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Role of cGMP and cGMP-dependent protein kinase in nitrovasodilator inhibition of agonist-evoked calcium elevation in human platelets

(protein phosphorylation/cyclic nucleotides/calcium signaling/receptor-operated calcium channels)

JÖRG GEIGER*, CHRISTINE NOLTE*, ELKE BUTT*, STEWART O. SAGE†, AND ULRICH WALTER*‡

*Medizinische Universitätsklinik, Klinische Forschergruppe, Josef-Schneider-Strasse 2, D-8700 Würzburg, Federal Republic of Germany, and †Physiological Laboratory, Downing Street, Cambridge, CB2 3EG, United Kingdom

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ABSTRACT **Most platelet agonists activate and elevate the cytosolic free calcium concentration in human platelets through receptor-dependent mechanisms that are antagonized by cAMP- and cGMP-elevating agents. Nitrovasodilators such as nitroprusside and endothelium-derived relaxing factor are potent cGMP-elevating platelet inhibitors. In the present study, the role of cGMP and cGMP-dependent protein kinase in nitrovasodilator inhibition of ADP- and thrombin-evoked calcium elevation and activation of human platelets was investigated. Preincubation of platelets with 8-(4-chlorophenylthio)guanosine 3′,5′-cyclic monophosphate (8-pCPT-cGMP; a membrane-permeant selective activator of the cGMP-dependent protein kinase that does not significantly affect cGMP-regulated phosphodiesterases) inhibited the thrombin-induced phosphorylation mediated by myosin light chain kinase and protein kinase C. Nitrovasodilator-induced protein phosphorylation in human platelets was distinct from that induced by cAMP-elevating prostaglandins and could be mimicked by 8-pCPT-cGMP. Preincubation of human platelets with nitrovasodilators or 8-pCPT-cGMP inhibited the ADP- and thrombin-evoked calcium elevation in the presence and absence of external calcium. Nitrovasodilators and 8-pCPT-cGMP also inhibited the agonist-induced Mn^{2+} influx, but stopped-flow experiments indicated that the ADP receptor-operated cation channel was not significantly inhibited. These results suggest that in human platelets nitrovasodilators inhibit the agonist-induced calcium mobilization from intracellular stores and the secondary store-related calcium influx but not the ADP receptor-operated cation channel. The results also suggest that these nitrovasodilator effects are mediated by cGMP and the cGMP-dependent protein kinase.**

The activation of platelets is stimulated or inhibited by numerous hormones, drugs, eicosanoids, and other vasoactive substances. Agonists such as thrombin, thromboxane, vasopressin, platelet activating factor, and ADP elevate the cytosolic free Ca^{2+} and stimulate the activity of myosin light chain kinase (MLCK) and protein kinase C (PKC) resulting in platelet adhesion, aggregation, and degranulation (1–3). Most platelet agonists activate phospholipase C (PLC) and elevate cytosolic calcium by an inositol 1,4,5-trisphosphate-dependent release of calcium from intracellular stores, as well as stimulation of the entry of extracellular Ca^{2+} (3–7). ADP is the only platelet agonist known to cause a fast Ca^{2+} entry mediated by a receptor-operated cation channel. This rapid influx precedes the ADP-evoked release of calcium from intracellular stores and a second phase of store-related calcium entry (3–7).

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In contrast to platelet agonists, many platelet inhibitors elevate the level of cAMP, cGMP, or both and antagonize the activator-evoked calcium elevation (2, 3, 8–11). Considerable evidence suggests that cAMP-elevating agents such as prostacyclin [prostaglandin (PG)-I_2], PGE_1, and forskolin are effective inhibitors of platelet activation and of the agonist-evoked calcium discharge from intracellular stores. This cAMP-mediated effect is most likely due to the inhibition of PLC activation, although other mechanisms have also been considered (2, 3, 6, 8–12). The role and mechanism of action of cGMP in platelet function is less well defined. Nitrovasodilators [sodium nitroprusside (SNP) and other nitric oxide-generating drugs] and endothelium-derived relaxing factor inhibit the activation of platelets, elevate cGMP levels, and stimulate cGMP-dependent protein kinase (cGMP-PK) in platelets (9, 10, 12–15). However, cGMP also directly regulates enzymes and proteins other than the cGMP-PK—e.g., cGMP-stimulated and cGMP-inhibited phosphodiesterases (PDEs) and ion channels (9, 16). It has also been suggested that cGMP may achieve its antagonistic effect on rabbit platelet activation by inhibiting the cGMP-inhibited PDE, resulting in elevated cAMP levels and activation of the cAMP signal transduction cascade (17, 18). cGMP-elevating platelet inhibitors also antagonize the agonist-induced increase of cytosolic calcium in platelets (3, 9, 19, 20) although it is not clear whether this effect is mediated by cGMP-PK and which of the distinct calcium mobilization pathways is affected. In the present study, we have, therefore, examined the role of cGMP and cGMP-PK in the regulation of cytosolic calcium in human platelets. In particular, the role of cGMP and cGMP-PK in the regulation of Ca^{2+} entry through receptor-operated cation channels and for the regulation of calcium mobilization from intracellular stores was investigated.

MATERIALS AND METHODS

Materials. PGE_1, SNP, thrombin, ADP, aspirin, and apyrase were from Sigma. Fura-2 tetrakis(acetoxymethyl)ester was from Molecular Probes or from Sigma, and 8-(4-chlorophenylthio)guanosine 3′,5′-cyclic monophosphate (8-pCPT-cGMP) was from Biolog (Bremen, F.R.G.). The nitric oxide donor 3-morpholinosydnonimine hydrochloride (SIN-1) (21) was a kind gift of M. Just (Casella, Frankfurt am Main, F.R.G.). Radiochemicals ([γ-^{32}P]ATP, ^{125}I-labeled protein A, and $^{32}PO_4^{3-}$) were from Amersham. All other

Abbreviations: MLCK, myosin light chain kinase; PKC, protein kinase C; PLC, phospholipase C; PG, prostaglandin; SNP, sodium nitroprusside; cGMP-PK, cGMP-dependent protein kinase; cAMP-PK, cAMP-dependent protein kinase; PDE, phosphodiesterase; 8-pCPT-cGMP, 8-(4-chlorophenylthio)guanosine 3′,5′-cyclic monophosphate; VASP, vasodilator-stimulated phosphoprotein; SIN-1, 3-morpholinosydnonimine hydrochloride.

‡To whom reprint request should be addressed.

chemicals were reagent grade and obtained from commercial sources.

Phosphorylation Experiments with Intact Human Platelets. ^{32}P-labeled human platelets were prepared and used in phosphorylation experiments as described (13, 15). Quantitative phosphorylation of the vasodilator-stimulated phosphoprotein (VASP) was measured by SDS/PAGE and Western blot (quantitation of the shift of VASP from the 46- to 50-kDa form) as described (14, 15).

Fluorescence Experiments with Fura-2-Loaded Platelets. Platelets were prepared from whole blood as described (4), and the experimental conditions for the fluorescence measurements were essentially as reported (4–6). Briefly, venous blood obtained from healthy volunteers was mixed with 107 mM citrate buffer (pH 6.5) [4:1 (vol/vol) ratio] containing 0.1 mM aspirin and then centrifuged at 300 × *g* for 20 min. The platelet-rich plasma was incubated with 4 μM fura-2 at 37°C for 45 min and then centrifuged at 500 × *g* for 20 min. The platelet pellet was resuspended in 10 mM Hepes buffer (pH 7.4) containing apyrase (0.1 unit/ml). Before experiments, the platelet suspension was diluted to 1×10^8 cells per ml. Fluorescence was measured using a Perkin–Elmer Luminescence spectrometer LS50 fitted with a thermostated cuvette holder. Samples were stirred and maintained at 37°C throughout the experiment. The external medium contained 1 mM Ca^{2+}, 0.5 mM Ca^{2+} plus 0.5 mM Mn^{2+}, or 1 mM EGTA as indicated. The fluorescence excitation wavelength was 340 nm with a bandwidth of 2.5 nm, and the emission was collected at 510 nm with a bandwidth of 6 nm. In the manganese influx experiments (5, 6), the emission wavelength was identical, whereas the excitation wavelength was 360 nm with a bandwidth of 2.5 nm. Stopped-flow fluorimetry was performed as described (5, 6).

Test reagents were dissolved in water except for 8-pCPT-cGMP, which was dissolved in dimethyl sulfoxide (final concentration in experiments, 1%). The SIN-1 solution was prepared immediately before use. For each experimental condition and test reagent, control experiments were performed with the vehicle alone. In most experiments, platelets were preincubated with SNP, SIN-1, and 8-pCPT-cGMP as indicated and were then stimulated with agonists (20 or 40 μM ADP or thrombin at 0.1 unit/ml).

RESULTS AND DISCUSSION

8-pCPT-cGMP as Selective Activator of cGMP-PK. In agreement with previous studies (22, 23), 8-pCPT-cGMP was found to be a potent activator of the cGMP-PK. Half-maximal activation of purified cGMP-PK or purified type II cAMP-dependent protein kinase (cAMP-PK) was observed with 0.04 μM or 7.0 μM 8-pCPT-cGMP, respectively (data not shown). Additional data to be reported in detail elsewhere (E.B., J. A. Beavo, and U.W., unpublished data) suggested that 8-pCPT-cGMP may be a useful and selective activator for the cGMP-PK in intact cell preparations. (*i*) In contrast to other cyclic nucleotide analogs including 8-bromo-cGMP, 8-pCPT-cGMP was not significantly hydrolyzed by purified PDEs (cGMP-stimulated and -inhibited PDEs and calmodulin-dependent PDE) and had little if any effect on cGMP-regulated PDEs (i.e., no detectable effect on cGMP-stimulated PDE and half-maximal inhibition of cGMP-inhibited PDE at 385 μM 8-pCPT-cGMP). (*ii*) In membranes of human platelets, 1 μM 8-pCPT-cGMP reproduced the selective activation of the endogenous cGMP-PK observed with 1 μM cGMP (13, 24). (*iii*) The lipophilicity of 8-pCPT-cGMP was substantially higher when compared with cGMP or 8-bromo-cGMP, suggesting good membrane-permeant properties. These properties were tested in phosphorylation experiments with intact platelets.

Regulation of Protein Phosphorylation in Intact Human Platelets by Nitrovasodilators and 8-pCPT-cGMP. Preincubation of platelets with PGE_1 or SNP (12) or 8-pCPT-cGMP (Fig. 1*A*) strongly inhibited the thrombin-induced phosphorylation of a 20-kDa and a 44-kDa protein. Since the 20-kDa protein (myosin light chain) and the 44-kDa protein of platelets are phosphorylated during thrombin stimulation by activated MLCK or PKC, respectively (1–3), the results shown in Fig. 1*A* suggest that not only activation of cAMP-PK but also activation of cGMP-PK inhibits the activation pathways for both PKC and MLCK. As shown in Fig. 1*B*, PGE_1 alone stimulated the phosphorylation of several proteins (proteins with apparent molecular masses of 240 kDa, 68 kDa, 50 kDa, 24 kDa, and 22 kDa), an effect previously shown to be mediated by the cAMP-PK (9, 13, 24, 25). In contrast, SNP caused significant phosphorylation of only the 50-kDa protein (Fig. 1*B*), an effect previously shown to be mediated by the cGMP-PK (9, 13) and now found to be reproduced by

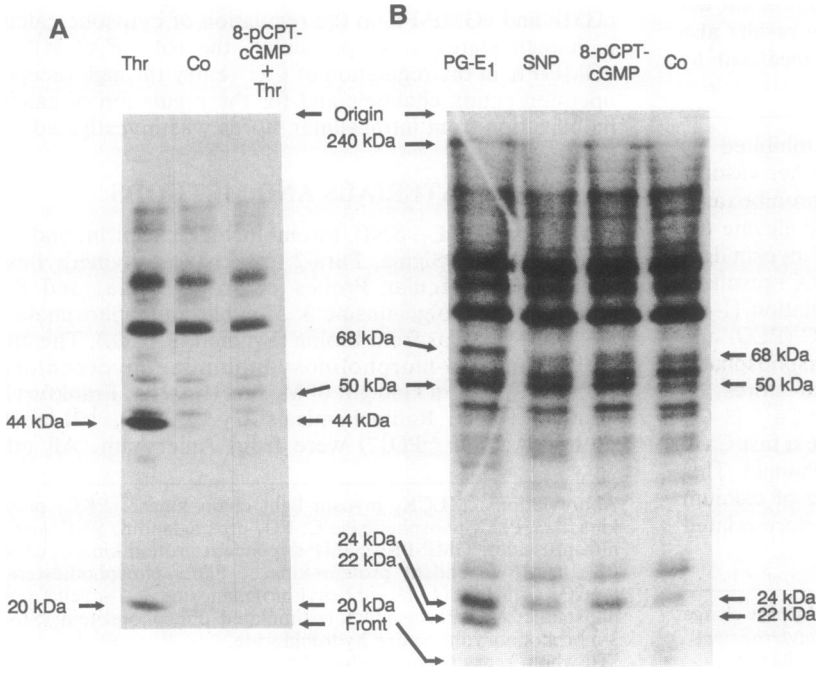

FIG. 1. Autoradiograph showing the effects of thrombin, PGE_1, SNP, and 8-pCPT-cGMP on protein phosphorylation in human platelets. (*A*) Effect of 8-pCPT-cGMP pretreatment on thrombin-induced phosphorylation. ^{32}P-labeled platelets were incubated without additions (control, Co) or with thrombin (Thr) at 0.2 unit/ml for 1 min. When two agents were studied, the 1-min incubation with thrombin at 0.2 unit/ml was preceded by a 10-min incubation with 0.5 mM 8-pCPT-cGMP. (*B*) Comparison of the effects of PGE_1, SNP, and 8-pCPT-cGMP on protein phosphorylation. ^{32}P-labeled platelets were incubated for 30 min without additions (control, Co) or with 10 μM PGE_1, 0.1 mM SNP, or 0.5 mM 8-pCPT-cGMP. Arrows indicate the positions of the phosphoproteins affected by the various agents.

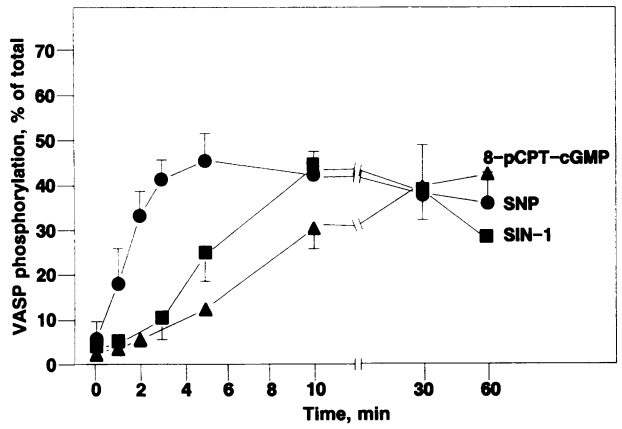

FIG. 2. Quantitative analysis of VASP phosphorylation in platelets incubated with SNP, SIN-1, or 8-pCPT-cGMP. Intact human platelets were incubated with 0.1 mM SNP (●), 0.1 mM SIN-1 (■), or 0.5 mM 8-pCPT-cGMP (▲) for the times indicated. Platelets were analyzed for VASP phosphorylation on a Western blot. Phosphorylated VASP (50-kDa protein) is expressed as a percentage of total VASP (46-kDa plus 50-kDa protein). Data represent the mean ± SEM of three experiments.

8-pCPT-cGMP. Most importantly, SNP and 8-pCPT-cGMP did not cause significant phosphorylation of the 68-, 24-, and 22-kDa proteins, which are established substrates of the cAMP-PK in intact human platelets (13, 24, 25). Recently, the 50-kDa phosphoprotein (also termed VASP, a protein phosphorylated in response to both cAMP- and cGMP-elevating vasodilators) has been purified (26). Furthermore, an antiserum against VASP has been developed that detects the phosphorylation-induced mobility shift of VASP in SDS/PAGE from a 46-kDa dephosphorylated form to the 50-kDa phosphorylated form permitting the quantitative analysis of VASP phosphorylation in intact platelets by using Western blots (14, 15). These advances enabled us to quantitatively compare VASP phosphorylation induced by nitrovasodilators and 8-pCPT-cGMP. As shown in Fig. 2, treatment of intact human platelets with 0.1 mM SNP, 0.1 mM SIN-1, and 0.5 mM 8-pCPT-cGMP converted >30% of VASP to its phosphorylated form within 2–5 min (SNP), 5–10 min (SIN-1), or 10–30 min (8-pCPT-cGMP). As demonstrated with SNP (14), VASP phosphorylation induced by SNP and SIN-1 was preceded by a 10-fold increase in platelet cGMP level whereas cAMP levels were not elevated (data not shown). These results demonstrate that in human platelets cGMP-elevating nitrovasodilators and 8-pCPT-cGMP do not significantly activate the cAMP-PK but selectively activate the cGMP-PK resulting in the inhibition of agonist-induced activation of MLCK and PKC.

Regulation of Agonist-Induced Calcium Mobilization by SNP, SIN-1, and 8-pCPT-cGMP. The ADP-induced calcium

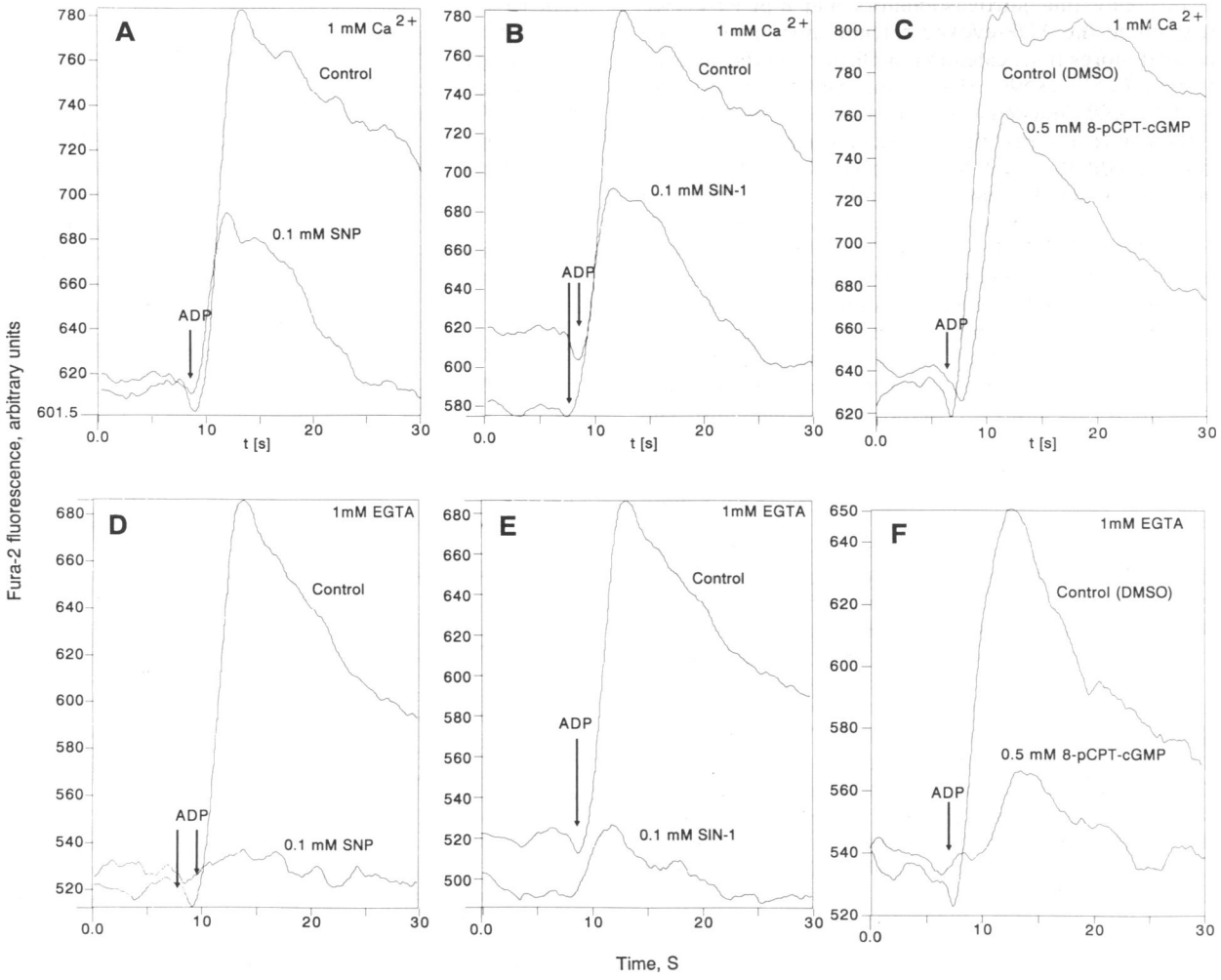

FIG. 3. Effects of SNP, SIN-1, and 8-pCPT-cGMP on the ADP-induced calcium response in human platelets. Fura-2-loaded platelets were incubated in the presence of 1 mM external Ca^{2+} (*A–C*) or in the absence of external Ca^{2+} (1 mM EGTA) (*D–F*). When indicated (arrows), platelets were mixed with 20 μM ADP (final concentration). Platelets were preincubated for 10 min with 0.1 mM SNP, 0.1 mM SIN-1, or 0.5 mM 8-pCPT-cGMP or without additions (controls) as indicated.

Table 1. Quantitative effects of SNP, SIN-1, and 8-pCPT-cGMP on ADP- and thrombin-evoked calcium response in fura-2-loaded human platelets

		Change in fura-2 fluorescence, % of control						
		SNP		SIN-1		8-pCPT-cGMP		
Agonist	Condition	Mean	SEM	Mean	SEM	Mean	SEM	*n*
Thrombin	Ca^{2+} (1 mM)	33.3	15.7	47.0	12.4	43.3	15.8	12
	EGTA (1 mM)	20.8	7.7	51.3	12.9	28.3	9.5	12
	Mn^{2+} (0.5 mM) + Ca^{2+} (0.5 mM)	49.1	8.2	43.8	6.5	47.5	11.1	8
ADP	Ca^{2+} (1 mM)	44.9	9.8	53.2	12.9	52.3	9.2	13
	EGTA (1 mM)	13.2	6.2	23.0	7.4	22.8	9.7	13
	Mn^{2+} (0.5 mM) + Ca^{2+} (0.5 mM)	52.8	12.0	50.2	15.6	58.3	12.4	8

For each experimental condition, platelets were preincubated for 10 min with buffer alone (control) or with test reagent (0.1 mM SNP, 0.1 mM SIN-1, or 0.5 mM 8-pCPT-cGMP) before the agonist (thrombin at 0.1 unit/ml or 20 μM ADP) was added. The agonist-induced calcium response (maximal change of fura-2 fluorescence over basal level) obtained with test reagent (SNP, SIN-1, or 8-pCPT-cGMP) is expressed as a percent of the response observed in control experiments. The agonist-evoked response with the external medium containing 1 mM Ca^{2+} or 1 mM EGTA was an increase of fluorescence, whereas the response with the medium containing 0.5 mM Mn^{2+} plus 0.5 mM Ca^{2+} was a decrease due to Mn^{2+}-quenched fluorescence. The mean values, SEM, and number of experiments (*n*) are indicated.

response of fura-2-loaded platelets was inhibited (in the presence of 1 mM external calcium) or nearly abolished (in the absence of external calcium; 1 mM EGTA) when platelets were preincubated with 0.1 mM SNP, 0.1 mM SIN-1, or 0.5 mM 8-pCPT-cGMP (Fig. 3). SNP, SIN-1, and 8-pCPT-cGMP did not significantly affect the basal fluorescence of fura-2-loaded platelets (data not shown). These results demonstrate that cGMP-elevating nitrovasodilators and 8-pCPT-cGMP strongly inhibit the ADP-evoked calcium mobilization from intracellular stores (i.e., calcium mobilization in the absence of external Ca^{2+}). SNP, SIN-1, and 8-pCPT-cGMP also inhibited the ADP-induced Mn^{2+} influx (Table 1) measured as Mn^{2+} quenching of fura-2 fluorescence. These data indicate that nitrovasodilators and 8-pCPT-cGMP partially inhibit calcium entry in platelets since Mn^{2+} influx is an indicator of Ca^{2+} influx (3, 5, 6). A quantitative analysis of the SNP, SIN-1, and 8-pCPT-cGMP effects on both ADP- and thrombin-induced calcium mobilization in platelets is shown in Table 1. Calcium mobilization from intracellular stores alone (EGTA experiments) was more strongly inhibited than calcium influx (measured as Mn^{2+} influx) or the calcium elevation due to both Ca^{2+} influx and intracellular mobilization (experiments with 1 mM Ca^{2+} in the external medium). The inhibitory effect of SNP and 8-pCPT-cGMP on thrombin-induced calcium response and protein phosphorylation was variable when higher thrombin concentrations (e.g., 1 unit/ml) were used (data not shown).

We also investigated which phase of the ADP-evoked calcium entry is affected by nitrovasodilators. Stopped-flow fluorimetry has been used (5, 6) to demonstrate that ADP (at an optimal concentration of 40 μM) evokes an increase of cytosolic Ca^{2+} that, in the presence of external Ca^{2+}, can be resolved into two phases. The first fast phase commences without measurable delay and is due to Ca^{2+} influx, since it is abolished by the removal of external Ca^{2+} or by the entry blocker Ni^{2+}. This phase appears to be mediated by a receptor-operated cation channel that has been demonstrated in cell-attached and whole-cell patch clamp recordings (7, 27). Mn^{2+} quench studies indicated that the second phase of the ADP-evoked increase of cytosolic Ca^{2+} is also associated with Ca^{2+} entry and that this delayed entry is related to the release of Ca^{2+} from intracellular stores (6). In the absence of external Ca^{2+}, ADP evokes a delayed increase in cytosolic Ca^{2+} that is delayed in onset, as expected for the release of Ca^{2+} from intracellular stores by a diffusible messenger. The effects of SNP and 8-pCPT-cGMP on the early kinetics of ADP-evoked increase in cytosolic Ca^{2+} as recorded using stopped-flow fluorimetry are shown in Fig. 4.

In the presence of external Ca^{2+}, ADP caused an increase in Ca^{2+} under control conditions that commenced without measurable delay and showed some acceleration after 100–200 ms (Fig. 4 *A* and *B*). Treatment with 0.1 mM SNP or 0.5 mM 8-pCPT-cGMP did not abolish the first fast phase of ADP-evoked Ca^{2+} increase, although in some experiments the rate of increase was slowed (Fig. 4*A*). With both SNP and 8-pCPT-cGMP, the second phase of Ca^{2+} increase commenced later than in the controls, the rate of increase was decreased and the time to peak was increased (Table 2). In the absence of external Ca^{2+}, ADP evoked an increase in cytosolic Ca^{2+} that was delayed in onset (Fig. 4 *C* and *D*). Treatment with SNP or 8-pCPT-cGMP increased the delay in onset of the ADP-evoked Ca^{2+} increase, slowed the rate of increase, and increased the time to peak (Fig. 4 *C* and *D* and Table 2). The extent of Ca^{2+} increase in the presence of the inhibitors was noticeably reduced compared to controls in the absence rather than in the presence of external Ca^{2+}, in

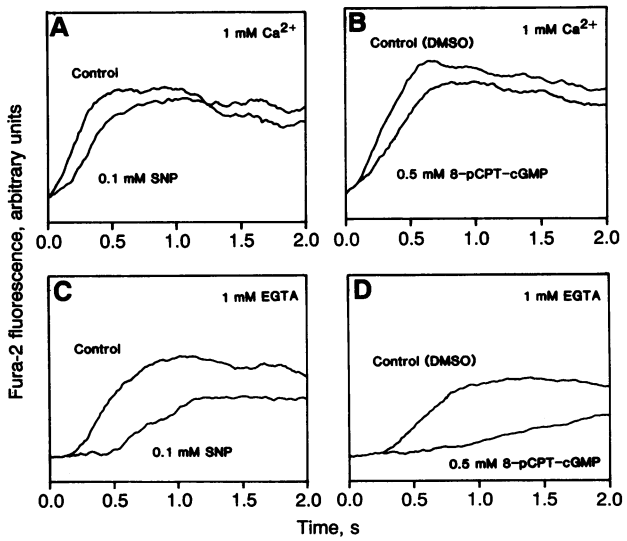

FIG. 4. Effects of SNP and 8-pCPT-cGMP on the early kinetics of ADP-evoked increases in cytosolic calcium in human platelets. Fura-2-loaded platelets were rapidly mixed with ADP at a final concentration of 40 μM at time zero and fluorescence was recorded by stopped-flow fluorimetry. Platelets were preincubated with 0.1 mM SNP (*A* and *C*), 0.5 mM 8-pCPT-cGMP (*B* and *D*), or vehicles (dimethyl sulfoxide, control) for 10 min and then 1 mM $CaCl_2$ (*A* and *B*) or 1 mM EGTA (*C* and *D*) was added to suspensions and agonist solutions as indicated. Fura-2 fluorescence was recorded at an excitation wavelength of 340 nm with emission at 500 nm.

Table 2. Effects of SNP and 8-pCPT-cGMP on the fast kinetics of ADP-evoked calcium response in fura-2-loaded human platelets

Condition	Inhibitor	Delay in onset, ms		Time to peak, ms		n
		Mean	SEM	Mean	SEM	
Ca^{2+}	Control	0	0	480	70	7
	SNP	0	0	890	170	8
	Control	0	0	580	30	6
	8-pCPT-cGMP	0	0	730	50	9
EGTA	Control	190	20	800	50	10
	SNP	390	60	1390	140	10
	Control	180	20	860	40	14
	8-pCPT-cGMP	370	30	1400	90	14

Data represent delays in onset and time to peak of fura-2 fluorescence at an excitation wavelength of 340 nm in the presence of 1 mM external Ca^{2+} or 1 mM EGTA. Platelets were treated as indicated with 0.1 mM SNP, 0.5 mM 8-pCPT-cGMP, or the vehicle buffer (control) for 10 min at 37°C before stimulation with 40 μM ADP. The mean, SEM, and number of experiments (n) are indicated.

agreement with the results shown in Fig. 3 and Table 1. These results indicate that cGMP-elevating nitrovasodilators and 8-pCPT-cGMP increase the delay in onset and slow and reduce the ADP-evoked release of Ca^{2+} from intracellular stores. This effect may account for the delaying and slowing of the second phase of ADP-evoked Ca^{2+} entry since the two events may be causally related (3, 6). The fast phase of the ADP-evoked Ca^{2+} increase (Ca^{2+} entry) in the presence of external Ca^{2+} was relatively resistant to inhibition by SNP or 8-pCPT-cGMP.

These data indicate that nitrovasodilators and 8-pCPT-cGMP under the conditions used do not inhibit the ADP receptor-operated cation channel but inhibit the discharge of intracellular Ca^{2+} stores and the secondary phase of Ca^{2+} entry associated with intracellular Ca^{2+} discharge. Our results do not support the suggestion (20) that ADP-evoked Ca^{2+} entry is more sensitive to inhibition by cGMP than the release of Ca^{2+} from intracellular stores since we observed, both in conventional and stopped-flow experiments, the opposite result.

Since the conditions for the use of nitrovasodilator and 8-pCPT-cGMP in these experiments were similar to the phosphorylation experiments with intact platelets (Figs. 1 and 2), it appears likely that the inhibitory effects of these reagents on the calcium mobilization are mediated by the cGMP-PK. Our present results with cGMP-elevating platelet inhibitors and numerous previous studies with cAMP-elevating platelet inhibitors (for reviews, see refs. 3 and 9–11) suggest that inhibition of intracellular Ca^{2+} discharge is an effect of both cGMP- and cAMP-elevating agents, mediated by cGMP-PK and cAMP-PK, respectively. Interestingly, phosphorylation of VASP, a substrate for both cGMP-PK and cAMP-PK in intact human platelets, correlates very well with the inhibition of platelet function (9, 10, 12–15) although we have at present no evidence that VASP is directly involved in the vasodilator inhibition of calcium discharge in platelets. Also, this inhibition could be caused by one or more independent mechanisms including inhibition of PLC, stimulation of Ca^{2+} reuptake by intracellular stores, stimulation of Ca^{2+} extrusion from the platelets, or inhibition of inositol 1,4,5-trisphosphate-induced Ca^{2+} release from intracellular stores. Vasodilator inhibition of agonist-dependent PLC activation would result in decreased formation of both inositol 1,4,5-trisphosphate and 1,2-diacylglycerol, reduced intracellular Ca^{2+} discharge, and then a decreased activation of both Ca^{2+}/calmodulin-dependent MLCK and 1,2-diacylglycerol-dependent PKC, most of which were observed in our present study (Figs. 1, 3, and 4). These results support the view that a major effect of cGMP-elevating platelet inhibitors is the inhibition of PLC activation, which is also considered to be the major effect of cAMP-elevating platelet inhibitors (2, 3, 9, 10). However, other sites of action of both cAMP- and cGMP-elevating platelet inhibitors cannot be excluded.

In conclusion, our present results indicate that cGMP-elevating nitrovasodilators do not significantly inhibit the ADP receptor-operated cation channel in intact human platelets but strongly inhibit the ADP-evoked Ca^{2+} discharge from intracellular stores and the secondary store-related Ca^{2+} entry from the external medium. Additional data suggest that inhibition of agonist-induced PLC activation is the mechanism of action of cGMP-elevating nitrovasodilators whose effects are mediated by the cGMP-PK. Future experiments will have to identify the precise molecular mechanism for the nitrovasodilator inhibition of Ca^{2+} discharge from the intracellular stores and whether cGMP-PK-mediated phosphorylation of VASP is an important component in this regulatory event.

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