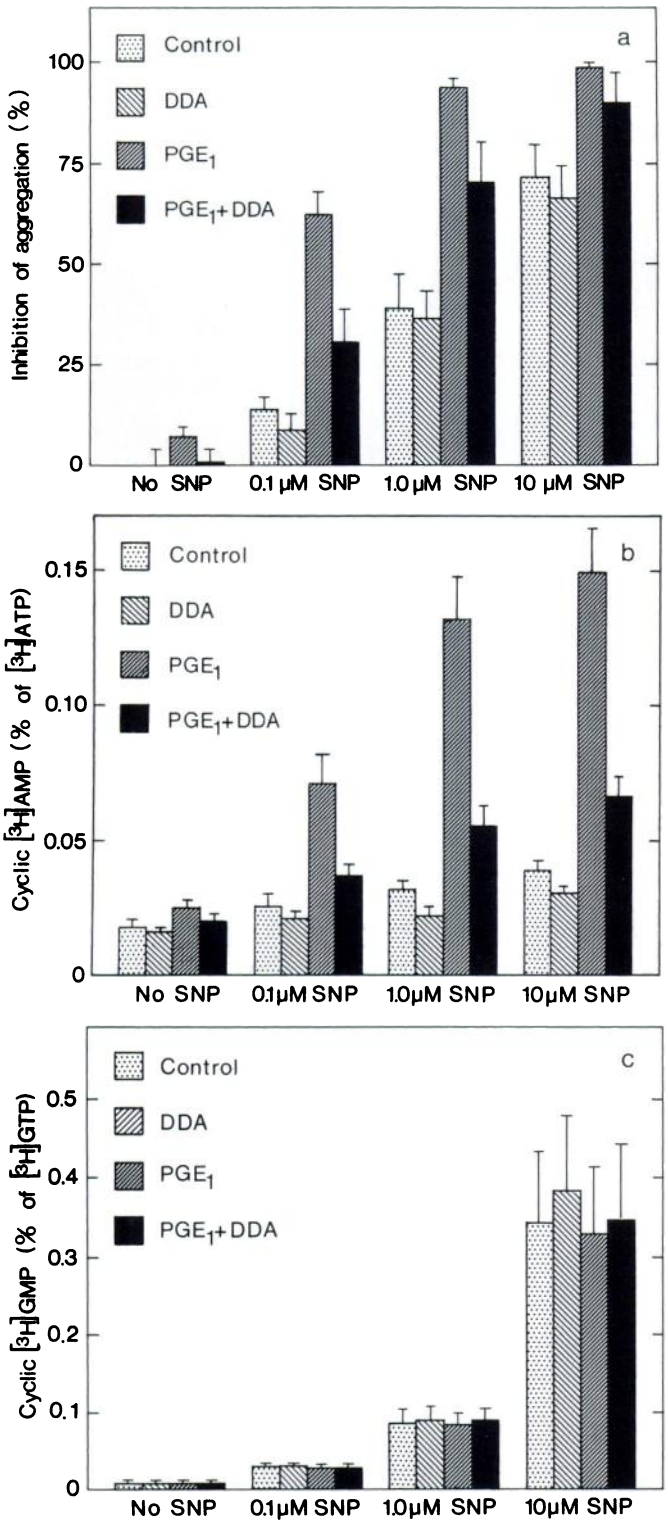

Fig. 3. Effects of different concentrations of SNP on platelet aggregation, cyclic [^{3}H]AMP, and cyclic [^{3}H]GMP in the absence and presence of DDA and PGE_1. Platelets were labeled with [^{3}H]guanine and [^{3}H]adenine (see Experimental Procedures). Samples of the suspension were incubated at 37° for 0.5 min with SNP (0–10 μM), in the absence or presence of 0.02 μM PGE_1, after an initial 0.5-min incubation with 200 μM DDA or vehicle, as indicated. a, After these incubations, part of each sample (0.5 ml, 2×10^8 platelets) was transferred to an aggregometer tube containing PAF (final concentration, 10 nM), and the platelets were stirred for 0.5 min. Aggregation was recorded (see Fig. 2) and the percentage inhibition was determined from the changes in absorbance after 0.5 min in the presence and absence of the compounds studied. Values are means ± standard errors from five separate experiments with different platelet preparations. b and c, After the above incubations, another part of each sample (0.5 ml, 2×10^8 platelets) was extracted with trichloroacetic acid. Cyclic [^{3}H]AMP (b) and cyclic [^{3}H]GMP (c) were isolated and expressed as percentages of the corresponding [^{3}H]nucleoside triphosphates; values are means ± standard errors from four and five separate experiments, respectively.

platelet cyclic [^{3}H]AMP no more than did 1 μM SNP, but together these compounds had a much more marked effect on cyclic [^{3}H]AMP accumulation (Table 2), comparable with that observed in the presence of both PGE_1 and SNP (Table 1). Adenosine had no significant effect on platelet cyclic [^{3}H]GMP. These findings suggest that nitrovasodilators may potentiate the effects on platelets of all compounds that activate platelet adenylate cyclase.

Effects of hemoglobin on the actions of SNP. Addition of hemoglobin, which binds nitric oxide (40), completely prevented the increases in both cyclic [^{3}H]GMP and cyclic [^{3}H]AMP caused by addition of 1 μM SNP to labeled platelets (Fig. 6). Hemoglobin was also effective when added 0.5 min after SNP and reversed these increases in cyclic [^{3}H]nucleotides, indicating that their maintenance required the continued presence of nitric oxide. The synergistic effect of SNP and PGE_1 on cyclic [^{3}H]AMP accumulation was likewise prevented or reversed by hemoglobin (Fig. 6). Similar effects of hemoglobin were obtained in experiments with 10 μM SIN-1 in the presence or absence of PGE_1 (not shown). These results indicate that formation of nitric oxide is responsible for the increases in both cyclic [^{3}H]GMP and cyclic [^{3}H]AMP caused by nitrovasodilators and that these effects are freely reversible.

Comparison of the effects of cilostamide and SNP. Preliminary experiments showed that cilostamide, a selective inhibitor of the low K_m cyclic AMP phosphodiesterase of human and rabbit platelets (41), increased platelet cyclic [^{3}H]AMP in a dose-dependent manner, causing a near-maximal effect at 10

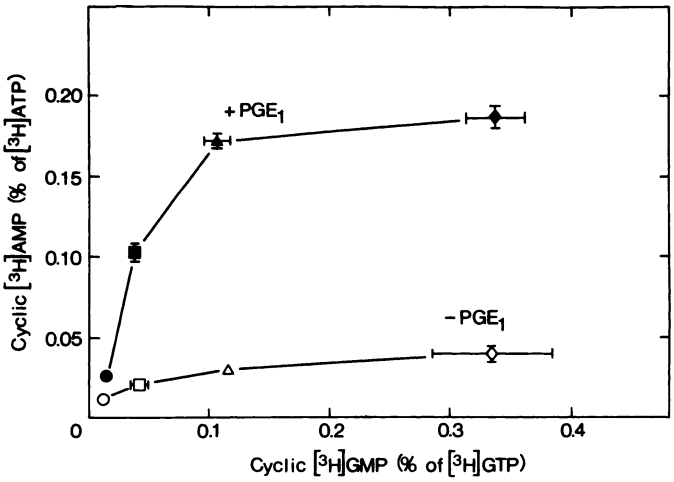

Fig. 4. Relationships between the increases in platelet cyclic [^{3}H]GMP and cyclic [^{3}H]AMP caused by SNP in the absence and presence of PGE_1. Platelets were labeled with [^{3}H]guanine and [^{3}H]adenine (see Experimental Procedures). Samples of the suspension of labeled platelets were incubated for 0.5 min at 37° with no SNP (*circles*), 0.1 μM SNP (*squares*), 1.0 μM SNP (*triangles*), or 10 μM SNP (*diamonds*), in the absence (*open symbols*) or presence (*closed symbols*) of 0.02 μM PGE_1. Incubations were then terminated by the addition of trichloroacetic acid. Cyclic [^{3}H]GMP and cyclic [^{3}H]AMP were isolated and expressed as percentages of the corresponding [^{3}H]nucleoside triphosphates; values are means ± standard errors from three identical incubation mixtures.

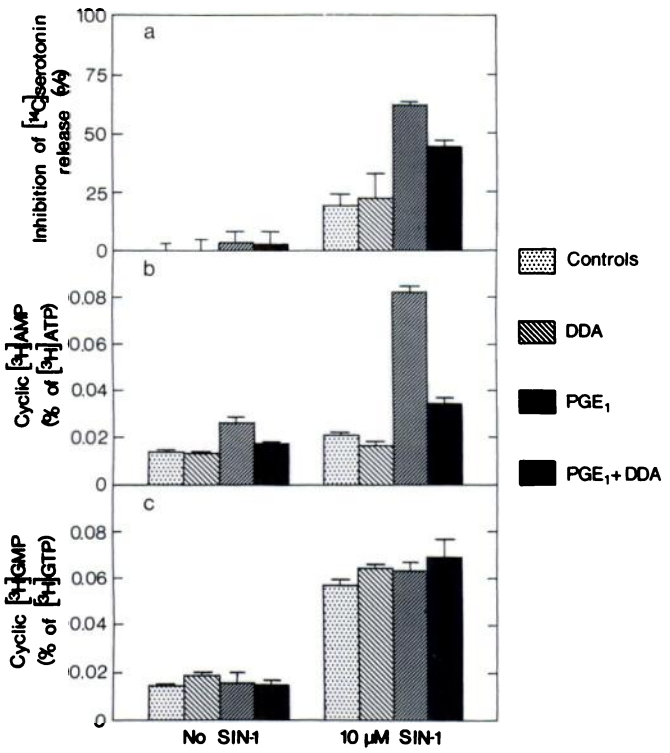

Fig. 5. Effects of SIN-1 on release of platelet [^{14}C]5-HT and on platelet cyclic [^{3}H]AMP and cyclic [^{3}H]GMP: synergism between SIN-1 and PGE_1 and reversal of the effects of PGE_1 by DDA. Platelets were labeled with [^{3}H]guanine, [^{3}H]adenine, and [^{14}C]5-HT (see Experimental Procedures). Samples of the suspension were incubated at 37° for 0.5 min with 10 μM SIN-1, in the absence or presence of 0.02 μM PGE_1, after an initial 0.5-min incubation with 200 μM DDA or vehicle, as indicated. a, After these incubations, part of each sample (0.5 ml, 2 × 10^8 platelets) was transferred to an aggregometer tube containing PAF (final concentration, 10 nM) and the platelets were stirred for 0.5 min, at which time release of [^{14}C]5-HT was measured (see Experimental Procedures). The percentage inhibition of release of [^{14}C]5-HT was determined with respect to controls containing PAF alone. Values are means ± standard errors from three identical incubation mixtures. b and c, After the above incubations, another part of each sample (0.5 ml, 2 × 10^8 platelets) was extracted with trichloroacetic acid. Cyclic [^{3}H]AMP (b) and cyclic [^{3}H]GMP (c) were isolated and expressed as percentages of the corresponding [^{3}H]nucleoside triphosphates; values are means ± standard errors from three identical incubation mixtures.

TABLE 2

Synergism between SNP and adenosine with respect to increases in platelet cyclic [^{3}H]AMP but not cyclic [^{3}H]GMP

Platelets were labeled with [^{3}H]guanine and [^{3}H]adenine (see Experimental Procedures). Samples of suspension containing 2 × 10^8 platelets were incubated for 0.5 min at 37° with 1.0 μM SNP and/or 1.0 μM adenosine, as indicated (final volume, 0.5 ml). Incubations were terminated with trichloroacetic acid. Cyclic [^{3}H]GMP and cyclic [^{3}H]AMP were isolated and each was expressed as a percentage of the corresponding [^{3}H]nucleoside triphosphate (see Experimental Procedures). Values are means ± standard errors from four identical incubation mixtures. Significant increases in cyclic [^{3}H]nucleotides are shown.

Additions	Cyclic [^{3}H]AMP	Cyclic [^{3}H]GMP
	% of [^{3}H]ATP	*% of [^{3}H]GTP*
None	0.014 ± 0.001	0.007 ± 0.000
SNP	0.036 ± 0.001*	0.053 ± 0.006*
Adenosine	0.031 ± 0.001*	0.010 ± 0.003
SNP + adenosine	0.276 ± 0.005*	0.037 ± 0.005*

* $p < 0.005$.

μM. These increases in platelet cyclic [^{3}H]AMP were comparable in magnitude to those observed in the presence of 0.1–10 μM SNP. Moreover, cilostamide and SNP each greatly potentiated the increase in cyclic [^{3}H]AMP caused by a low concentration of PGE_1 (Table 3). Finally, in the presence of 10 μM cilostamide, SNP had no additional effect on platelet cyclic [^{3}H]AMP, whether PGE_1 was included in the incubation medium or not (Table 3). These results indicate that the increases in platelet cyclic [^{3}H]AMP caused by SNP result from an inhibition of the cyclic AMP phosphodiesterase that is also the target of cilostamide. The effects of SNP and cilostamide differ in that the latter does not affect basal cyclic [^{3}H]GMP levels in platelets and, at 10 μM, only slightly increased the cyclic [^{3}H]GMP that accumulated in response to SNP (Table 3). The above findings raise the possibility that the cyclic GMP formed in response to SNP increases platelet cyclic AMP by inhibiting the low K_m cyclic AMP phosphodiesterase. Such an effect has been well documented in studies with the purified enzyme (30) but has not previously been observed in intact cells.

Effects of M&B 22,948 on platelet cyclic [^{3}H]nucleotides. To investigate the possible role of cyclic GMP in the regulation of platelet cyclic AMP phosphodiesterase, we studied the changes in platelet cyclic [^{3}H]GMP and cyclic [^{3}H]AMP caused by M&B 22,948, a selective inhibitor of cyclic GMP phosphodiesterase (42). At 10 μM, M&B 22,948 had no significant effect on cyclic [^{3}H]GMP levels in resting platelets, suggesting that there is little turnover of cyclic GMP under basal conditions. Platelet cyclic [^{3}H]AMP was also unaffected by 10 μM M&B 22,948 alone. However, in the presence of SNP, 1–10 μM M&B 22,948 caused significant increases in both the cyclic [^{3}H]GMP and cyclic [^{3}H]AMP that accumulated in labeled platelets (Fig. 7). The increases in platelet cyclic [^{3}H]AMP and cyclic [^{3}H]GMP observed with all combinations of SNP and M&B 22,948 conformed to a single hyperbolic relationship, which yielded a linear Hill plot with a slope close to 1 (Fig. 8). A half-maximal increase in cyclic [^{3}H]AMP required an increase in platelet cyclic [^{3}H]GMP equivalent to 0.1% of platelet [^{3}H]GTP. Because the same relationship was found between the increases in platelet cyclic [^{3}H]AMP and cyclic [^{3}H]GMP caused by SNP, whether M&B 22,948 was present or not, M&B 22,948 is likely to have enhanced cyclic [^{3}H]AMP accumulation solely by inhibiting cyclic GMP phosphodiesterase and so increasing the amount of cyclic GMP present. These results support the hypothesis that the increases in platelet cyclic [^{3}H]AMP caused by SNP are secondary to increases in cyclic GMP, which acts by inhibiting the low K_m cyclic AMP phosphodiesterase.

Discussion

By using both sensitive prelabeling techniques and radioimmunoassays, we have shown that the nitrovasodilator SNP causes small, at most 3-fold, increases in cyclic AMP in rabbit platelets, in addition to the expected large increases in cyclic GMP. These two assays gave equivalent results, but the prelabeling assay, which was less subject to error, was used in the majority of experiments. The increases in platelet cyclic [^{3}H]AMP caused by SNP were of a similar magnitude to those previously measured by radioimmunoassay in human and rabbit platelets (12, 15) and there is no reason to doubt their reality, although some other workers have failed to detect any change (13). However, the attenuation of these increases in cyclic [^{3}H]AMP by DDA, an inhibitor of adenylate cyclase (38),

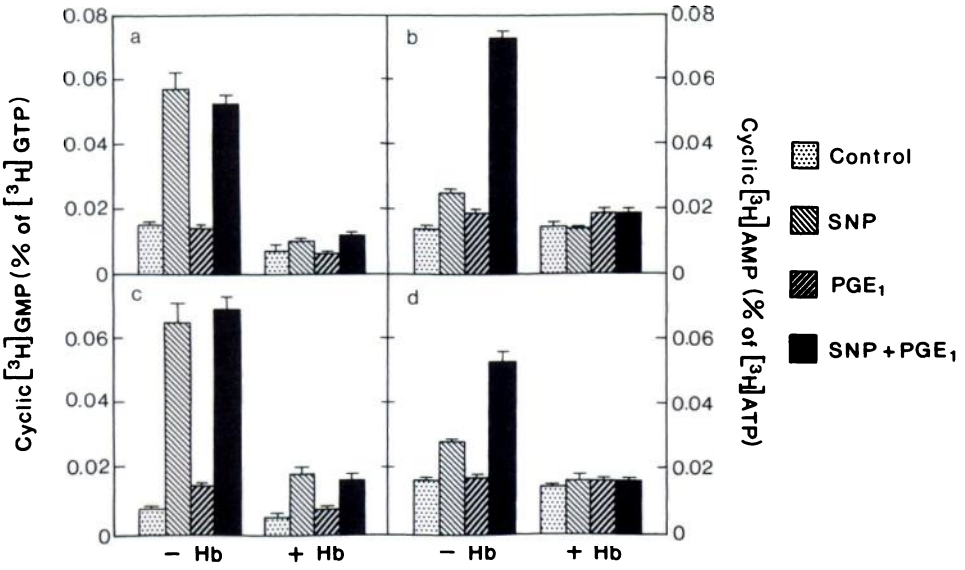

Fig. 6. Inhibition by hemoglobin of the increases in cyclic [^{3}H]GMP and cyclic [^{3}H]AMP induced by SNP in the absence or presence of PGE_1. Platelets were labeled with [^{3}H]guanine and [^{3}H]adenine (see Experimental Procedures). a and b, Samples of suspension (0.5 ml, 2 × 10^8 platelets) were incubated without hemoglobin or with 10 μM hemoglobin for 0.5 min at 37° and then for an additional 0.5 min with either no further addition, 1.0 μM SNP, 0.02 μM PGE_1, or 1.0 μM SNP and 0.02 μM PGE_1, as indicated. c and d, Samples of suspension (0.5 ml, 2 × 10^8 platelets) were incubated for 0.5 min at 37° with SNP and/or PGE_1 (as above) but in the absence of hemoglobin. Following this incubation, 10 μM hemoglobin or an equal volume of 0.154 M NaCl was added to the samples, which were then incubated for an additional 1.5 min before addition of trichloroacetic acid. Cyclic [^{3}H]GMP (a and c) and cyclic [^{3}H]AMP (b and d) were determined; values are means ± standard errors from three identical incubation mixtures.

TABLE 3

Comparison of the effects of SNP and cilostamide on platelet cyclic [^{3}H]nucleotides

Platelets were washed and labeled with [^{3}H]guanine and [^{3}H]adenine (see Experimental Procedures). Samples of suspension containing 4 × 10^8 platelets were incubated for 0.5 min at 37° with 1.0 μM SNP, 10 μM cilostamide, and 0.02 μM PGE_1, as indicated (final volume, 1.0 ml). All samples contained 0.2% (v/v) dimethyl sulfoxide. Incubations were terminated by addition of trichloroacetic acid. Cyclic [^{3}H]GMP and cyclic [^{3}H]AMP were isolated and each was expressed as a percentage of the corresponding [^{3}H]nucleoside triphosphate (see Experimental Procedures). Values are means ± standard errors from three identical incubation mixtures. Significant increases in cyclic [^{3}H]nucleotides are shown.

Additions	Cyclic [^{3}H]AMP	Cyclic [^{3}H]GMP
	% of [^{3}H]ATP	% of [^{3}H]GTP
None	0.011 ± 0.000	0.014 ± 0.000
SNP	0.022 ± 0.002*	0.166 ± 0.007*
Cilostamide	0.025 ± 0.000*	0.015 ± 0.002
SNP + cilostamide	0.026 ± 0.001*	0.208 ± 0.004*
PGE_1	0.028 ± 0.001*	0.014 ± 0.050
PGE_1 + SNP	0.241 ± 0.005*	0.153 ± 0.007*
PGE_1 + cilostamide	0.391 ± 0.012*	0.016 ± 0.002
PGE_1 + SNP + cilostamide	0.399 ± 0.015*	0.198 ± 0.003*

*$p < 0.01$.

without a corresponding effect on platelet aggregation, indicates that cyclic AMP did not play a significant role in the inhibition of platelet function by SNP, when this compound alone was incubated with rabbit platelets. Presumably, cyclic GMP mediated the inhibitory action of SNP under these conditions. This conclusion contrasts with preliminary results from similar experiments with human platelets, in which DDA reduced the effects of SNP (16). The full reason for this species difference is not clear, although it presumably reflects a difference in the relative effectiveness of cyclic AMP and cyclic GMP as inhibitory second messengers in human and rabbit platelets. In the presence of a low PGE_1 concentration, the role of cyclic AMP was much clearer; SNP then caused very large additional increases in platelet cyclic AMP, which with the lower concentrations of this compound were associated with much more marked inhibitions of platelet aggregation and degranulation. Because DDA reduced both these increases in platelet cyclic [^{3}H]AMP and the associated inhibitions of platelet function without affecting cyclic [^{3}H]GMP levels, it follows that the synergistic effects of low concentrations of SNP and PGE_1 were mediated by cyclic AMP. Similar results were obtained when SIN-1, the active metabolite of molsidomine, was used in place of SNP, suggesting that the phenomenon we have observed reflects a general property of nitrovasodilator drugs.

In an earlier paper from this laboratory (12), it was suggested that the increases in platelet cyclic AMP caused by SNP might result from the reported ability of guanylate cyclase to make some use of ATP as a substrate (43). This cannot be the case for three reasons. First, cyclic [^{3}H]AMP formation lagged behind that of cyclic [^{3}H]GMP; second, DDA decreased the accumulation of cyclic [^{3}H]AMP but not that of cyclic [^{3}H]GMP; third, the relationship between cyclic [^{3}H]AMP and cyclic [^{3}H]GMP formation at different SNP concentrations was hyperbolic rather than linear. Moreover, our present studies suggest an entirely different mechanism for the increases in platelet cyclic AMP caused by nitrovasodilators. Because these compounds greatly enhanced the accumulation of cyclic [^{3}H]AMP that occurred in the presence of activators of adenylate cyclase, such as PGE_1 or adenosine, but caused no further increase in the presence of cilostamide, an inhibitor of platelet cyclic AMP phosphodiesterase (41), they must act by inhibiting cyclic AMP breakdown and not by promoting cyclic AMP formation. Presumably, this reflects a direct or indirect inhibition of the low K_m cyclic AMP phosphodiesterase, which is the target of cilostamide and accounts for most of cyclic AMP breakdown in both human and rabbit platelets (30, 41). A proteolyzed form of this enzyme has been purified from human platelets (28) and was subsequently shown to be related immunologically to a low K_m cyclic AMP phosphodiesterase from bovine heart (29). In both instances, the purified enzyme was found to be inhibited by submicromolar concentrations of cyclic GMP (28, 44), and this enzyme has now been designated the cyclic GMP-inhibited phosphodiesterase (30). These findings suggested to us that increases in platelet cyclic AMP caused by nitrovasodilators might be mediated indirectly by cyclic GMP. A number of our experiments support this hypothesis. Thus, addition of hemoglobin, which binds nitric oxide released by nitrovasodilators and so blocks the activation of guanylate

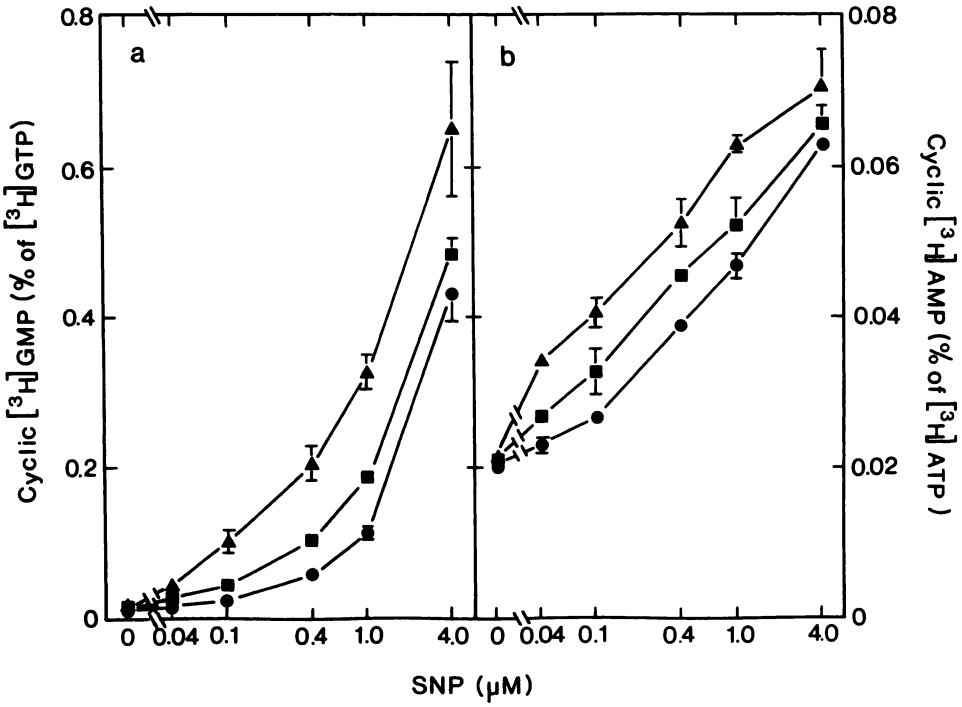

Fig. 7. Effects of SNP and M&B 22,948 on platelet cyclic [^{3}H]GMP and cyclic [^{3}H]AMP. Platelets were labeled with [^{3}H]guanine and [^{3}H]adenine (see Experimental Procedures). Samples of the suspension (4×10^8 platelets) were incubated for 0.5 min at 37° with 0–4.0 μM SNP and the following additions (final volume, 1.0 ml): no M&B 22,948 (●), 2.5 μM M&B 22,948 (■), or 10 μM M&B 22,948 (▲). All samples contained 0.2% (v/v) dimethyl sulfoxide. Incubations were then terminated by addition of trichloroacetic acid. Cyclic [^{3}H]GMP and cyclic [^{3}H]AMP were isolated and expressed as percentages of the corresponding [^{3}H]nucleoside triphosphates; values are means ± standard errors from three identical incubation mixtures.

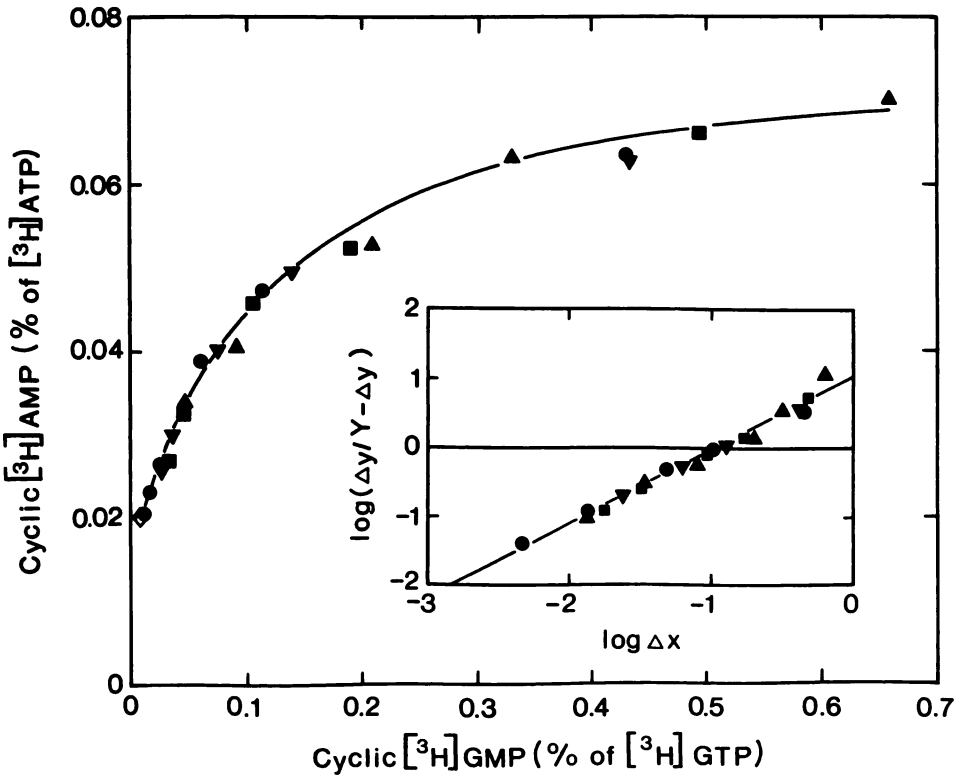

Fig. 8. Relationship between the increases in platelet cyclic [^{3}H]GMP and cyclic [^{3}H]AMP caused by SNP in the absence and presence of M&B 22,948. This figure is based on the results shown in Fig. 7, from incubations with 0.04–4 μM SNP, with the addition of values for cyclic [^{3}H]GMP and cyclic [^{3}H]AMP obtained in the same experiment in incubations with SNP and 1.0 μM M&B 22,948 (▼). The initial values of the cyclic [^{3}H]nucleotides are also shown (◇). *Inset*, Hill plot relating the increases in cyclic [^{3}H]AMP (Δy) to the increases in cyclic [^{3}H]GMP (Δx). Linear regression gave a Hill slope of 1.06 and a log Δx intercept of −0.97 ($r^2 = 0.984$).

cyclase by these compounds (10), prevented the accumulation of both cyclic GMP and cyclic AMP. This shows that SNP and SIN-1 do not directly inhibit cyclic AMP phosphodiesterase but does not eliminate the formal possibility that nitric oxide does so. More convincingly, we found that a selective inhibitor of platelet cyclic GMP phosphodiesterase, M&B 22,948 (42), enhanced the accumulation of cyclic [^{3}H]AMP caused by low SNP concentrations, although, in contrast to cilostamide, it did not affect basal platelet cyclic [^{3}H]AMP levels. Moreover, the relationship between the cyclic [^{3}H]GMP and cyclic [^{3}H]AMP accumulating in response to increasing SNP concentrations was unaffected by addition of M&B 22,948. These observations are most simply explained if cyclic [^{3}H]AMP accumulation is a function of cyclic [^{3}H]GMP accumulation. Indeed, the hyperbolic relationship observed suggests that cyclic GMP acts at a site that is saturable at low concentrations of this cyclic nucleotide. Thus, a half-maximal effect of cyclic [^{3}H]GMP on cyclic [^{3}H]AMP accumulation was seen with an in-

crease in the former that was equivalent to only about 0.1% of the [^{3}H]GTP present (Fig. 8), which corresponds to some 12 pmol of cyclic GMP/10^9 platelets (from Table 1). These results provide the first evidence that cyclic GMP inhibits cyclic AMP breakdown in intact platelets and confirms speculations that the cyclic GMP-inhibited phosphodiesterase might represent a target through which cyclic GMP mediates some of its biological effects (28, 30). Our findings are complementary to indications that cyclic GMP may enhance cyclic AMP breakdown by a cyclic GMP-stimulated cyclic nucleotide phosphodiesterase in some other cell types (30, 45). Recent studies have shown that the cyclic GMP-inhibited phosphodiesterase from human platelets can be stimulated by cyclic AMP-dependent phosphorylation (46, 47). However, our results cast some doubt on the importance of this effect in intact rabbit platelets. Thus, the markedly synergistic effects of SNP and PGE_1 on cyclic [^{3}H]AMP accumulation indicate that inhibition of cyclic AMP phosphodiesterase activity by cyclic GMP is the dominant regulatory mechanism. Because a similar synergism is seen in human platelets (16), the same conclusion applies in that species.

PGE_1 can be regarded as a stable analog of PGI_2, because both compounds stimulate platelet adenylate cyclase through the same receptor (23). It is PGI_2, synthesized by the vascular endothelium, that is likely to be one of the most important local inhibitors of platelet aggregation under physiological conditions (23, 36). Nitric oxide, either released from nitrovasodilator drugs (10) or generated endogenously by the vascular endothelium as the major component of EDRF (17–19), also appears to decrease platelet responsiveness *in vivo* (6–9, 48, 49). It follows that, because both PGI_2 and nitrovasodilators (24, 27) and PGI_2 and EDRF (25, 26) act synergistically to inhibit platelet aggregation, the cyclic AMP-dependent mechanism that we have described may have important effects on platelet function, both enhancing the antithrombotic action of nitrovasodilator drugs and providing a basis for the physiological limitation of the growth of hemostatic plugs at sites of vascular injury. A similar mechanism could operate in other cells exposed to both nitric oxide and an activator of adenylate cyclase, provided cyclic AMP breakdown depends largely on the cyclic GMP-inhibited phosphodiesterase. An important question is whether this is the case in vascular smooth muscle. The limited evidence available is equivocal; a synergistic effect of PGI_2 and EDRF on relaxation has been observed with pig coronary artery (50) but no synergism between a PGI_2 analog and SNP was seen with rabbit coeliac and mesenteric arteries (51), suggesting species differences. Cyclic nucleotide measurements in vascular smooth muscle have indicated that cyclic GMP rather than cyclic AMP mediates relaxation induced by nitrovasodilators (10), but the possibly that the latter may also increase the cyclic AMP accumulation caused by PGI_2 or adenosine does not appear to have been investigated. If such synergism does not occur in human vascular smooth muscle, appropriate combinations of low doses of nitrovasodilators and PGI_2 analogs could, in principle, exert selective effects on platelet function that might have therapeutic applications.

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