0026-895X/96/020367-13$3.00/0

MOLECULAR PHARMACOLOGY, 50:367-379 (1996).

Thrombin-Stimulated Phospholipase C Activity Is Inhibited without Visible Delay by a Rapid Increase in the Cyclic GMP Levels Induced by Sodium Nitroprusside

F. J. AZULA, E. S. ALZOLA, M. CONDE, M. TRUEBA, J. M. MACARULLA, and A. MARINO

Department of Biochemistry and Molecular Biology, Faculty of Sciences, University of Basque Country, 48080 Bilbao, Spain

Received September 13, 1995; Accepted April 15, 1996

SUMMARY

Different drugs that elevate the cGMP levels inhibit the agonist-induced platelet activation. The mechanisms of action of cGMP probably include inhibition of both phospholipase C and the increase in intracellular Ca^{2+} concentration, and these effects seem to be mediated by cGMP-dependent protein kinases. However, in most studies, cells were preincubated with nitrovasodilators before stimulation. The effect of the preincubation with sodium nitroprusside before stimulation or the simultaneous addition of sodium nitroprusside and thrombin has been compared. The simultaneous addition of sodium nitroprusside and thrombin was able to inhibit without any significant delay the platelet aggregation. This rapid effect was correlated with an inhibition of both the maximum increase in intracellular Ca^{2+} concentration and the phospholipase C activity. Also, the simultaneous addition of sodium nitroprusside and thrombin clearly accelerated the decline in the Ca^{2+} signal, which was not observed in platelets preincubated with sodium nitroprusside. The rapid inhibition induced by sodium nitroprusside was correlated with a rapid and significant increase in the cGMP levels and reversed when platelets were pretreated with methylene blue. The inhibitor of cAMP-dependent protein kinase Rp-8-(4-chlorophenylthio)-adenosine-3′,5′-cyclic monophosphorothioate was able to abolish nearly completely the inhibitory effect induced by sodium nitroprusside independent of the protocol used. Thus, the rapid inhibition induced by sodium nitroprusside seems to be induced by a rapid phosphorylation-dependent mechanism. In addition, both cGMP- and cAMP-dependent protein kinase seem to be involved; however, the cAMP-dependent protein kinase seems to be more important.

Aggregation of human platelets has been shown to be a critically important event in the processes of hemostasis and thrombosis. Thrombin, a serine protease, is a potent platelet-activating agent. Platelet activation by thrombin involves a series of rapid biochemical changes that lead to shape change, granule secretion, and aggregation. Stimulation of human platelets by thrombin is associated with the hydrolysis of phosphatidylinositol-4,5-bisphosphate, generating the two second messengers Ins(1,4,5)P3 and 1,2-diacylglycerol (for a review, see Ref. 1). Ins(1,4,5)P3 releases Ca^{2+} from intracellular stores. In addition, in platelets the thrombin-induced increase in $[Ca^{2+}]_i$ results from Ca^{2+} entry from the extracellular space. Platelet stimulation also produces 1,2-diacylglycerol, which activates protein kinase C.

On the other hand, many platelet inhibitors elevate the level of cyclic nucleotides and inhibit the platelet activation. Therefore, the preincubation with different drugs that elevate the cGMP levels or cGMP analogs inhibits shape change (2), secretion (3, 4), and platelet aggregation (2, 5–7) induced by agonists. The mechanism of action of cGMP in platelet function is poorly defined and probably includes inhibition of both (a) phospholipase C (3, 4, 8) and (b) Ca^{2+} mobilization from intracellular stores and Ca^{2+} influx (2, 3, 7, 9–11). In addition, the phospholipase A_2/arachidonic acid pathway seems to be an important target for the physiological actions of cGMP and for the pharmacological action of nitrovasodi-

This work was supported by funds from the Ministerio de Educación y Ciencia/Dirección General de Investigación Científica y Tecnológica (Grants PB94-1357 and PM92-0109), IBERDROLA S. A., and J. Gangoiti-Barrera Foundation.

ABBREVIATIONS: Ins(1,4,5)P3, inositol-1,4,5-trisphosphate; $[Ca^{2+}]_i$, intracellular calcium concentration; PKG, cGMP-dependent protein kinase; PKA, cAMP-dependent protein kinase; SNP, sodium nitroprusside; SNAP, *S*-nitroso-*N*-acetylpenicillamine; SIN-1, 3-morpholinosydnomidine; OPT, ophthalaldehyde; PGE_1, prostaglandin E_1; EGTA, ethylene glycol bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; FLP, Fura-2-loaded platelets; NFLP, non-Fura-2-loaded platelets; PMA, phorbol-12-myristate-13-acetate; VASP, vasodilator-stimulated phosphoprotein; 8-pCPT-cGMP, 8-(4-chlorophenyl-thio)cGMP; Rp-8-pCPT-cGMPS, Rp-8-(4-chlorophenylthio)-guanosine-3′,5′-cyclic monophosphorothioate; Rp-8-CPT-cAMPS, Rp-8-(4-chlorophenylthio)-adenosine-3′,5′-cyclic monophosphorothioate; Sp-5,6-DCl-cBIMPS, Sp-5,6-dichloro-1-β-ribofuranosyl-benzimidazole-3′,5′-monophosphorothioate; $InsP_1$, inositol monophosphate; $InsP_2$, inositol bisphosphate; $InsP_3$, inositol trisphosphate.

lators (12). However, the molecular basis of the inhibition of platelet function by cGMP-elevating agents is not well understood. These effects seem to be mediated by PKG (10, 13–15). It has been purified a 46-kDa platelet protein termed VASP, which is phosphorylated by both PKA and PKG in human platelets and support the view that VASP phosphorylation is an important component of the intracellular mechanism of action of these vasodilators in human platelets (10, 16–19). More recently, VASP has been characterized as a novel focal adhesion protein (20), and the VASP phosphorylation by both PKA and PKG seems to be closely associated with the inhibition of aggregation by regulation of the fibrinogen receptor (21). In addition, cGMP may achieve its intracellular effects by other mechanisms. Nitrovasodilators such as SNP that increase the cGMP levels can increase the cAMP levels, probably through a cGMP-inhibited cAMP phosphodiesterase, an enzyme known to be present in many cells, including platelets (5, 22–24). Therefore, the cAMP can contribute to the inhibitory effects of nitrovasodilators in human platelets by activation of the cAMP signal transduction cascade. In addition, PKG-deficient platelets (obtained from patients with chronic myelogenous leukemia) represent another example of cGMP effects being mediated by PKA. The treatment of these platelets with SNP caused a small phosphorylation of a substrate protein, presumably via PKA (14). Also, cGMP exerts major effects in some cells through other mechanisms (for a review, see Ref. 25).

It is known that the rapid interaction between platelets and endothelial cells is an important event in the regulation of the platelet reactivity. In addition to other factors, endothelial cells release endothelium-derived relaxing factor (now thought to be nitric oxide), which, via specific stimulation of the guanylate cyclase, increases the cGMP levels and blocks the platelet activation. Therefore, the addition of indomethacin-treated endothelial cells to activated platelets rapidly induced platelet disaggregation (26). Although it is known that the nitrovasodilators inhibit the platelet activation, in most of these studies the cells were preincubated with nitrovasodilators before stimulation with different agonists. To the best of our knowledge, only Brüne and Ullrich (27) have observed that 12-hydroperoxy-5,8,10,14-eicosatetranoic acid inhibited the aggregatory response and significantly increased the cGMP levels without an apparent time delay. In the current study, the effect of preincubation with SNP before stimulation or the simultaneous addition of SNP and thrombin have been compared. SNP inhibited the thrombin-induced platelet aggregation without significant time delay. We discuss the mechanisms involved in this rapid inhibition.

Experimental Procedures

Materials. Fura-2 acetoxymethylester was obtained from Boehringer Mannhein; the reagent OPT, the serotonin for standard, KT-5720, and KT-5823 were obtained from Calbiochem (San Diego, CA); thrombin, SNP, methylene blue, PGE_1, EDTA, EGTA, HEPES, SNAP, and SIN-1 were obtained from Sigma Chemical (St. Louis, MO); *myo*-[2-^{3}H]inositol and enzyme immunoassay kits for cAMP and cGMP were obtained from Amersham International (Buckinghamshire, UK); and 8-pCPT-cGMP, Rp-8-pCPT-cGMPS, Rp-8-CPT-cAMPS, and Sp-5,6-DCl-cBiMPS were obtained from Biolog (Bremen, Germany). The other materials were purchased from various sources and were reagent grade.

Measurement of $[Ca^{2+}]_i$ levels. Levels of $[Ca^{2+}]_i$ were monitored as described previously (28, 29). Briefly, washed human platelets at 2.5×10^8 cells/ml were loaded with Fura-2 by incubation at 37° for 45 min with 1 μM Fura-2. Cells were washed by centrifugation and resuspended at 2.5×10^8 cells/ml. Aliquots (0.5 ml) of cells were transferred to a cuvette and prewarmed at 37°. Fluorescence was measured with a Perkin-Elmer spectrofluorimeter model LS-5B equipped with a thermostatically controlled cuvette holder and magnetic stirrer. Fura-2 fluorescence was monitored continuously using monochromator settings of 340 nm (excitation) and 505 nm (emission). The $[Ca^{2+}]_i$ levels were calculated as described previously (28, 29). Fluorescence from external dye, assessed by the addition of excess EGTA to separate samples, was subtracted from the total fluorescence signal.

Platelet aggregation measurement. Platelet aggregation measurements were realized as described previously (28, 29). Briefly, a Menarini model Aggrecorder II PA-3220 was used. Washed human platelets (0.5 ml) at 2.5×10^8 cells/ml were placed in siliconized glass aggregometer cuvettes (0.5 ml) and prewarmed to 37° without stirring. The samples were then placed in the 37°-thermostated aggregometer and stirred (1000–1050 rpm) before the addition of the effectors. Aggregation was measured as the maximum change percentage in light transmission against a buffer blank for 10 min. For experiments of platelet aggregation with FLP, the cells were loaded with Fura-2 as described above.

Measurement of secretion of serotonin. The secretion of serotonin was measured by the fluorimetric method as described previously by Holmsen and Dangelmaier (30). The reagent OPT, at 0.5% (w/v) in ethanol, was mixed with 10 volumes of 8 N HCl. Serotonin creatinine sulfate (for standard) was dissolved to 1 mM in 1 N HCl and stored protected from light in small portions at −20°. Standard solutions of serotonin (from 0 to 1 μM) were made in deionized water just before measurement. Washed human platelets at 3×10^8 cells/ml were placed in siliconized glass aggregometer cuvettes (0.9 ml) and prewarmed at 37°. Subsequently, samples were placed in the aggregometer (37°) and stirred (1000–1050 rpm). After stimulation, 100 μl of 50 mM ice-cold EDTA, pH 7.4, was added. All samples were then centrifuged for 30 sec at 4° and 12,000 × *g* except the samples used to determine the total content of serotonin. Trichloroacetic acid (120 μl of 6 M) was added to 600 μl of supernatant. The samples were centrifuged for 2 min at room temperature and 12,000 × *g* in an Eppendorf centrifuge. OPT reagent (2 ml) was added to the supernatants, and the samples were placed in a boiling water bath for 10 min. The samples were cooled in ice and washed twice with chloroform. Fluorescence was measured with a spectrofluorimeter (LS-5B, Perkin-Elmer) with excitation at 360 nm and emission at 475 nm at room temperature in quartz cuvettes. Standards and blanks were processed as the samples. The secretion of serotonin was calculated as described previously (30).

Measurement of [^{3}H]inositol phosphates. Measurement of [^{3}H]inositol phosphates was carried out as described previously (28). Briefly, washed human platelets were incubated with *myo*-[2-^{3}H]-inositol at 37° for 3 hr. Subsequently, cells were washed to remove unincorporated radiolabeled inositol and resuspended in HEPES buffer at 6–8 10^8 cells/ml. Platelet suspensions (0.5 ml) were prewarmed at 37° for 5 min. Subsequently, samples were placed in the aggregometer (37°) and stirred (1000–1050 rpm). After stimulation, the reaction was stopped by the addition of 0.5 ml of ice-cold 10% (v/v) perchloric acid. Samples were centrifuged at 1000 × *g* for 5 min, and the supernatant was neutralized with 1.5 M KOH/75 mM HEPES and recentrifuged at 1500 × *g* for 5 min. Then, the supernatant was diluted with 10 mM HEPES (pH 7.4)/2 mM EDTA. The [^{3}H]inositol phosphates were separated by anion-exchange chromatography on a Dowex AG1-X8 column (Bio-Rad Laboratories) as described previously (28). The radioactivity of the column eluates was quantified with a Tri-Carb liquid scintillation spectrometer (model 2000 CA; Packard Instrument Company, Meriden, CT).

Determination of cGMP and cAMP. Washed human platelets (3×10^8 cells/ml) were placed in siliconized glass aggregometer cuvettes (0.5 ml), warmed to 37°, and stirred (1000–1050 rpm) before the addition of the effectors. After stimulation, the reaction was stopped by the addition of 0.5 ml of ice-cool 20% (w/v) trichloroacetic acid. Samples were kept in ice for 30 min and centrifuged at 10,000 × *g* and 4° for 10 min. After centrifugation, the supernatants were washed four times with 1 ml of water-saturated diethyl ether. The aqueous phase was lyophilized, and the residue was stored at −20°. Residues were dissolved in 0.05 M acetate buffer, pH 5.8, 0.02% (w/v) bovine serum albumin, and 0.005% (w/v) thimerosal. cGMP and cAMP concentrations were determined by enzyme immunoassay using commercial kits. Cyclic nucleotide was acetylated with acetic anhydride/triethylamine (1:2, v/v) to increase the sensitivity of the assay.

Statistical Analysis. Except when indicated, values in figures are presented as mean ± standard error. Statistical significance of the results was determined using a Student's *t* test.

Results

SNP inhibits the thrombin-induced platelet aggregation without any significant time delay. Two different protocols were used in these experiments. In protocol 1, cells (prewarmed at 37°) were incubated for 5 min with SNP or vehicle (control samples) (at 37° and stirring at 1000 rpm) and then stimulated with thrombin. In protocol 2, SNP and thrombin or vehicle and thrombin were premixed and immediately added to platelets prewarmed at 37°. We examined the effect of both protocols on NFLP and FLP aggregation induced by 0.1 unit/ml thrombin (Fig. 1). The control aggregations (in the absence of SNP) of NFLP and FLP obtained with both protocols were different (~95% and 75% of aggregation, respectively). This effect probably would be caused by the chelation of $[Ca^{2+}]_i$ by Fura-2 in FLP. Therefore, the increase in $[Ca^{2+}]_i$ induced by thrombin in NFLP would be higher than that in FLP.

Independent of the protocol used, SNP inhibited the NFLP aggregation in a similar degree and in a dose-dependent manner (p = NS between protocols 1 and 2). In the same manner as NFLP, SNP inhibited FLP aggregation, dose-dependently and independent of the protocol used; however, at low doses of SNP (0.025 and 1 μM), the inhibition observed with protocol 1 was slightly greater than that with protocol 2.

The effect of SNP was clearly different in NFLP and FLP (Fig. 1). In FLP, low doses of SNP strongly inhibited the aggregation. Therefore, 1 μM SNP inhibited the FLP aggregation by 75% and 53% (protocol 1 and 2, respectively), whereas the NFLP aggregation was inhibited by 15% (both protocols) (values are percentages of inhibition with respect to the control aggregations). High doses of SNP completely inhibited the FLP aggregation, whereas SNP was unable to completely inhibit the NFLP aggregation. The different effects of SNP on thrombin-induced NFLP and FLP aggregation may be due to the chelation of intracellular Ca^{2+} by Fura-2 in FLP. The increase in $[Ca^{2+}]_i$ would be lower in FLP than in NFLP, and the FLP would be more sensible to an inhibitory mechanism. This hypothesis was supported by the effect of 0.5 mM $NiCl_2$ (to block receptor-mediated Ca^{2+} entry across the platelet plasma membrane) on the inhibition of the NFLP aggregation induced by SNP. Low doses of SNP were required to inhibit significantly and completely the NFLP aggregation in the presence of 1 mM $CaCl_2$/0.5 mM $NiCl_2$ (data not shown).

In addition, the curves of aggregation of NFLP and FLP exhibited two entirely different profiles (Fig. 1). SNP delayed the onset of NFLP aggregation (Fig. 1). Therefore, 1 μM SNP remarkably decreased the rate of aggregation, whereas the maximum aggregation was only slightly inhibited (~15%; Fig. 1). Concentrations of ≥5 μM SNP clearly delayed the onset of aggregation; however, after 4–5 min of stimulation, a sharp aggregation was observed. SNP did not delay the onset of the FLP aggregation induced by thrombin (Fig. 1).

To verify whether the rapid inhibition induced by SNP is

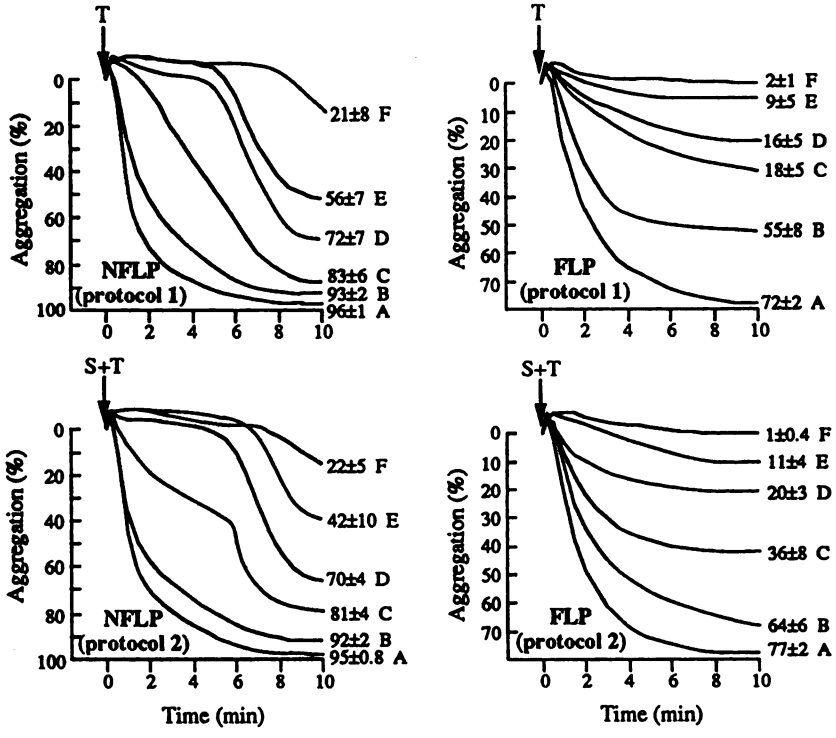

Fig. 1. Inhibition by SNP of platelet aggregation induced by thrombin. NFLP and FLP were incubated for 5 min with different concentrations of SNP and then stimulated with thrombin (protocol 1) or with SNP plus thrombin (protocol 2). In both cases, platelets were stimulated in the presence of 1 mM $CaCl_2$. SNP concentrations were 0 μM (A), 0.025 μM (B), 1 μM (C), 5 μM (D), 100 μM (E), and 500 μM (F) (additional SNP concentrations were studied; however, for clarity, only these SNP concentrations are shown). The thrombin concentration used was 0.1 unit/ml. *T*, addition of thrombin; *S+T*, simultaneous addition of SNP plus thrombin. Aggregation traces were obtained in a representative experiment. Values represent maximum aggregation (mean ± standard error) of seven to nine (NFLP) and six to nine (FLP) experiments with different platelet preparations.

obtained with other nitric oxide donors, we studied the inhibitory effect of the nitric oxide donors SNAP and SIN-1 on the thrombin-induced NFLP aggregation. Thus, SNAP was able to inhibit the maximum aggregation induced by thrombin in a similar degree and in a dose-dependent manner, independent of the protocol used (Fig. 2). The curves of aggregation of NFLP in the presence of SNAP and SNP exhibited similar profiles (Figs. 2 and 1, respectively). However, SNP seems to be more potent than SNAP. For a similar concentration, SNP more strongly inhibited the NFLP aggregation. Therefore, 5 and 500 μM SNP inhibited the maximum aggregation by 25% and 75%, respectively (both protocols), whereas SNAP inhibited the maximum aggregation by ~10% and ~55%, respectively (both protocols) (values are percentages of inhibition with respect to the control aggregations) (Figs. 1 and 2). Similar results were obtained with the nitric oxide donor SIN-1 (data not shown). In a similar manner to SNP and SNAP, SIN-1 inhibited the NFLP aggregation induced by thrombin without any significant time delay. The curves of aggregation obtained with SIN-1 were similar to those obtained with SNP and SNAP (data not shown). The order of potency was SNP > SNAP = SIN-1 (data not shown).

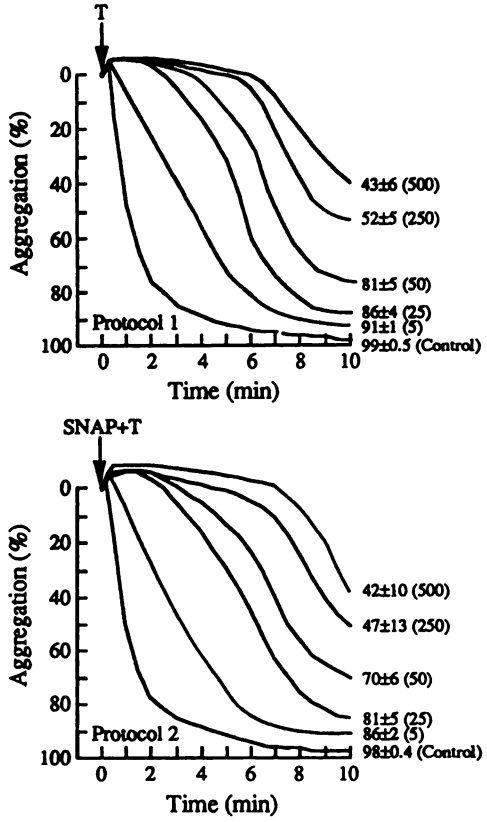

Fig. 2. Inhibition by SNAP of platelet aggregation induced by thrombin. NFLP were incubated for 5 min with different concentrations of SNAP and then stimulated with thrombin (protocol 1) or with SNAP plus thrombin (protocol 2). In both cases, platelets were stimulated in the presence of 1 mM $CaCl_2$. The thrombin concentration used was 0.1 unit/ml. *T*, addition of thrombin; *SNAP+T*, simultaneous addition of SNAP and thrombin. SNAP concentrations (in μM) are indicated in parentheses. Aggregation traces were obtained in a representative experiment. Values represent maximum aggregation (mean ± standard error) of five experiments with different platelet preparations. Similar results were obtained with the nitric oxide donor SIN-1 (data not shown).

Effect of SNP on thrombin-induced serotonin secretion. To understand the delay in the onset of the NFLP aggregation shown in Fig. 1, we studied the serotonin secretion induced by 0.1 unit/ml thrombin (Fig. 3). NFLP were incubated for 5 min with 5 μM SNP before stimulation with 0.1 unit/ml thrombin (protocol 1). In the absence of SNP, the serotonin secretion induced by thrombin is quick and peaks at ~1 min. However, in the presence of 5 μM SNP, the secretion was slower than that in the absence of SNP. In fact, no secretion was obtained in the first minute after stimulation, and the secretion was clearly delayed. The delay in the onset of NFLP aggregation and the subsequent sharp aggregation observed in Fig. 1 (at 5 μM SNP) were associated with a similar effect on the secretion of serotonin. Also, the maximum serotonin secretion induced by 0.1 unit/ml thrombin in the presence of SNP was lower than that in the absence of SNP. In fact, 5 μM SNP induced a similar inhibition of the maximum secretion and maximum aggregation values (obtained at 10 min), ~25% (Figs. 1 and 3). Minimum secretion was observed in control platelets (addition of vehicle) in both the absence and the presence of SNP. The total content of serotonin in platelets in both the absence and the presence of SNP was similar, 292 ± 18 and 261 ± 26 nmol/10^{11} cells, respectively (mean ± standard error).

Effects of SNP on increases in $[Ca^{2+}]_i$ induced by thrombin. Independent of the protocol used, SNP inhibited the FLP aggregation induced by thrombin. To understand the rapid inhibition induced by SNP on FLP aggregation, the effect of both protocols on increases in $[Ca^{2+}]_i$ induced by 0.1 unit/ml thrombin was studied in parallel experiments (Fig. 4). As previously reported (2, 11), during the period of preincubation (5 min) with different concentrations of SNP (protocol 1), the basal Ca^{2+} levels were not altered significantly (Fig. 4A). Independent of the protocol used, SNP inhibited, to a similar magnitude and in a dose-dependent manner, the

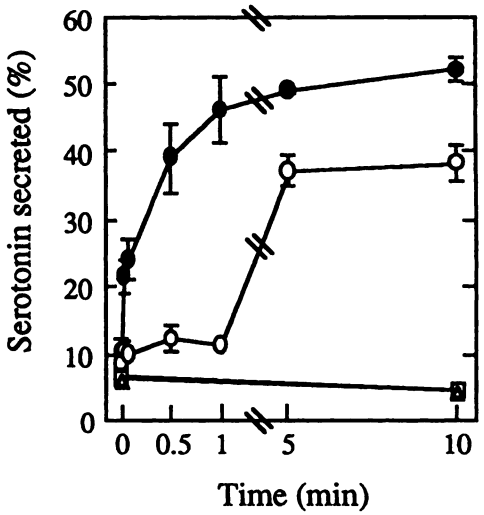

Fig. 3. Effect of SNP on secretion of serotonin induced by thrombin. NFLP were incubated for 5 min with vehicle (●) or 5 μM SNP (○) in the presence of 1 mM $CaCl_2$. Then, cells were stimulated with 0.1 unit/ml thrombin (protocol 1). Reaction was stopped at the indicated times. Values are mean ± standard error of three experiments performed in duplicate with different platelet preparations. The secretion is expressed as percentage of secretion with respect to the total content of serotonin (100%). Secretion of control platelets (stimulation with vehicle) in the absence (□) and the presence of SNP (△) is also shown. Error bars are omitted when smaller than the symbol.

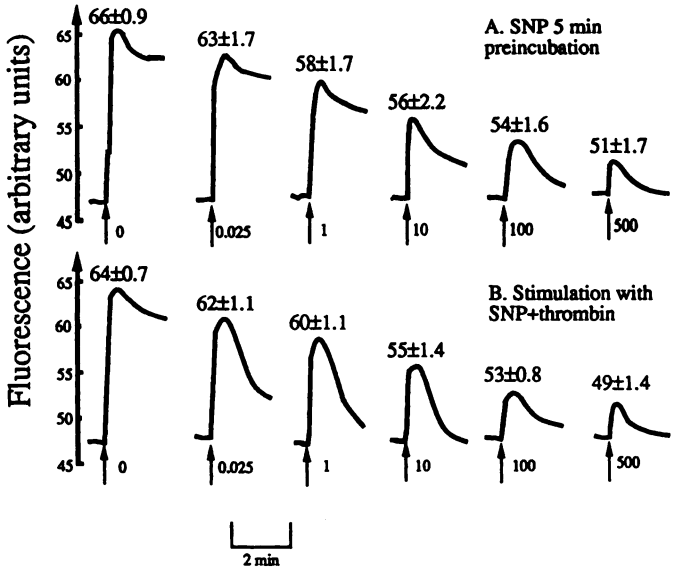

Fig. 4. SNP inhibits the increases in $[Ca^{2+}]_i$ induced by thrombin without a significant time delay. FLP were incubated for 5 min with different concentrations of SNP and then stimulated with thrombin (protocol 1; A) or with SNP plus thrombin (protocol 2; B). In both cases, platelets were stimulated in the presence of 1 mM $CaCl_2$. The thrombin concentration used was 0.1 unit/ml. *Arrows*, addition of thrombin (A) or SNP plus thrombin (B). *Bottom numbers*, SNP concentrations (in μM) (additional SNP concentrations were studied; however, for clarity, only these SNP concentrations are shown). *Top numbers*, increase in fluorescence (arbitrary units) (mean ± standard error of six to nine experiments with different platelet preparations). Fluorescence traces were obtained in a representative experiment.

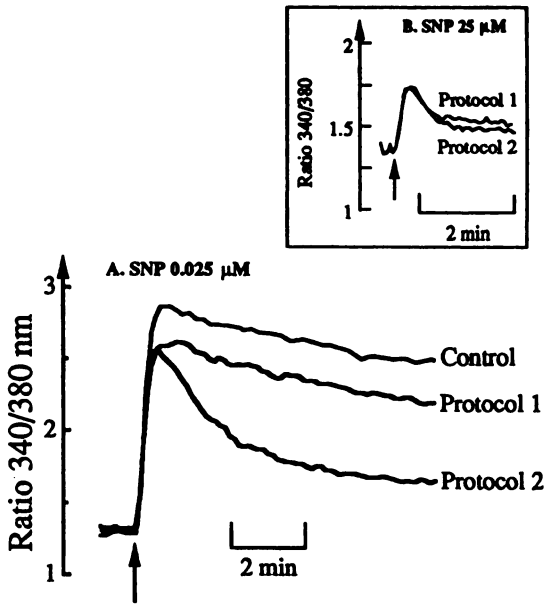

Fig. 5. SNP accelerated the decline of the Ca^{2+} levels. FLP were incubated for 5 min with SNP and then stimulated with thrombin (protocol 1) or with SNP plus thrombin (protocol 2) in the presence of 1 mM $CaCl_2$. The thrombin concentration used was 0.1 unit/ml, whereas the SNP concentrations were 0.025 μM (A) and 25 μM (B, *insert*). *Arrows*, addition of thrombin or SNP plus thrombin (protocols 1 and 2, respectively). In A, for clarity, only the control trace obtained with protocol 1 (incubation with vehicle for 5 min before stimulation with thrombin) is shown. A similar control trace was obtained with protocol 2 (stimulation with vehicle plus thrombin). In B, the control traces are not shown, and only the early minutes are represented. The traces were obtained in a representative experiment. Similar results were obtained in four different experiments. This experiment was performed by the ratio method. The fluorescence signals from two excitation wavelengths (340 and 380 nm) at a single emission wavelength (505 nm) were measured. The autofluorescence was measured at both wavelengths and subtracted from all data before calculation of the ratios.

maximum increase in $[Ca^{2+}]_i$ (Fig. 4), which supports the inhibition of FLP aggregation shown in Fig. 1.

It is clear that the activation by thrombin is rapid. The addition of thrombin and other agonists induced a sharp increase in $[Ca^{2+}]_i$, and the peak was reached within a few seconds. In this way, the addition of SNP plus thrombin was able to inhibit the rapid increase in $[Ca^{2+}]_i$ induced by thrombin. However, different fluorescence traces were observed (Fig. 4). Thrombin caused a sharp rise in $[Ca^{2+}]_i$, and then a slow decline in the Fura-2 signal was observed (Fig. 4, control traces). A slow decline in the Fura-2 signal was also observed when platelets were incubated with SNP for 5 min before stimulation (particularly in the presence of a low dose of SNP, 0.025 and 1 μM) (Fig. 4A). When cells were stimulated with SNP plus thrombin, the decline in the Fura-2 signal was clearly accelerated (Fig. 4B). However, blood platelets aggregate in response to many agonists, and this produces a loss in the fluorescence signal from Fura-2 and looks, based on a single wavelength (340 nm excitation and 505 nm emission), like a fall in $[Ca^{2+}]_i$. However, if the ratio method (340/380 nm excitation and 505 nm emission) is used, the loss of signal is proportionately the same at both wavelengths in a dual recording so that the ratio of the two signals can measure $[Ca^{2+}]_i$ even during the formation of aggregates (31). The ratio method was used to study this effect (Fig. 5). SNP (0.025 μM) inhibited the maximum increase in $[Ca^{2+}]_i$ induced by thrombin in a similar magnitude independent of the protocol used (Fig. 5A). Also, when platelets were stimulated with SNP plus thrombin (protocol 2), the decline in the $[Ca^{2+}]_i$ was clearly accelerated (Fig. 5A). Similar results were obtained with 1, 5, 25, and 100 μM SNP (data not shown); however, the effect was less evident at high doses of SNP because the increase in the $[Ca^{2+}]_i$ was strongly inhibited. Thus, an accelerated decline in the Fura-2 fluorescence was clearly observed at a low dose of SNP (0.025 and 1 μM) (Figs. 5A and 4 and data not shown), whereas at high SNP concentrations, the accelerated decline in the Fura-2 fluorescence was not as evident because the maximum increase in the $[Ca^{2+}]_i$ was strongly inhibited (Fig. 4 and data not shown). An example based on the use of 25 μM SNP is shown in Fig. 5B (*insert*).

It is known that the thrombin-induced platelet activation probably has properties of both an enzyme-mediated reaction and an agonist-receptor equilibrium (for a review, see Ref. 32). In fact, a mechanism for the activation by thrombin has been described that involves proteolytic cleavage of the receptor (33). To discard the possibility of a direct effect of SNP on the enzymatic activity of thrombin, we performed an assay of the enzymatic activity of thrombin using one of its natural substrates (fibrinogen) by visual estimation of the clotting time. The esterase activity of thrombin was estimated using the chromogenic substrate Tos-Gly-L-Pro-L-Arg-pNA. The addition of different SNP concentrations to the reaction medium before thrombin or the addition of SNP plus thrombin did not affect the enzymatic activity of thrombin based on visual estimation of the clotting time (data not shown). Esterase activity of thrombin, using the chromogenic substrate

Tos-Gly-L-Pro-L-Arg-pNA, was not affected by SNP at doses of ≤200 μM (SNP added before thrombin or SNP plus thrombin). However, in both cases, 500 μM SNP slightly inhibited the esterase activity of thrombin (statistical significance was reached) (data not shown). We cannot exclude that at this concentration of SNP, the inhibition (or part of the inhibition) could be due to this effect.

Effect of SNP added after thrombin on NFLP and FLP activation. We studied the effect of 10 μM SNP added 20 sec after 0.1 unit/ml thrombin on the NFLP and FLP aggregation (Fig. 6). The addition of SNP 20 sec after thrombin significantly inhibited the NFLP and FLP aggregation (Fig. 6). The inhibition of NFLP aggregation was smaller than that of FLP aggregation. This result indicates that the addition of SNP after thrombin was able to block the cellular activation. In addition to the effect on platelet aggregation, we studied the effect on Ca^{2+} levels using the ratio method (data not shown). The addition of 10 μM SNP after thrombin rapidly accelerated the decline in the Fura-2 signal. Similar effects on the Ca^{2+} signal have been observed previously in platelets stimulated with platelet-activating factor (11) and thrombin (9).

SNP rapidly inhibits the thrombin-stimulated phospholipase C activity. It has been described that the preincubation for several minutes with both SNP and metabolically stable analogues of cGMP inhibited the intracellular Ca^{2+} mobilization (3, 10, 15) and the phospholipase C activity stimulated by thrombin (3, 4, 8). Using $NiCl_2$ to block Ca^{2+} influx (28), we also observed that SNP inhibited the intracellular Ca^{2+} mobilization induced by thrombin (data not shown). With the purpose of understanding the rapid inhibition induced by SNP on the maximum increase in $[Ca^{2+}]_i$, the effect on the phospholipase C activity stimulated by thrombin was studied (Fig. 7). In the absence of SNP, thrombin induced rapid rises in $InsP_2$ and $InsP_3$. Thus, at 15 and 30 sec after thrombin addition, the levels of $InsP_2$ and

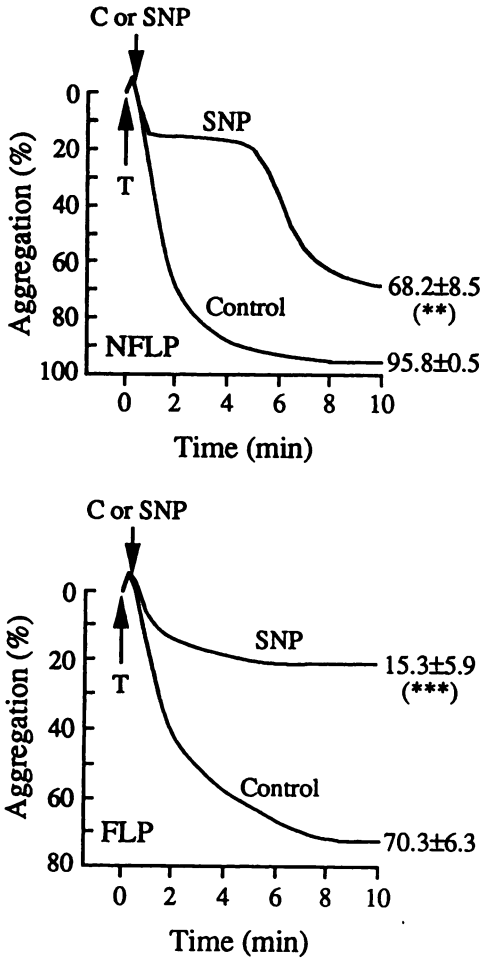

Fig. 6. Effect of SNP added after thrombin on platelet aggregation. NFLP or FLP were stimulated with 0.1 unit/ml thrombin. Twenty seconds later, vehicle (*C*) or 10 μM SNP was added. *T*, addition of thrombin. Experiments were performed in the presence of 1 mM $CaCl_2$. Values represent maximum aggregation (mean ± standard error) of nine (NFLP) and four (FLP) experiments with different platelet preparations. **, $p < 0.01$, and ***, $p < 0.001$, compared with the respective value for control.

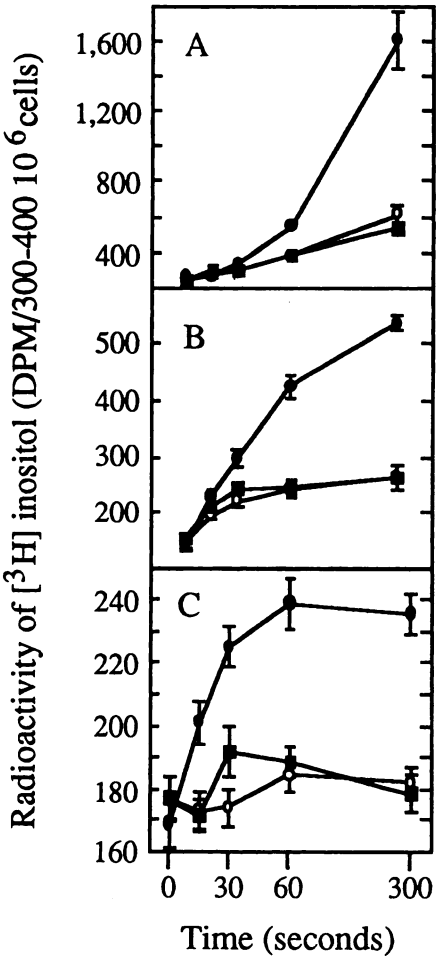

Fig. 7. Effect of SNP on the thrombin-stimulated phospholipase C activity. [^{3}H]Inositol-labeled platelets were incubated for 5 min with SNP and then stimulated with thrombin (protocol 1) (○) or with SNP plus thrombin (protocol 2) (■) in the presence of 1 mM $CaCl_2$. The stimulation control (in the absence of SNP) obtained with both protocols was similar; for this reason, the stimulation control shown (●) is the mean ± standard error of the control stimulation obtained under the two different conditions. The reaction was stopped at the times indicated. The thrombin concentration used was 0.1 unit/ml, whereas the SNP concentration was 25 μM. Values are mean ± standard error of two experiments performed in triplicate. $InsP_1$ (A), $InsP_2$ (B), and $InsP_3$ (C) were analyzed by anion-exchange chromatography on a Dowex AG1-X8 column and eluted. Error bars are omitted when smaller than the symbol.

$InsP_3$ rose significantly. The level of $InsP_1$ rose less rapidly than the levels of $InsP_2$ and $InsP_3$. Without previous preincubation with SNP (protocol 2), SNP inhibited the rapid increase in the $InsP_3$ levels. In fact, $InsP_3$ levels obtained at 15, 30, and 60 sec were significantly lower than the values obtained in the absence of SNP. The $InsP_2$ level at 15 sec was similar but levels at 30 and 60 sec were lower than values obtained in the absence of SNP. $InsP_1$ levels at 15 and 30 sec were similar but $InsP_1$ levels at 60 and 300 sec were lower than values obtained in the absence of SNP. Therefore, the simultaneous addition of SNP and thrombin was able to rapidly inhibit the phospholipase C activity stimulated by thrombin. In addition, we confirm previous observations in which the preincubation with SNP before stimulation with thrombin inhibited the phospholipase C activity.

Increases in cGMP levels induced by SNP. We have shown that the addition of SNP plus thrombin (protocol 2) or preincubation for 5 min with SNP before thrombin (protocol 1) inhibited NFLP and FLP aggregation and the maximum increase in $[Ca^{2+}]_i$ induced by 0.1 unit/ml thrombin (Figs. 1 and 4, respectively). It is clear that the activation by thrombin is very rapid. Therefore, NFLP aggregation was fast during the first 2 min after thrombin addition (aggregation of ≥70%), reaching maximal values after 5–6 min of incubation (Fig. 1, control traces). Also, the addition of thrombin and other agonists induced a sharp increase in $[Ca^{2+}]_i$ and the peak was reached within a few seconds. Therefore, with both protocols, SNP was able to inhibit the maximum increase of Ca^{2+} stimulated by thrombin compared with the Ca^{2+} signal observed in the absence of SNP (Fig. 4). In contrast to protocol 1, when cells were stimulated with SNP plus thrombin (protocol 2), an accelerated decline in the thrombin-induced Ca^{2+} signal was observed (Figs. 4 and 5A). However, this effect was not as evident at high SNP concentrations (Fig. 5B, *insert*). In addition, the phospholipase C activity stimulated by thrombin was inhibited by SNP independent of the protocol used (Fig. 7). To understand the rapid effect of SNP on platelet activation, we determined cGMP levels (Fig. 8). In this experiment, three different conditions were used: (a) SNP was added, and the reaction was immediately stopped; (b) SNP and thrombin were added, and the reaction was immediately stopped (protocol 2); and (c) platelets were incubated with SNP for 5 min before the reaction was stopped (protocol 1). SNP or SNP plus thrombin quickly increased the cGMP levels in a dose-dependent manner (Fig. 8). These results show that SNP was able to immediately increase cGMP levels and that the addition of SNP plus thrombin did not alter the levels of cGMP in comparison with the addition of only SNP. Although it has been described previously that thrombin increases the cGMP levels, the increase induced by thrombin is severalfold lower than that induced by SNP (34) and is probably hidden by the SNP-induced increase. When the platelets were incubated for 5 min with SNP (protocol 1), 10 and 25 μM SNP induced cGMP levels similar to those obtained under the other two conditions. However, the cGMP level obtained with 100 μM SNP was smaller than those obtained under the other two conditions, which is probably due to the breakdown of cGMP by phosphodiesterases.

The time-dependent effect of 10 μM SNP on cGMP levels is shown in Fig. 9. The maximum cGMP level was obtained at 3 min of incubation and then declined (probably via the action of phosphodiesterases). Interestingly, when 10 μM

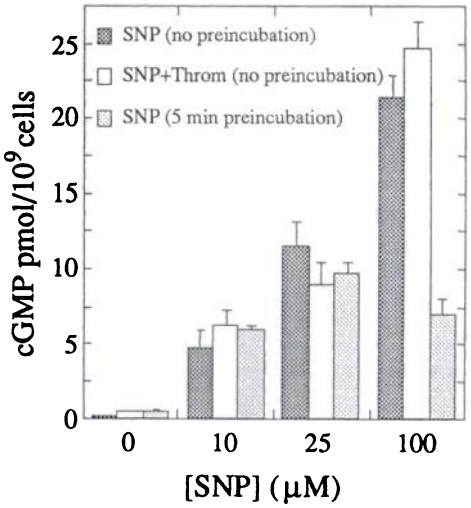

Fig. 8. Rapid increase in the cGMP level induced by SNP. NFLP were prepared and the cGMP levels were measured as described in Experimental Procedures. Three different conditions were used: (a) SNP was added, and the reaction was stopped immediately; (b) SNP plus thrombin (*Throm*) were added, and the reaction was stopped immediately (protocol 2); and (c) cells were incubated for 5 min with SNP before the reaction was stopped (protocol 1). All of the experiments were performed in the presence of 1 mM $CaCl_2$. Values are mean ± standard error of two experiments performed in duplicate with different platelet preparations.

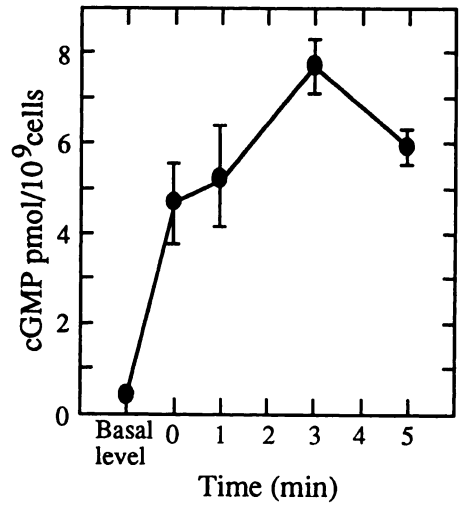

Fig. 9. Time course of effects of SNP on cGMP levels in NFLP. Washed human platelets were incubated with 10 μM SNP for 1, 3, or 5 min before the reaction was stopped. *Time 0*, addition of 10 μM SNP; the reaction was stopped immediately. The basal level of cGMP is also indicated. The experiments were performed in the presence of 1 mM $CaCl_2$. Values represent mean ± standard error of two experiments performed in duplicate with different platelet preparations. Error bars are omitted when smaller than the symbol.

SNP was added and the reaction was immediately stopped (Fig. 9, *time 0*), the cGMP level was quickly and significantly increased with respect to basal level (Fig. 9). The rapid and significant increase in the cGMP levels supports the inhibition of the thrombin-induced platelet response without any significant delay time (especially regarding Ca^{2+} levels and phospholipase C activity).

Several investigators have noted a SNP-induced elevation in cAMP levels in human and rabbit platelets (5, 22–24). In addition to the increase in cGMP levels, the inhibition of the

platelet response by SNP could be due to an increase in the cAMP levels. The use of 0.1 μM PGE_1 (a known activator of the adenylate cyclase) rapidly (≤30 sec) elevated the platelet cAMP level (from ~13 to 136 pmol/10^9 cells) and then declined (Fig. 10). The use of 25 μM SNP was not able to significantly increase the cAMP level within 5 min (from ~12 to 14.8 pmol/10^9 cells within 1 min) (Fig. 10).

Methylene blue reversed the rapid inhibition induced by SNP on NFLP aggregation. The effect of 25 μM SNP on NFLP aggregation was studied in cells preincubated with methylene blue, a known inhibitor of soluble guanylate cyclase (Fig. 11). Platelets were pretreated with 2 μM methylene blue for 15 min at room temperature, and then cells were stimulated in accordance with protocol 1 or 2. Fig. 11A shows the effect of the methylene blue on the traces of aggregation, whereas Fig. 11B shows the effect of methylene blue on the maximum aggregation. Preincubation with SNP or the simultaneous addition of SNP and thrombin inhibited the maximum aggregation and delayed the onset of the aggregation. Pretreatment with methylene blue reversed the inhibition of aggregation induced by 25 μM SNP toward normal values, independent of the protocol used (Fig. 11, A and B). However, methylene blue nearly completely reversed the inhibition of the maximum aggregation induced by SNP, whereas the inhibition of the slope of aggregation did not revert completely (a small inhibition was still observed compared with the control trace) (Fig. 11A). This result supports that the inhibition of the NFLP aggregation without any significant time delay observed with protocol 2 is principally due to a rapid increase in the cGMP levels mediated by guanylate cyclase.

Role of PKG and PKA in the inhibition of platelet aggregation induced by SNP. The inhibition by agents that elevate the cGMP or cAMP levels seems to be due to the activation of PKG and PKA, respectively. However, it has also been shown that cGMP can inhibit the platelet activation by preventing the breakdown of cAMP by the cGMP-inhibited cAMP phosphodiesterase (5, 22–24) and that the PKA activation may be responsible, at least in part, for the inhibition of platelet function by SNP. In fact, low intracellular levels of cGMP effectively inhibit type III cGMP-inhibited phosphodiesterase in human platelets (35). From this standpoint, the inhibition of platelet activation could be related to the cGMP-dependent activation of the PKA. To further examine this possibility, selective inhibitors of PKG and PKA were used to elucidate the role of these kinases in the platelet inhibition. The effect of KT-5823, an inhibitor of the PKG (36), on the inhibition of the platelet aggregation induced by SNP was examined (data not shown). NFLP were preincubated for 10 min at 37° with three different concentrations of KT-5823 (125, 250, and 500 nM). Then, cells were stimulated in accordance with protocol 1 or 2. Three different SNP concentrations were used (1, 10, and 100 μM). At any concentration used, KT-5823 did not reduce the inhibitory effect induced by SNP on NFLP aggregation, either on the maximum aggregation or on the profile of aggregation (data not shown). This result could suggest inhibition of platelet aggregation induced by SNP by PKG-independent mechanisms. However, it is also possible that an effective inhibition of the PKG in platelets can require higher dose of inhibitor and/or longer periods of incubation. Thus, we studied the effect of 20 μM KT-5823 on the inhibition induced by 8-pCPT-cGMP, a potent and selective activator of PKG in intact cell

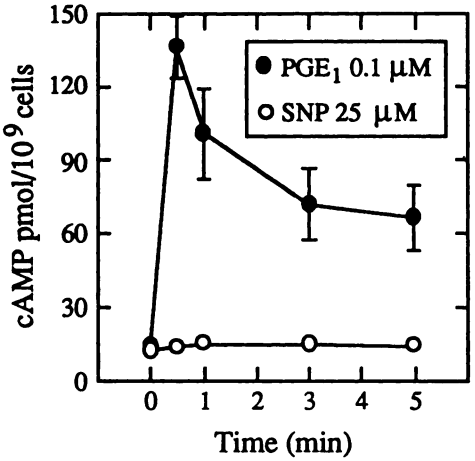

Fig. 10. Time course of effects of SNP on cAMP levels in platelets. Platelets were incubated with 0.1 μM PGE_1 (●) or 25 μM SNP (○) in the presence of 1 mM $CaCl_2$ for times indicated. The cAMP levels were measured. *Data*, mean ± standard error of two experiments done in triplicate. Error bars are omitted when smaller than the symbol.

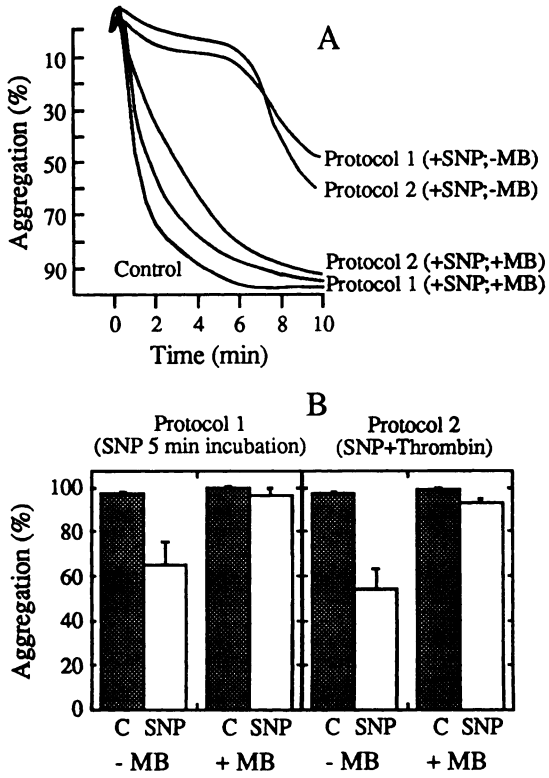

Fig. 11. Effect of methylene blue on the inhibition induced by SNP. NFLP were pretreated with 2 μM methylene blue (+*MB*) or vehicle (−*MB*) for 15 min at room temperature. Then, the cells were stimulated in accordance with protocol 1 [preincubation with SNP or vehicle (*C*) for 5 min before stimulation] or protocol 2 [stimulation with SNP plus thrombin (*SNP*) or vehicle plus thrombin (*C*)]. A, Effect on aggregation traces. The control aggregations (in the absence of SNP) were not affect by methylene blue. For this reason and for clarity, only the control aggregation obtained with protocol 1 in the absence of methylene blue is shown. Traces represented were obtained in a representative experiment. B, Effect on the maximum aggregation. The SNP concentration was 25 μM, and the thrombin concentration was 0.1 unit/ml. Values are mean ± standard error of 10 experiments with different platelet preparations.

preparations (13) (data not shown). NFLP were preincubated for 45 min at 37° with 20 μM KT-5823 or vehicle. Then, cells were also incubated for 15 min with 0.5 mM 8-pCPT-cGMP or vehicle and stimulated with 0.1 unit/ml thrombin. In the absence of KT-5823, 8-pCPT-cGMP weakly inhibited the maximum aggregation, whereas it clearly inhibited the velocity of platelet aggregation (see Fig. 12C). Therefore, 8-pCPT-cGMP produced a change in the velocity of platelet aggregation induced by thrombin. The preincubation with 20 μM KT-5823 did not affect the inhibitory effect (velocity of platelet aggregation) induced by 8-pCPT-cGMP (data not shown).

We have also studied the effect of the KT-5720, a specific inhibitor of PKA (36), on the inhibition induced by PGE_1 and SNP (both protocols) (Fig. 13). In the absence of PGE_1 or SNP, 25 μM KT-5720 did not affect the thrombin-induced platelet aggregation (data not shown). In fact, similar aggregations were observed in both the absence (vehicle) and the presence of KT-5720 (~95% of aggregation). In the absence of KT-5720, PGE_1 and SNP (both protocols) inhibited the maximum aggregation induced by thrombin (Fig. 13). Therefore, the maximum aggregations in the presence of PGE_1 and SNP (both protocols) were 22% and 45% of aggregation, respectively (75% and 53% of inhibition with respect to value controls). In the presence of KT-5720, maximum aggregations induced by thrombin in the presence of PGE_1 and SNP (protocols 1 and 2) were 52%, 73%, and 65%, respectively (Fig. 13) (45%, 24%, and 31% of inhibition with respect to value controls). Therefore, KT-5720 was able to reduce partially the inhibitory effects of PGE_1 and SNP (both protocols).

The effects of Rp-8-pCPT-cGMPS and Rp-8-CPT-cAMPS, inhibitors of PKG and PKA, respectively (37, 38), on the inhibition induced by SNP was also examined (see Fig. 12).

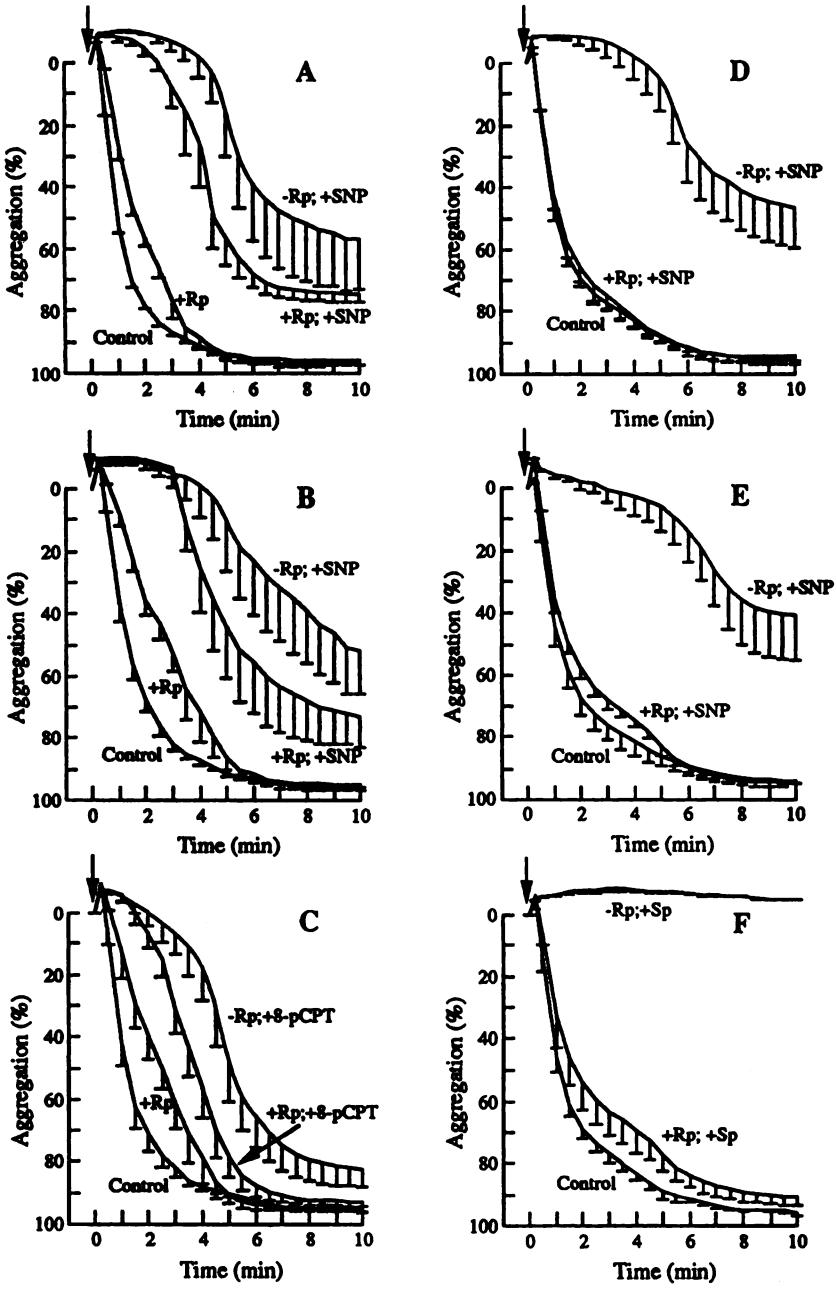

Fig. 12. The inhibitory effect of SNP was abolished by Rp-8-CPT-cAMPS. NFLP were previously preincubated with 0.8 mM Rp-8-pCPT-cGMPS or vehicle (A–C) or with 0.5 mM Rp-8-CPT-cAMPS or vehicle (D–F) for 45 min at 37°. Subsequently, cells were also preincubated with 10 μM SNP or vehicle for 5 min at 37° before stimulation with 0.1 unit/ml thrombin (protocol 1) (A and D); stimulated with SNP plus thrombin or vehicle plus thrombin (protocol 2) (B and E); or also preincubated with 0.5 mM 8-pCPT-cGMP or vehicle (C) and 0.1 mM Sp-5,6-DCl-cBIMPS or vehicle (F) for 15 min at 37° before stimulation with 0.1 unit/ml thrombin. D–F, Traces in the presence of only Rp-8-CPT-cAMPS were omitted for clarity but were similar to the control traces. Platelets were stimulated in the presence of 1 mM $CaCl_2$. *Arrows*, addition of thrombin (A, C, D, and F) and the addition of SNP plus thrombin or vehicle plus thrombin (B and E). Traces represent the average curves ± standard error of four (A), five (B), eight (C), and three (D–F) experiments with different platelet preparations.

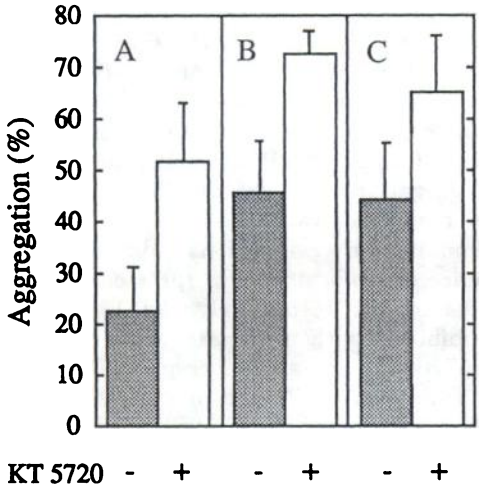

Fig. 13. Effect of KT-5720 on the inhibition induced by PGE_1 and SNP. NFLP were preincubated with 25 μM KT-5720 (+) or vehicle (−) for 45 min at 37°. Subsequently, cells were incubated with 5×10^{-7} M PGE_1 for 5 min before stimulation with 0.1 unit/ml thrombin (A); incubated with 10 μM SNP for 5 min before stimulation with 0.1 unit/ml thrombin (protocol 1) (B); or stimulated with SNP plus thrombin (protocol 2) (C). Platelets were stimulated in the presence of 1 mM $CaCl_2$. Values represent maximum aggregations (mean ± standard error) of three experiments with different platelet preparations.

The preincubation with 0.8 mM Rp-8-pCPT-cGMPS slightly inhibited the velocity of the platelet aggregation induced by 0.1 unit/ml thrombin (Fig. 12, A–C, *traces +Rp*). The inhibitory effects of SNP observed with both protocol 1 (Fig. 12A) and protocol 2 (Fig. 12B) were only partially reduced by Rp-8-pCPT-cGMPS. In a similar manner to SNP, the inhibitory effect of 8-pCPT-cGMP (principally on the velocity of the aggregation) was also partially reduced by Rp-8-pCPT-cGMPS (Fig. 12C). It is clear for the standard error shown in Fig. 12, A–C, that a high variability was observed. Thus, in some experiments, a clearer effect of the Rp-8-pCPT-cGMPS on the inhibition induced by SNP or 8-pCPT-cGMP was observed, whereas in other experiments, no effects of Rp-8-pCPT-cGMPS were observed. However, even in the experiments with a clearer effect, Rp-8-pCPT-cGMPS induced only partial reversions. Similar results were obtained with 0.2 mM Rp-8-pCPT-cGMPS (data not shown). In contrast to Rp-8-pCPT-cGMPS, Rp-8-CPT-cAMPS nearly completely abolished the inhibitory response induced by SNP (both protocols) and Sp-5,6-DCl-cBiMPS, a specific activator of PKA (39) (Fig. 12, D–F, respectively). Thus, in the absence of SNP or Sp-5,6-DCl-cBiMPS, the preincubation with 0.5 mM Rp-8-CPT-cAMPS did not affect the platelet aggregation induced by 0.1 unit/ml thrombin (for this reason and for clarity, the curves in the presence of only Rp-8-CPT-cAMPS have been omitted in Fig. 12). The PKA activator Sp-5,6-DCl-cBiMPS completely inhibited the thrombin-induced platelet aggregation (Fig. 12F). However, in the presence of Rp-8-CPT-cAMPS, the inhibitory response induced by Sp-5,6-DCl-cBiMPS was nearly completely abolished (Fig. 12F). Surprisingly and in a similar manner to Sp-5,6-DCl-cBiMPS, the inhibitory effect of SNP (independent of the protocol used) was nearly completely abolished by Rp-8-CPT-cAMPS (Fig. 12, D and E).

Discussion

The preincubation for 5 min with SNP before stimulation with thrombin or the simultaneous addition of SNP and thrombin inhibited the NFLP and FLP aggregations and the increases in $[Ca^{2+}]_i$ in FLP in a dose-dependent manner (Figs. 1 and 4, respectively). Other nitric oxide donors (SNAP and SIN-1) also inhibited the NFLP aggregation induced by thrombin without a significant time delay and to a similar order of magnitude (Fig. 2 and data not shown). The inhibition induced by SNP of the increase in $[Ca^{2+}]_i$ correlates better with the inhibition of FLP aggregation than with the inhibition of NFLP aggregation (Figs. 1 and 4). In addition, the simultaneous addition of SNP and thrombin clearly accelerated the decline in the Fura-2 signal (Figs. 4 and 5A). However, this effect was evident at low doses of SNP (0.025 and 1 μM), whereas it was not as evident at major SNP concentration (Figs. 4 and 5B, *insert*) (probably because the increase in $[Ca^{2+}]_i$ was strongly inhibited). The rapid inhibition observed was accompanied by a rapid and significant increase in cGMP levels (Figs. 8 and 9) and reversed when platelets were pretreated with the inhibitor of guanylate cyclase, methylene blue (Fig. 11). Thus, the inhibition by SNP of the platelet activation without a significant time delay seems to be principally due to a rapid increase in the cGMP levels mediated by guanylate cyclase. Considerable evidence suggests that the inhibition of the platelet response by cAMP- and cGMP-elevating agents in human platelets is primarily due to the inhibition of agonist-induced phospholipase C activation (3, 4, 8). In these, as in other many studies, cells were preincubated for ≥1 min with SNP before stimulation. In our study, the inhibition of aggregation and the Ca^{2+} response without any significant time delay and the rapid increase in cGMP levels support that cGMP-elevating vasodilators inhibit the platelet response at an early step at the activation cascade. We confirm a previous observation in which the preincubation with SNP before stimulation with thrombin inhibited the phospholipase C activity. Surprisingly, the simultaneous addition of SNP and thrombin was also able to inhibit the rapid activation of the phospholipase C induced by thrombin (Fig. 7). However, the mechanism of phospholipase C inhibition by SNP in platelets has not been elucidated at the molecular level.

Considerable evidence suggests that the effects of drugs that elevate cAMP and cGMP are mediated by PKA and PKG, respectively (10, 13–15). A protein has been purified and characterized, VASP, whose vasodilator-stimulated phosphorylation in intact cells is mediated by PKA or PKG, and VASP phosphorylation seems to be an important component of the intracellular mechanism of action of the nitrovasodilators in human platelets (10, 13, 16–19). However, the mechanisms by which PKG and VASP phosphorylation are involved in platelet inhibition are not clear. Very recently, Horstrup *et al.* (21) proposed that VASP phosphorylation in intact platelets is more closely associated with the inhibition of aggregation by regulating the fibrinogen receptor than by inhibiting Ca^{2+} mobilization. In addition, a crossover effect between cyclic nucleotides and the protein kinases that they activate may occur. It is clear that SNP increases cGMP levels and induces PKG activation. Also, it is well established that SNP is able to induce small increases (in percentages) in platelet cAMP levels, probably through a cGMP-inhibited

cAMP phosphodiesterase (5, 22–24) and in this way activates the cAMP signal transduction cascade. In addition, in PKG-deficient platelets, SNP caused a small phosphorylation, presumably via PKA (14). In our study, SNP was not able to significantly increase the cAMP level (Fig. 10). Other investigators have not observed effects of SNP on cAMP levels (2, 17). However, low intracellular levels of cGMP can effectively inhibit the phosphodiesterase in human platelets despite that high levels of PKG present in this cell type. The cGMP-binding site of the cGMP-inhibited phosphodiesterase can successfully compete with the cGMP-binding site of type I PKG for the available cGMP in intact platelets (35). In addition, inhibitors of the cGMP-inhibited cAMP phosphodiesterase cause small increases in cAMP levels and activation of PKA, and these effects seem to be associated with the inhibition of the platelet aggregation (40, 41). Therefore, it is possible that small increases in cAMP levels cause inhibition of platelet aggregation by PKA activation. In our experiments, SNP, in addition to a cGMP/PKG mechanism, could activate the cAMP/PKA mechanism. To further examine this possibility, selective inhibitors of PKA and PKG were used to elucidate the role of these kinases in the inhibition induced by SNP of the platelet aggregation.

KT-5823 seems to be an effective inhibitor of PKG in intact cells (42, 43). KT-5823 concentrations at 0.1–1 μM are usually used in these studies. In our study, the preincubation for 10 min with 0.5 μM as a maximal concentration did not prevent the inhibitory effect of SNP on platelet aggregation. This result could suggest inhibition of platelet aggregation by independent mechanisms of PKG. However, there is some discrepancy between *in vitro* and *in vivo* systems regarding the effective dose of protein kinase inhibitors. Therefore, the potency of an inhibitor in intact cells is dependent on its permeability. An effective inhibition can require a longer period of incubation and/or a higher dose of inhibitor. Also, a high dose of inhibitor can have nonspecific actions. However, the preincubation for 45 min with 20 μM KT-5823 did not affect the inhibitory effect induced by 8-pCPT-cGMP, a specific activator of PKG (13). This result suggests inhibition by PKG-independent mechanisms. However, in our opinion, with the use of 8-pCPT-cGMP, KT-5823 could be expected to revert, at least partially, the inhibitory effect. In addition, it has been reported that KT-5823 did not inhibit the phosphorylation of vimentin mediated by PKG and that the activation of neutrophils by KT-5823 may not involve PKG activation (44, 45). Thus, it is possible that under our experimental conditions, KT-5823 could not be an effective inhibitor of PKG in intact platelets. This hypothesis was supported by the effect of the Rp-8-pCPT-cGMPS, which has been described as a selective inhibitor of PKG (37). In fact, Rp-8-pCPT-cGMPS antagonized the activation of PKG by 8-pCPT-cGMP. In our study, Rp-8-pCPT-cGMPS partially reverted the inhibitory effect induced by SNP and 8-pCPT-cGMP. However, although in some experiments a more evident effect (Fig. 12) was observed, in other experiments no effect of Rp-8-pCPT-cGMPS was observed. Although we cannot explain this variability, the results obtained suggest a partial inhibition of platelet aggregation by PKG-dependent mechanisms.

On the other hand, we also evaluated the effect of PKA inhibitors. Thus, KT-5720 partially reverted the inhibitory effect induced by SNP, suggesting an important role for PKA in the inhibitory effects induced by SNP. This result was supported by the results obtained with Rp-8-CPT-cAMPS, an inhibitor of PKA (38). Surprisingly, the inhibitory effects induced by SNP were nearly completely abolished by Rp-8-CPT-cAMPS. In contrast to the partial and highly variable effects of Rp-8-pCPT-cGMPS, the clear effect of Rp-8-CPT-cAMPS seems to indicate that the activation of PKA by SNP could be the more important mechanism in the inhibition of platelet aggregation induced by SNP. However, the specificity of Rp-8-CPT-cAMPS remains to be elucidated.

Independent of the protocol used, SNP inhibited the maximum increase in $[Ca^{2+}]_i$ induced by thrombin. The inhibition could be caused by one or more mechanisms, including inhibition of phospholipase C, stimulation of Ca^{2+} reuptake by intracellular stores, stimulation of Ca^{2+} extrusion from the platelets, or inhibition of Ins(1,4,5)P3-induced Ca^{2+} release from intracellular stores. With protocol 1, the inhibition of the maximum increase in $[Ca^{2+}]_i$ induced by thrombin could be due to inhibition of phospholipase C, whereas an accelerated decline in the Ca^{2+} signal was not observed. However, with protocol 2, inhibition of the maximum increase in $[Ca^{2+}]_i$ could be due to both an accelerated decline in the Ca^{2+} signal and an inhibition of phospholipase C. An accelerated decline in the Fura-2 fluorescence was clearly observed at low dose of SNP, whereas at high SNP concentrations, the accelerated declined in the Fura-2 signal was not as evident, and the inhibition of phospholipase C could be more significant at a high dose of SNP.

The addition of SNP after thrombin inhibited the platelet aggregation (Fig. 6) and rapidly accelerated the decline in the Fura-2 signal (data not shown). Probably, the effect of the addition of SNP after thrombin on the decline in $[Ca^{2+}]_i$ reflects sequestration and/or extrusion of Ca^{2+} rather than inhibition of persisting Ca^{2+} influx and/or mobilization of internal Ca^{2+}. Whether Ca^{2+}/ATPases are involved in this process is not clear.

On the other hand, SNP clearly delayed the onset of NFLP aggregation induced by thrombin. A similar profile in human platelets incubated with *S*-nitrosothiols and stimulated with sodium arachidonate has been reported (6). The delay and the sharp aggregation observed may be due to a similar effect on the serotonin secretion (Fig. 3). Wu *et al.* (34) observed a similar profile in human platelets incubated with SNP and stimulated with thapsigargin and PMA. Aggregation was not observed during the first 3 min after the addition of thapsigargin and PMA. Then, a sharp aggregation was observed. Thapsigargin and PMA promoted substantial cGMP efflux at 3 min after stimulation. Therefore, cGMP escape from platelets seems to be facilitated by the two critical events in the activation of platelet signaling, increase in $[Ca^{2+}]_i$, and activation of protein kinase C (34). The delay observed in the onset of the platelet aggregation and the sharp aggregation could be due to the cGMP efflux; however, whether cGMP efflux exists under our conditions must be determined.

In conclusion, we confirmed previous observations in which the preincubation with SNP inhibited the platelet activation by thrombin. In addition, we have shown that SNP was able to inhibit the thrombin-induced platelet activation without a significant time delay. This inhibition seems to be due to a quick increase in the cGMP levels and supports that the inhibition occurs in the first steps of the activation cascade. In fact, the simultaneous addition of SNP and thrombin

inhibited the phospholipase C activity without affecting the enzymatic properties of thrombin. The results obtained with protein kinase inhibitors suggest that SNP, independent of the protocol used, principally inhibited the platelet activation by phosphorylation-dependent mechanisms (suggesting that SNP seems to be able to induce a very rapid phosphorylation). Also, both PKG and PKA seem to be involved in the inhibition induced by SNP; however, the PKA-dependent mechanisms seem to be more important than the PKG-dependent mechanisms in the inhibition induced by SNP. This hypothesis was supported by the effect of KT-5720 and, principally, by the effect of Rp-8-CPT-cAMPS. In our laboratory, additional studies are under way to determine the specificity of Rp-8-CPT-cAMPS and clarify the biochemical mechanisms of action of nitric oxide donors in human platelets.

Acknowledgments

We thank Drs. I. Zuazua Verde and A. Ibarra Fontan (Cruces Blood Center Hospital, Bilbao, Spain) for the platelet preparations. We also thank the Technical Service of the University of Basque Country for technical assistance.

References

1. Siess, W. Molecular mechanisms of platelet activation. *Physiol. Rev.* **69:** 58–178 (1989).
2. Morgan, R. O., and A. C. Newby. Nitroprusside differentially inhibits ADP-stimulated calcium influx and mobilization in human platelets. *Biochem. J.* **258:**447–454 (1989).
3. Nakashima, S., T. Tohmatsu, H. Hattori, Y. Okano, and Y. Nozawa. Inhibitory action of cyclic GMP on secretion, polyphosphoinositide hydrolysis and calcium mobilization in thrombin-stimulated human platelets. *Biochem. Biophys. Res. Commun.* **135:**1099–1104 (1986).
4. Takai, Y., K. Kaibuchi, T. Matsubara, and Y. Nishizuka. Inhibitory action of guanosine 3′,5′-monophosphate on thrombin-induced phosphatidylinositol turnover and protein phosphorylation in human platelets. *Biochem. Biophys. Res. Commun.* **101:**61–67 (1981).
5. Andersson, T. L. G., and E. Vinge. Interactions between isoprenaline, sodium nitroprusside, and isozyme-selective phosphodiesterase inhibitors on ADP-induced aggregation and cyclic nucleotide levels in human platelets. *J. Cardiovasc. Pharmacol.* **18:**237–242 (1991).
6. Mellion, B. T., L. J. Ignarro, C. B. Myers, E. H. Ohlstein, B. A. Ballot, A. L. Hyman, and P. J. Kadowitz. Inhibition of human platelet aggregation by *S*-nitrosothiols: heme-dependent activation of soluble guanylate cyclase and stimulation of cyclic GMP accumulation. *Mol. Pharmacol.* **23:653–664** (1983).
7. Menshikov, M. Y., K. Ivanova, M. Schaefer, C. Drummer, and R. Gerzer. Influence of the cGMP analog 8-PCPT-cGMP on agonist-induced increases in cytosolic ionized Ca^{2+} and on aggregation of human platelets. *Eur. J. Pharmacol.* **245:**281–284 (1993).
8. Waldmann, R., and U. Walter. Cyclic nucleotides elevating vasodilators inhibit platelet aggregation at an early step of the activation cascade. *Eur. J. Pharmacol.* **159:**317–320 (1989).
9. Brüne, B., and V. Ullrich. Cyclic nucleotides and intracellular-calcium homeostasis in human platelets. *Eur. J. Biochem.* **207:**607–613 (1992).
10. Geiger, J., C. Nolte, E. Butt, S. O. Sage, and U. Walter. Role of cGMP and cGMP-dependent protein kinase in nitrovasodilator inhibition of agonists-evoked calcium elevation in human platelets. *Proc. Natl. Acad. Sci. USA* **89:**1031–1035 (1992).
11. MacIntyre, D. E., M. Bushfield, and A. M. Shaw. Regulation of platelet cytosolic free calcium by cyclic nucleotides and protein kinase C. *FEBS Lett.* **188:**383–388 (1985).
12. Sane, D. C., A. Bielawska, C. S. Greenberg, and Y. A. Hannun. Cyclic GMP analogs inhibit *gamma* thrombin-induced arachidonic acid release in human platelets. *Biochem. Biophys. Res. Commun.* **165:**708–714 (1989).
13. Butt, E., C. Nolte, S. Schulz, J. Beltman, J. A. Beavo, B. Jastorff, and U. Walter. Analysis of the functional role of cGMP-dependent protein kinase in intact human platelets using a specific activator 8-para-chlorophenylthio-cGMP. *Biochem. Pharmacol.* **43:**2591–2600 (1992).
14. Eigenthaler, M., H. Ullrich, J. Geiger, K. Horstrup, P. Hönig-Liedl, D. Wiebecke, and U. Walter. Defective nitrovasodilator-stimulated protein phosphorylation and calcium regulation in cGMP-dependent protein kinase-deficient human platelets of chronic myelocytic leukemia. *J. Biol. Chem.* **268:**13526–13531 (1993).
15. Geiger, J. C. Nolte, and U. Walter. Regulation of calcium mobilization and entry in human platelets by endothelium-derived factors. *Am. J. Physiol.* **267:**C236–C244 (1994).
16. Eigenthaler, M., C. Nolte, M. Halbrügge, and U. Walter. Concentration and regulation of cyclic nucleotides, cyclic-nucleotide-dependent protein kinases and one of their major substrates in human platelets: estimating the rate of cAMP-regulated and cGMP-regulated protein phosphorylation in intact cells. *Eur. J. Biochem.* **205:**471–481 (1992).
17. Halbrügge, M., C. Friedrich, M. Eigenthaler, P. Schanzenbächer, and U. Walter. Stoichiometric and reversible phosphorylation of a 46-kDa protein in human platelets in response to cGMP- and cAMP-elevating vasodilators. *J. Biol. Chem.* **265:**3088–3093 (1990).
18. Waldmann, R., M. Nieberding, and U. Walter. Vasodilator-stimulated protein phosphorylation in platelets is mediated by cAMP- and cGMP-dependent protein kinases. *Eur. J. Biochem.* **167:441–448** (1987).
19. Halbrügge, M., and U. Walter. Purification of a vasodilator-regulated phosphoprotein from human platelets. *Eur. J. Biochem.* **185:**41–50 (1989).
20. Reinhard, M., M. Halbrügge, U. Scheer, C. Wiegand, B. M. Jockusch, and U. Walter. The 46/50 kDa phosphoprotein VASP purified from human platelets is a novel protein associated with actin filaments and focal contacts. *EMBO J.* **11:**2063–2070 (1992).
21. Horstrup, K., B. Jablonka, P. Hönig-Liedl, M. Just, K. Kochsiek, and U. Walter. Phosphorylation of focal adhesion vasodilator-stimulated phosphoprotein at Ser157 in intact human platelets correlates with fibrinogen receptor inhibition. *Eur. J. Biochem.* **225:**21–27 (1994).
22. Maurice, D. H., and R. J. Haslam. Molecular basis of the synergistic inhibition of platelet function by nitrovasodilators and activators of adenylate cyclase: inhibition of cyclic AMP breakdown by cyclic GMP. *Mol. Pharmacol.* **37:**671–681 (1990).
23. Bowen, R., and R. J. Haslam. Effects of nitrovasodilators on platelet cyclic nucleotide levels in rabbit blood: role for cyclic AMP in synergistic inhibition of platelet function by SIN-1 and prostaglandin E_1. *J. Cardiovasc. Pharmacol.* **17:**424–433 (1991).
24. Haslam, R. J., and M. M. L. Davidson. Roles of cyclic AMP and cyclic GMP in the inhibition of platelet function by nitroprusside and phosphodiesterase inhibitors. *Circulation* **66** (Suppl. II):II-291 (1982).
25. Lincoln, T. M., and T. L. Cornwell. Intracellular cyclic GMP receptor proteins. *FASEB J.* **7:**328–338 (1993).
26. Hawkins, D. J., B. O. Meyrick, and J. J. Murray. Activation of guanylate cyclase and inhibition of platelet aggregation by endothelium-derived relaxing factor released from cultured cells. *Biochim. Biophys. Acta* **969:**289–296 (1988).
27. Brüne, B., and V. Ullrich: 12-Hydroperoxyeicosatetranoic acid inhibits main platelet functions by activation of soluble guanylate cyclase. *Mol. Pharmacol.* **39:**671–678 (1991).
28. Azula, F. J., R. Alonso, A. Marino, M. Trueba, and J. M. Macarulla. Ni^{2+} impairs thrombin-induced signal transduction by acting on the agonists and/or receptor in human platelets. *Am. J. Physiol.* **265:**C1681–C1688 (1993).
29. Font, J., F. J. Azula, A. Marino, N. Nieva, M. Trueba, and J. M. Macarulla. Intracellular Ca^{2+} mobilization and not calcium influx promotes phorbol ester-stimulated thromboxane A_2 synthesis in human platelets. *Prostaglandins* **43:**383–395 (1992).
30. Holmsen, H., and C. A. Dangelmaier. Measurement of secretion of serotonin. *Methods Enzymol.* **169** (Part A):205–210 (1989).
31. Cobbold, P. H., and T. J. Rink. Fluorescence and bioluminescence measurement of cytoplasmic free calcium. *Biochem. J.* **248:**313–328 (1987).
32. De Caterina, R., and R. Sicari. Cellular effects of thrombin: pharmacology of the receptor(s) in various cell types and possible development of receptor antagonists. *Pharmacol. Res.* **27:**1–19 (1993).
33. Vu, T.-K. H., D. T. Hung, V. I. Wheaton, and S. R. Coughlin. Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. *Cell* **64:**1057–1068 (1991).
34. Wu, X.-B., B. Brüne, F. V. Appen, and V. Ullrich. Efflux of cyclic GMP from activated human platelets. *Mol. Pharmacol.* **43:**564–568 (1993).
35. Nolte, C., M. Eigenthaler, K. Horstrup, P. Hönig-Liedl, and U. Walter. Synergistic phosphorylation of the focal adhesion-associated vasodilator-stimulated phosphoprotein in intact human platelets in response to cGMP- and cAMP-elevating platelet inhibitors. *Biochem. Pharmacol.* **48:**1569–1575 (1994).
36. Kase, H., K. Iwahashi, S. Nakanishi, Y. Matsuda, K. Yamada, M. Takahashi, C. Murakata, A. Sato, and M. Kaneko. K-252 compounds, novel and potent inhibitors of protein kinase C and cyclic nucleotide-dependent protein kinases. *Biochem. Biophys. Res. Commun.* **142:**436–440 (1987).
37. Butt, E., M. Eigenthaler, and H.-G. Genieser. (Rp)-8-pCPT-cGMPS, a novel cGMP-dependent protein kinase inhibitor. *Eur. J. Pharmacol.* **269:** 265–268 (1994).
38. Dostmann, W. R. G., S. S. Taylor, H.-G. Genieser, B. Jastorff, S. O. Doskeland, and D. Ogreid. Probing of the cyclic nucleotide binding sites of cAMP-dependent protein kinases I and II with analogs of adenosine 3′,5′-cyclic phosphorothioates. *J. Biol. Chem.* **265:**10484–10491 (1990).
39. Sandberg, M., E. Butt, C. Nolte, L. Fischer, M. Halbrügge, J. Beltman, T. Jahnsen, H.-G. Genieser, B. Jastorff, and U. Walter. Characterization of Sp-5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole-3′,5′-monophosphorothioate (Sp-5,6-DCl-cBiMPS) as a potent and specific activator of cyclic-AMP-dependent protein kinase in cell extracts and intact cells. *Biochem. J.* **279:**521–527 (1991).
40. Murray, K. J., R. J. Eden, J. S. Dolan, D. C. Grimsditch, C. A. Stutchbury, B. Potel, A. Knowles, A. Worby, J. A. Lynham, and W. J. Coates. The

effects of SK&F 95654, a novel phosphodiesterase inhibitor, on cardiovascular, respiratory and platelet function. *Br. J. Pharmacol.* **107**:463–470 (1992).
41. Seiler, S., E. Gillespie, A. J. Arnold, C. L. Brassard, N. A. Meanwell, and J. S. Fleming. Imidazoquinoline derivates: potent inhibitors of platelet cyclic AMP phosphodiesterase which elevate cyclic AMP levels and activate protein kinase in platelets. *Thromb. Res.* **62**:31–42 (1991).
42. Wahler, G. M., and S. J. Dollinger. Nitric oxide donor SIN-1 inhibits mammalian cardiac calcium current through cGMP-dependent protein kinase. *Am. J. Physiol.* **268**:C45–C54 (1995).
43. Murthy, K. S., and G. M. Makhlouf. Interaction of cA-kinase and cG-kinase in mediating relaxation of dispersed smooth muscle cells. *Am. J. Physiol.* **268**:C171–C180 (1995).
44. Wyatt, T. A., T. M. Lincoln, and K. B. Pryzwansky. Vimentin is transiently co-localized with and phosphorylated by cyclic GMP-dependent protein kinase in formyl-peptide stimulated neutrophils. *J. Biol. Chem.* **266**: 21274–21280 (1991).
45. Wyatt, T. A., K. B. Pryzwansky, and T. M. Lincoln. KT-5823 activates human neutrophils and fails to inhibit cGMP-dependent protein kinase phosphorylation of vimentin. *Res. Commun. Chem. Pathol. Pharmacol.* **74**:3–14 (1991).

Send reprint requests to: Dr. A. Marino, Departamento de Bioquímica y Biología Molecular, Facultad de Ciencias, Universidad del País Vasco/ Euskal Herriko Universitatea, Apdo. 644, 48080 Bilbao, Spain. E-mail: gbpmasaa@lg.ehu.es