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Prostacyclin and Sodium Nitroprusside Inhibit the Activity of the Platelet Inositol 1,4,5-Trisphosphate Receptor and Promote Its Phosphorylation*

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Prostaglandin I_2 (PGI_2) and sodium nitroprusside (SNP) induce a rapid decay of the thrombin-promoted increase of $[Ca^{2+}]_i$ in aspirin-treated platelets incubated in the absence of external Ca^{2+}. The mechanism of their effect was studied with a new method which utilizes ionomycin to increase $[Ca^{2+}]_i$, followed by bovine serum albumin (BSA) to remove the Ca^{2+} ionophore. The rapid decay of $[Ca^{2+}]_i$ after BSA is mostly due to the reuptake into the stores, since it is strongly inhibited by the endomembrane Ca^{2+}-ATPase inhibitor thapsigargin. PGI_2 and SNP are without effect on the BSA-promoted decay both with and without thapsigargin, showing that they do not affect the activity of the Ca^{2+}-ATPases. The fast decay of $[Ca^{2+}]_i$ after BSA is decreased by thrombin which produces the Ca^{2+} releaser inositol 1,4,5-trisphosphate ($InsP_3$), thus counteracting the activity of the endomembrane Ca^{2+} pump. When added after thrombin, PGI_2 and SNP accelerate the BSA-activated decay of $[Ca^{2+}]_i$. However, under the same conditions, they do not decrease the concentration of $InsP_3$. In saponin-permeabilized platelets, cAMP and cGMP counteract the Ca^{2+} release induced by exogenous $InsP_3$. Their inhibitory effect disappears at high $InsP_3$ concentrations. This demonstrates that PGI_2 and SNP potentiate Ca^{2+} reuptake by inhibiting the $InsP_3$ receptor. Two bands of approximately 260 kDa are recognized by a monoclonal antibody recognizing the C-terminal region of the $InsP_3$ receptor. Both are phosphorylated rapidly, the heavier more intensely, in the presence of PGI_2 and SNP. The phosphorylation of the $InsP_3$ receptor is fast enough to be compatible with its involvement in the inhibition of the receptor by cyclic nucleotides.

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 and PKC[1] resulting in platelet adhesion, aggregation, and degranulation (1, 2).

Most platelet agonists activate phospholipase C and elevate $[Ca^{2+}]_i$ by an $InsP_3$-dependent release of Ca^{2+} from the intracellular stores, as well as stimulation of the entry of extracellular Ca^{2+} (2–5). The agonist-releasable stores appear to be of two different types, distinguishable by the sensitivity of their Ca^{2+}-ATPases to low thapsigargin (Tg) concentrations or to 2,5-di-*tert*-butylhydroquinone (or high Tg concentrations), respectively (6–8).

The modes of Ca^{2+} entry are controversial. It is generally agreed that a substantial influx is activated by a signal generated by the depletion of the intracellular stores (capacitative Ca^{2+} influx) (9) also in the absence of agonist, *e.g.* by treatment with the endomembrane Ca^{2+}-ATPase inhibitor Tg (10, 11); such a signal could be a small molecule released together with the Ca^{2+} ions from the stores (12, 13), but also other possibilities cannot be excluded (14–16). Receptor-mediated influx systems are also operative. ADP induces a very fast Ca^{2+} entry which precedes the release from the intracellular stores and the subsequent second phase of Ca^{2+} entry (4). We recently found that the occupancy of the thrombin receptor activates a store-independent Ca^{2+} influx (17).

Ca^{2+} efflux from platelets is operated by a Ca^{2+}-ATPase, whose activity is potentiated by the activation of PKC (18, 19) as well as by the depletion of the stores (19).

The cyclic nucleotides cAMP and cGMP exert multiple inhibitory actions on platelet activation. cAMP was shown to decrease the binding of thrombin to its receptor on human platelets (20). In the presence of cAMP, the activation of phospholipase C (and hence the production of $InsP_3$ and diacylglycerol) by the agonist is depressed, leading to the inhibition of the increment of $[Ca^{2+}]_i$ and of PKC-dependent phosphorylations (21). The action of cAMP is also on events distal to the activation of phospholipase C since platelet aggregation and secretion induced by the Ca^{2+} ionophore ionomycin or by phorbol esters are also inhibited by the cyclic nucleotide (22–24). cAMP also increases the incorporation of diacylglycerol into phosphatidylinositol (25).

Similar inhibitory actions were reported for cGMP, which interferes with the agonist-induced activation of phospholipase C (26–30) and also affects events distal to the increase of $[Ca^{2+}]_i$ (31, 32). Furthermore, cGMP potentiates the action of cAMP elevating agents, by inhibiting the cAMP phosphodiesterase (33).

The cyclic nucleotides also interfere with the Ca^{2+} influx. We recently reported that cAMP and cGMP inhibit the thrombin-activated Ca^{2+} influx in platelets, without interfering with the Ca^{2+} influx secondary to the depletion of the intracellular

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[1] The abbreviation used are: PKC, protein kinase C; PKA, cAMP-dependent protein kinase; PKG, cGMP-dependent protein kinase; BSA, bovine serum albumin; $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; $InsP_3$, inositol 1,4,5-trisphosphate; PGI_2, prostaglandin I_2 or prostacyclin; SNP, sodium nitroprusside; Tg, thapsigargin.

stores (17). An inhibition by cAMP and cGMP has been reported recently on Ca^{2+} influx activated by thromboxane (34). On the contrary, the ADP-activated Ca^{2+} influx has been reported to be insensitive to cAMP (3).

The action of cAMP and cGMP on Ca^{2+} movements to and from the intracellular deposits is controversial and has received considerable attention. The activity of PKA was reported to be facilitatory, or even necessary, for Ca^{2+} release by $InsP_3$ from isolated platelet membrane vesicles (35). This conclusion was refuted (36). The catalytic subunit of PKA was reported to stimulate Ca^{2+} uptake by platelet membrane vesicles (35, 37, 38). This effect was correlated with the phosphorylation of a protein tentatively identified with phospholamban, the known promoter of Ca^{2+} transport in cardiac muscle membranes, but that identity was disputed (39). On the other hand, dibutyryl cAMP, and various prostaglandins and forskolin, are known to stimulate adenylate cyclase and reverse the Ca^{2+} mobilization produced by platelet agonists (40–42). Furthermore, the addition of cAMP to saponin-permeabilized platelets was reported to decrease the amount of Ca^{2+} released by the $InsP_3$-sensitive stores (43). Similar results were obtained with the catalytic subunit of PKA (4).

Finally, it was also proposed that cAMP and cGMP potentiate the action of the plasma membrane Ca^{2+}-ATPase (45).

The present study was performed to settle some controversy concerning the action of cAMP and cGMP on Ca^{2+} movements between cytosol and the stores. It is shown that both cyclic nucleotides are without effect on the Ca^{2+} pumps (including the plasma membrane Ca^{2+} pump) and that their action is to prevent the Ca^{2+}-releasing activity of $InsP_3$. Both cAMP and cGMP promote the phosphorylation of the $InsP_3$ receptor.

EXPERIMENTAL PROCEDURES

Materials—Thrombin, sodium nitroprusside, apyrase, hirudin, ionomycin, thapsigargin, staurosporine, cAMP, dibutyryl-cAMP, and cAMP-dependent protein kinase (PKA) (P 5511) were purchased from Sigma, GF 109 203X (2-[1-(3-dimethylaminopropyl)indol-3-yl]-3-(indol-3-yl)-maleimide) was from Boehringer Mannheim, 8-Br-cGMP, Fluo 3, and Indo 1/AM from Calbiochem, prostacyclin (PGI_2) from Cascade Biochem Ltd., United Kingdom. The anti-$InsP_3$ receptor (mouse monoclonal) antibody, prepared as in Ref. 46 and recognizing its C terminus cytoplasmic domain, was from Calbiochem (Catalogue No. 407140), the biotinylated anti-mouse antibody was from Dakopatts A/S, Denmark. The streptavidin-alkaline phosphatase complex and the D-*myo*-[^{3}H]Inositol 1,4,5-trisphosphate ($InsP_3$) assay system (the $InsP_3$ radioimmunoassay kit TRK 1000) were from Amersham Int. The chemiluminescent reagent was the chemiluminescent protein detection system Immun-Lite II (anti-mouse Catalogue No. 160 6478) from Bio-Rad. All other reagents were of analytical grade.

Platelet Preparation—Platelet-rich plasma and washed platelets were prepared and treated with aspirin as previously reported (22) from fresh blood drawn from healthy volunteers and mixed with acid citrate-dextrose anticoagulant supplemented with hirudin (50 milliunits/ml) apyrase (80 milliunits/ml) and PGI_2 (0.2 μg/ml).

Determination of Cytosolic Free Ca^{2+} Concentration—The intracellular Ca^{2+} concentration was determined with Indo 1/AM essentially according to Pollock and Rink (47). The Ca^{2+} fluorescent probe (2 μM) was added to aspirin-treated platelet-rich plasma, and loading was performed for 30 min at 37 °C. After centrifugation, the pellet was resuspended in Tyrode's buffer (145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 20 mM Na-Hepes, 10 mM glucose, pH 7.4) in the presence of 0.1 mM sulfinpyrazone (48), 40 milliunits/ml apyrase, and 5 milliunits/ml hirudin. The platelets were used at the concentration of 1×10^8 cells/ml. All experiments were performed in the presence 0.5 mM EGTA. Unless otherwise stated, fluorescence was measured at 37 °C in a thermostated, magnetically stirred cuvette, in a Shimadzu RL-5000 spectrofluorimeter with excitation and emission wavelengths set at 340 nm and 400 nm.

Determination of $InsP_3$—The amount of $InsP_3$ was determined on 0.5-ml aliquots of platelet suspensions (5–6 $\times$ 10^8 cells/ml) precipitated with 5% (v:v) ice-cold perchloric acid. After 20 min in ice, the deproteinized samples were centrifuged at 8000 $\times$ *g* for 5 min and neutralized with $KHCO_3$. The samples were used for the $InsP_3$ determination with the Amersham radioimmunoassay kit and following its specifications.

Platelet Permeabilization and Ca^{2+} Release by Exogenous $InsP_3$—For the detection of Ca^{2+} release induced by exogenous $InsP_3$, platelets were suspended in Tyrode's buffer (not supplemented with hirudin and apyrase) at 2×10^9/ml. Immediately before use, they were diluted five times with a KCl buffer (100 mM KCl, 100 mM sucrose, 1.4 mM $MgCl_2$, 20 mM K-Hepes, pH 7.4) and supplemented with (final concentrations): NaN_3 (1 mM), oligomycin (0.6 μg/ml), phosphocreatine (7.5 mM), creatine kinase (30 μg/ml), ATP (1 mM), KH_2PO_4 (1 mM), and Fluo 3 (4 μM). Free Ca^{2+} was decreased to approximately 300 nM with EGTA (based on a K_d for Ca^{2+} of Fluo 3 of 316 nM). Permeabilization was induced with saponin (20–30 μg/ml, the correct concentration had to be adjusted with each preparation). After uptake, Ca^{2+} was released by pulses of $InsP_3$ as described in the figures. Calibration were performed by back-titration of the Ca^{2+} traces with pulses of 2 μM EGTA. EDTA was removed from the PKA preparation by an ultrafiltration procedure (Minicent 10 Bio-Rad, 10 Kilodalton cutoff, filters). Fluorescence was measured at excitation and emission wavelengths set at 488 and 526 nm.

Immunodetection of the $InsP_3$ Receptor—Incubation of the platelet suspension (4×10^8 cells/ml) was terminated by the addition of 1/5 volume of modified Laemmli's buffer (1.46 M Tris, 11.5% (w/v) SDS, 7.7% mercaptoethanol, 23% glycerol, and 30 mM dithiothreitol) at pH 6.8 followed by overnight room temperature denaturation. One-dimensional SDS electrophoresis was performed on two-step (5 and 11%) polyacrylamide minigels. The gels were colored with Coomassie Blue for the identification of the protein pattern or subjected to electrophoretic transfer (4 h at 450 mA) onto nitrocellulose membranes (Hoefer Scientific Instruments TM-NC4-roll) for subsequent probing with an anti-$InsP_3$ receptor (mouse monoclonal) antibody. The blots were preincubated for 30 min with 5% (w/v) BSA in 0.1 M Tris, 0.15 M NaCl saline buffer, pH 7.5, containing 0.1% Tween (TBST buffer) to block residual protein binding sites. Immunodetection of $InsP_3$ receptor was achieved by using the monoclonal anti-$InsP_3$ receptor antibody for 1 h at 37 °C diluted 1:100 in TBST buffer. The primary antibody was then removed, and the blots washed three times in TBST containing 1% BSA. To detect the primary antibody, the blots were incubated with biotinylated anti-mouse antibody (diluted 1:3000) for 30 min, washed 3 times in TBST, and then incubated with streptavidin-alkaline phosphatase complex (1:3000), washed again 3 times in TBST, and then exposed to the Bio-Rad chemiluminescent reagents for 30 min (49). Blots were then revealed by exposition for a time ranging from 3 to 10 min to photographic film.

$^{32}P_i$ Loading and Analysis of Protein Phosphorylation—$^{32}P_i$ loading was performed by incubating platelets at a concentration of 2×10^9 cells with 300 μCi/ml $^{32}P_i$ for 60 min at room temperature. After diluting 10 times with Tyrode's buffer containing 0.2 μg/ml PGI_2 and 3 mg/ml BSA, platelets were centrifuged, and the pellet was resuspended in Tyrode's buffer for the subsequent stimulation at a concentration of 4×10^8 cells/ml. Platelets were incubated as specified in the legends to Figs. 7 and 8. The incubations were terminated with the Laemmli buffer, and electrophoresis was performed as described above. The gels were colored and counted for at least a 10-h exposition to the β emission sensitive scanner-Instant Imager (Packard Instruments). Occasionally, counting was performed on the transblots. Analysis of the p260 and p47 phosphorylation for data reported in Fig. 8 was performed on a single band integration of the counts/min patterns obtained by the Instant Imager.

RESULTS

PGI_2 and SNP Potentiate Ca^{2+} Reuptake into the Stores—As described by us (17, 19) and others (50, 51), the addition of the agonist thrombin to aspirin-treated platelets resuspended in an EGTA-supplemented medium elicits a large increase of $[Ca^{2+}]_i$ followed by a relatively rapid decrease close to the baseline, which is completed in 3–5 min. The decrease of $[Ca^{2+}]_i$ is attributable to a relatively large extent to a net reuptake into the intracellular stores, since the subsequent addition of the endomembrane Ca^{2+}-ATPase inhibitor Tg, together with a small amount of ionomycin to facilitate the release from the stores (17), releases into the cytosol a significant proportion of the disappeared Ca^{2+} (Fig. 1). The decrease of $[Ca^{2+}]_i$ is strongly stimulated if PGI_2 or SNP (which generate cAMP and cGMP, respectively) are added soon after the peak of the thrombin-induced increase of $[Ca^{2+}]_i$ (Fig. 1) (see also Refs. 17 and 51). In the latter situation, the increase of $[Ca^{2+}]_i$ upon

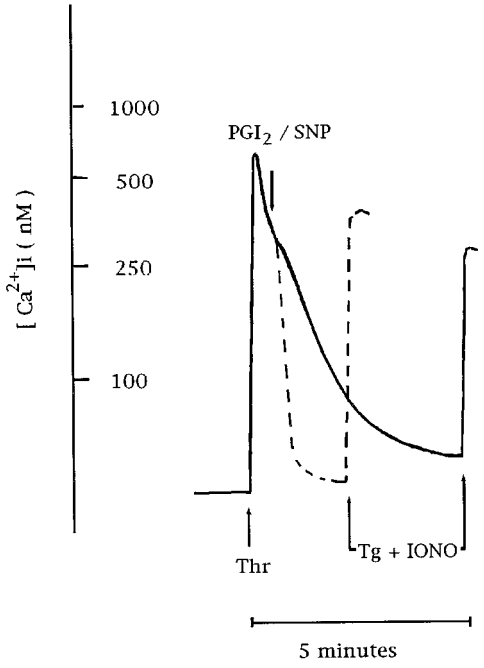

FIG. 1. **PGI_2 and SNP stimulate the decay of $[Ca^{2+}]_i$ elevated by thrombin.** Indicated are the additions of thrombin (*Thr*, 0.25 unit/ml), thapsigargin (*Tg*, 200 nM), and ionomycin (*IONO*, 50 nM). The *dotted line* indicates the progress of the Ca^{2+} trace upon addition of PGI_2 (0.6 μg/ml) or SNP (60 μM). The traces are representative of duplicate experiments with at least 5 different preparations with similar results.

addition of Tg *plus* ionomycin (a measure of the store-associated Ca^{2+}) is significantly higher than in the control. Similar results are obtained also with the membrane-permeable dibutyryl-cAMP and 8-Br-cGMP, although they stimulate the decrease of $[Ca^{2+}]_i$ somewhat less powerfully than PGI_2 and SNP (results not reported). These experiments show that increasing cAMP or cGMP potentiates Ca^{2+} reuptake into the stores, apparently without major effects on Ca^{2+} efflux from platelets. The pattern in Fig. 1 is unchanged by the specific PKC inhibitor GF 109 203X (3 μM) which decreases only somewhat the decay rate of $[Ca^{2+}]_i$ after thrombin; the stimulated decay by PGI_2 and SNP are on the contrary totally abolished by staurosporine (1–2 μM) which also inhibits PKA and PKG (results not reported).

PGI_2 and SNP Are without Effect on the Activity of Platelet Ca^{2+} Pumps—The potentiation of Ca^{2+} reuptake into the stores promoted by the cyclic nucleotides could depend in principle on the increased activity of the store- and plasma membrane-associated Ca^{2+}-ATPases or on the inhibition of Ca^{2+} release from the deposits. In order to distinguish between these possibilities, we introduced a new method which utilizes the Ca^{2+} ionophore ionomycin to mobilize Ca^{2+} without the intervention of agonists or inhibitors, followed by BSA to remove the ionophore. As described in Fig. 2, the addition of ionomycin to platelets suspended in EGTA-supplemented media induces a large increase of $[Ca^{2+}]_i$, released from the agonist-sensitive stores as well as from the secretory granules (19). At the ionomycin concentration used (400 nM), the agonist-sensitive deposits are largely depleted (as shown by the small increase of $[Ca^{2+}]_i$ upon addition of Tg at the top of the ionomycin-induced deflection, Fig. 2*B*). The subsequent addition of BSA, which binds the ionophore, allows the study of the decay of $[Ca^{2+}]_i$, operated by the Ca^{2+} pumps, in the absence of perturbing agents. As shown in Fig. 2*A*, the decay of $[Ca^{2+}]_i$ is very rapid upon addition of BSA (note that this experiment was performed at 20 °C to decrease the activity of the pumps). Two processes contribute to the depletion of $[Ca^{2+}]_i$, namely pumping into the stores and pumping into the extracellular space. The use of Tg to inhibit the endomembrane Ca^{2+}-ATPases allows quantitation of the contribution of each system to the overall decay rate. The decay of $[Ca^{2+}]_i$ is strongly depressed by the inclusion of Tg prior to BSA (Fig. 2, compare *B* and *A*) showing that pumping into the stores prevails over pumping out of the platelets.

The addition of PGI_2 or SNP prior to ionomycin has no effect on the ionophore-induced increase of $[Ca^{2+}]_i$ and importantly also has no effect on the rate of decrease of $[Ca^{2+}]_i$ upon addition of BSA, both in the absence (Fig. 2*A*) and presence (Fig. 2*B*) of Tg. Accordingly, the refilling of the stores after BSA (in the experiment without Tg, Fig. 2*A*), as measured by the increase of $[Ca^{2+}]_i$ following the addition of Tg, is unmodified by PGI_2 and SNP. This is more clearly shown (with an expanded time scale) in Fig. 3, where, after ionomycin, BSA was supplemented both at submaximal and at maximal concentrations. At the lower BSA concentrations, some ionomycin is left unbound and activates variable degrees of Ca^{2+} cycling. In these conditions, the combined action of the Ca^{2+} pumps and the ionomycin-induced leaks leads to the establishment of intermediate levels of Ca^{2+} accumulation in the stores and to intermediate $[Ca^{2+}]_i$. The rate of decay of $[Ca^{2+}]_i$ decreases and the steady state $[Ca^{2+}]_i$ increases with decreasing BSA. In no case does the inclusion of PGI_2 or SNP modify the decay rate or the final steady state. A stimulation of the pump would have resulted in an increased decay rate of $[Ca^{2+}]_i$ and a decreased final steady state. These experiments show that the cyclic nucleotides do not interfere with the operation of the two endomembrane Ca^{2+}-ATPases (6–8) nor of the plasma membrane Ca^{2+} pump.

PGI_2 and SNP Inhibit the $InsP_3$-activated Ca^{2+} Efflux from the Deposits—The decay rate of $[Ca^{2+}]_i$ upon removal of ionomycin with BSA, which is unaffected by increasing platelet cAMP and cGMP, is on the contrary strongly decreased if thrombin is supplemented together with BSA (Fig. 2*C*). This may be expected since the production of $InsP_3$ induced by thrombin activates a $InsP_3$-dependent (and BSA-insensitive) Ca^{2+} efflux from the deposits, which counteracts the action of the endomembrane Ca^{2+} pumps, thus decreasing the overall decay rate of $[Ca^{2+}]_i$. Accordingly, the amount of Ca^{2+} reaccumulated in the stores (shown by the increase of $[Ca^{2+}]_i$ upon addition of Tg) is significantly less in the presence of thrombin (Fig. 2, compare *C* and *A*). Adding PGI_2 or SNP along the $[Ca^{2+}]_i$ decay curve after BSA *plus* thrombin induces a strong acceleration of the rate of disappearance of $[Ca^{2+}]_i$, paralleled by a clearcut increase of the Tg-releaseable Ca^{2+}. This experiment shows that PGI_2 and SNP oppose the Ca^{2+} efflux from the deposits induced by the thrombin-produced $InsP_3$.

Since the cyclic nucleotides appear not to modify the activity of the Ca^{2+} pumps, but rather to oppose the effect of $InsP_3$, we measured the thrombin-induced production of $InsP_3$ and the effect of supplementing PGI_2 or SNP, after thrombin, on the platelet $InsP_3$ content. As shown in Fig. 4, $InsP_3$ increases sharply soon after the addition of thrombin, to progressively decrease to a lower steady state value. The addition of PGI_2 or SNP 10 s after thrombin, at a time when $InsP_3$ has reached its maximum, is without effect on the $InsP_3$ levels which remain undistinguishable from those observed with thrombin alone. This finding shows that the cAMP and cGMP potentiation of Ca^{2+} uptake into the stores (Fig. 1) is mediated by a decrease of Ca^{2+} efflux rather than by an increase in influx (Fig. 2), and this takes place with no variation of $InsP_3$ concentration.

To monitor directly the effect of cAMP and cGMP on the Ca^{2+} release promoted by exogenous $InsP_3$, we also performed experiments in saponin-permeabilized platelets. The amount of

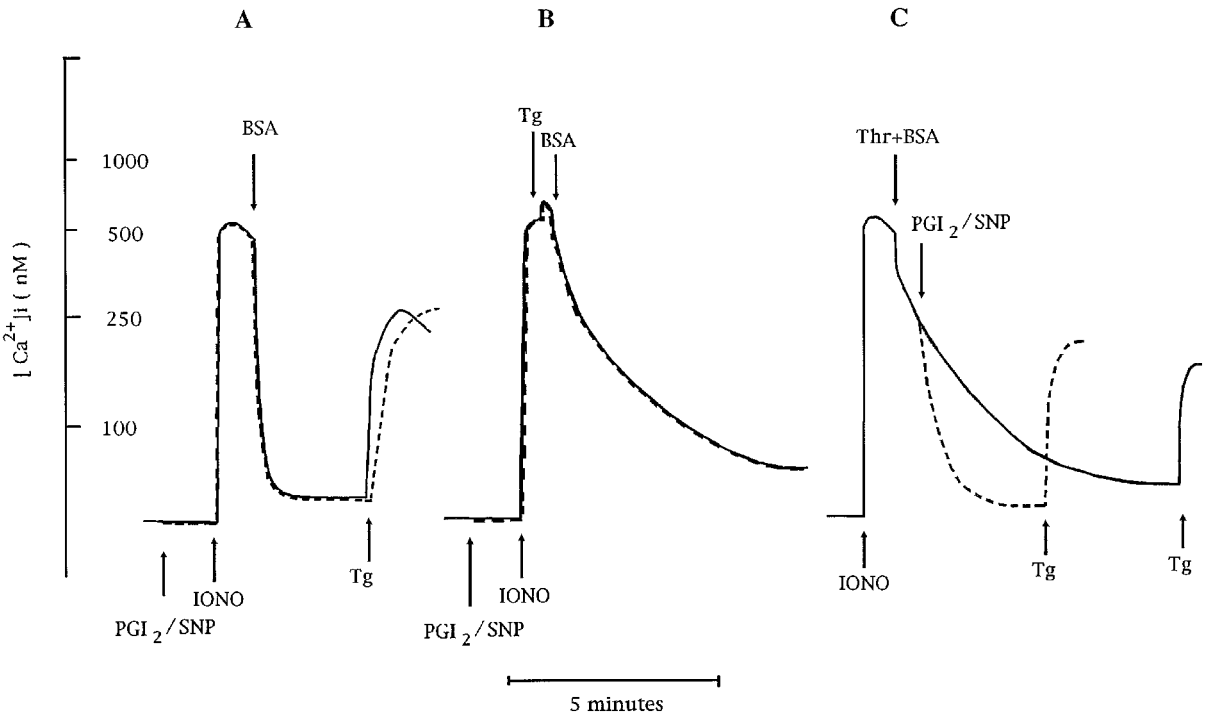

FIG. 2. **PGI_2 and SNP have no effect on the endoplasmic reticulum and plasma membrane Ca^{2+}-ATPases.** They inhibit the thrombin-induced increase of Ca^{2+} efflux from the stores. Ca^{2+} discharge from the intracellular stores was induced with ionomycin (*IONO*, 400 nM). The latter was then removed with BSA (*BSA*, 2 mg/ml). The traces following the addition of PGI_2 or SNP are indicated by the *dotted lines*. The temperature was 20 °C. The traces are representative of duplicate experiments with at least 5 different preparations with similar results.

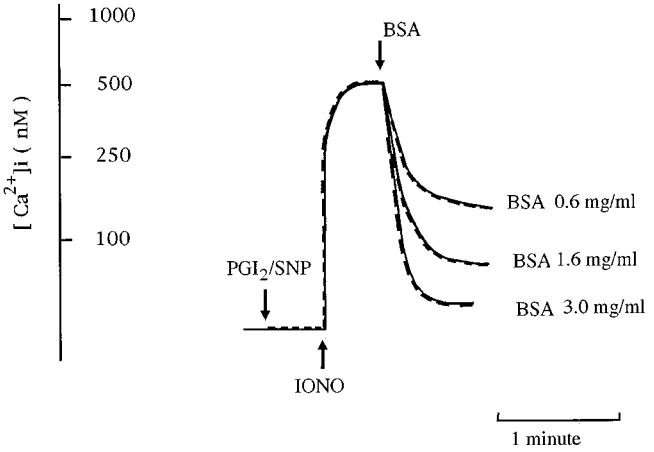

FIG. 3. **PGI_2 and SNP do not affect the rate of Ca^{2+} uptake by endoplasmic reticulum Ca^{2+}-ATPase.** Ca^{2+} discharge from the intracellular stores was induced with ionomycin (*IONO*, 500 nM). The latter was then partially or totally removed with different concentrations of BSA leading to variable degrees of Ca^{2+} cycling. The traces with PGI_2 or SNP are indicated by the *dotted lines*. The temperature was 20 °C.

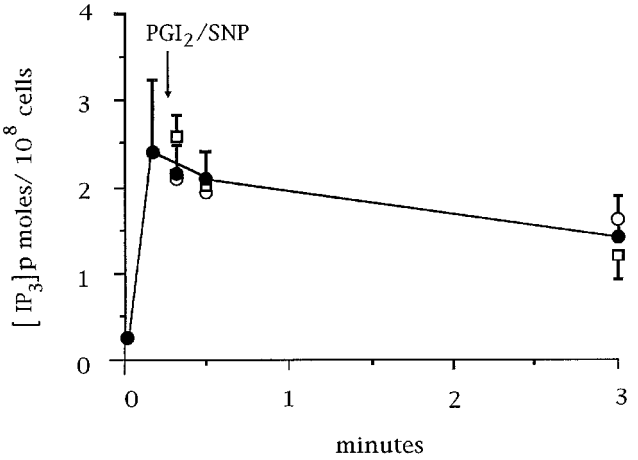

FIG. 4. **Platelet $InsP_3$ elevated by thrombin is not affected by the subsequent addition of PGI_2 or SNP.** Thrombin (●) was added at zero time followed at 10 s, where indicated, by PGI_2 (○) or SNP (□). Data are in triplicate from 2 different preparations.

saponin required for the permeabilization was variable and had to be adjusted in each platelet preparation. A large Ca^{2+} uptake into the endoplasmic reticulum is promoted by ATP upon permeabilization. Once the loading of the stores is completed, Ca^{2+} is released stepwise by graded pulses of $InsP_3$. The Ca^{2+} released by low concentrations of $InsP_3$ is strongly decreased by cAMP. The inhibition is frequently slightly potentiated further by the inclusion of PKA (the effect, however, was not appreciated in all preparations). This may be expected since saponin permeabilizes the plasma membrane to large molecules, thus allowing the loss into the medium of many proteins and cofactors. The effect of cAMP disappears at high $InsP_3$ concentrations. A typical experiment is reported in Fig. 5, and the cumulative results from several different platelet preparations are reported in Fig. 6 (the points are taken from experiments performed in the presence and absence of exogenous PKA). Similar experiments with cGMP gave essentially superimposable results.

PGI_2 and SNP Induce a Rapid Phosphorylation of the $InsP_3$ Receptor—The observation that increasing cAMP or cGMP counteracts the Ca^{2+}-releasing action of $InsP_3$ is consistent with the idea that PGI_2 and SNP modify the $InsP_3$ receptor in such a way that it becomes less sensitive to the action of $InsP_3$. Therefore, we attempted to separate and identify the $InsP_3$ receptor protein and to search for its cAMP- and cGMP-dependent phosphorylation. As shown in Fig. 7*A*, a double band of approximately 260 kDa is recognized by a monoclonal antibody to the $InsP_3$ receptor in transblots from SDS-polyacrylamide

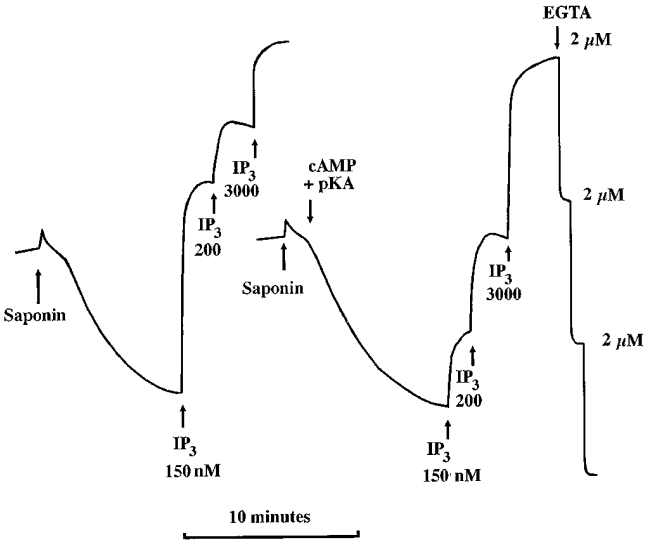

FIG. 5. **Fluo 3 measurement of the $InsP_3$-induced Ca^{2+} release from permeabilized platelets: inhibition by cAMP.** Platelets were treated as described under "Experimental Procedures." When present, cAMP was 20 μM, and PKA 100 units/ml.

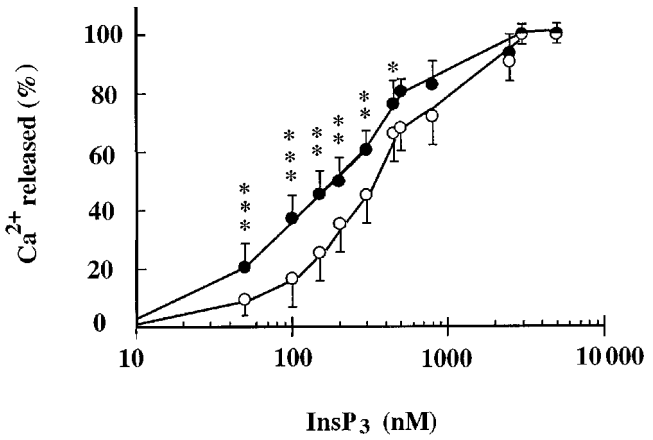

FIG. 6. **cAMP inhibition of the $InsP_3$-induced release of Ca^{2+} from permeabilized platelets is inhibited by cAMP.** The points were collected from experiments as in Fig. 7 from at least 13 different platelet preparations. Each point represents the mean value expressed as percent of total Ca^{2+} released ± S. D. (*bars*) of at least 9 different determinations: *, **, and *** indicate that cAMP and control are significantly different with a $p < 0.05$, < 0.01, < 0.005, respectively, calculated by the Student's *t* test. The points with cAMP are taken from experiments conducted in the presence as well as in the absence of added PKA, since the two conditions were not statistically significantly different overall. ●, control; ○, cAMP.

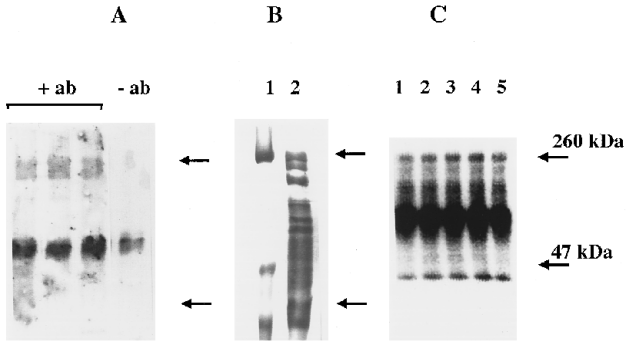

FIG. 7. **Western blot identification of the $InsP_3$ receptor (*A*) and its phosphorylation by PGI_2 (*C*).** *A*, recognition of the $InsP_3$ receptor with an anti-$InsP_3$ monoclonal antibody. *B*, Coomassie blue pattern of platelet proteins (*lane 2*); the monomeric form of hemocyanine from *Octopus vulgaris* (250 kDa) was used as high molecular mass standard protein in *lane 1*. *C*, autoradiographic pattern; platelets were incubated with 0.25 unit/ml thrombin for 10 s (*lane 1*), followed by 0.7 μg/ml PGI_2 for 10 s (*lane 2*), 30 s (*lane 3*), 60 s (*lane 4*), 300 s (*lane 5*). GF 109 203X (3 μM) was added 3 min before thrombin. The reported pattern is typical of at least 5 different experiments obtained from different platelet preparations.

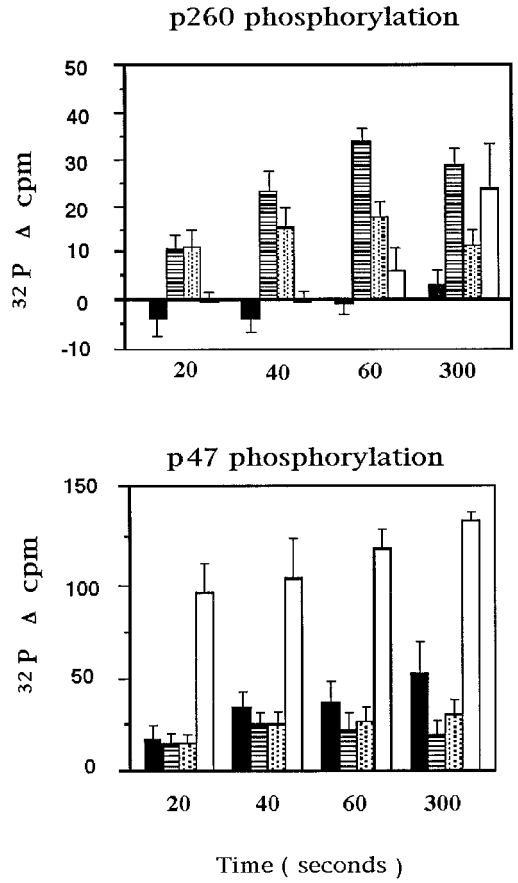

FIG. 8. **Time course of the 260-kDa ($InsP_3$) and of the 47-kDa protein phosphorylation by PGI_2, SNP, and thrombin.** ■, thrombin (0.25 unit/ml) added at zero time. GF 109 203X (3 μM) was added 3 min before thrombin. ▤, thrombin added at zero time followed 10 s later by PGI_2. GF 109 203X was added 3 min before thrombin. , thrombin added at zero time followed 10 s later by SNP. GF 109 203X was added 3 min before thrombin. □, thrombin added at zero time. The data are collected from at least 5 different experiments similar to those reported in Fig. 4*B*. The baseline counts/min prior to the addition of thrombin were subtracted from all the points.

gel electrophoresis separations of human platelets. Two bands of protein phosphorylation are rapidly induced by PGI_2 (or SNP) at the same levels (the higher molecular mass band is always more evident). This is shown in Fig. 7*C*, which reports the time course of the PGI_2-induced phosphorylation of the $InsP_3$ receptor in conditions identical with Fig. 1 (GF 109 203X was also included to prevent thrombin from slowly phosphorylating the receptor). The progress of the receptor phosphorylation (higher molecular mass band) induced by PGI_2, SNP, or thrombin (in the presence and absence of GF 109 203X) is presented in Fig. 8, which also reports for comparison the phosphorylation of the typical 47-kDa PKC substrate pleckstrin. The experiments presented in Fig. 8 show that the increment of the $InsP_3$ receptor phosphorylation by PGI_2 and SNP is rapid enough to be compatible with its involvement in the mechanism of the acceleration of Ca^{2+} reuptake into the stores depicted in Fig. 1. Noticeably, the PKC-dependent phosphorylation of the $InsP_3$ receptor induced by thrombin, and sensitive to GF 109 203X, is much slower than that promoted by PGI_2 and SNP.

DISCUSSION

The experiments reported in this study were performed in order to determine the relative potency and the regulation by the cyclic nucleotides cAMP and cGMP of the systems involved in the decay of platelet $[Ca^{2+}]_i$. The effect of the cyclic nucleotides is interesting since increasing their concentration after an agonist-dependent Ca^{2+} release from the stores potently stimulates the disappearance of $[Ca^{2+}]_i$. The experiments were performed in Ca^{2+}-free media to avoid interferences from Ca^{2+} influx.

In order to avoid unwanted interferences by agonists and inhibitors on Ca^{2+} movements, we introduced a new method which utilizes ionomycin to deplete the deposits and increase $[Ca^{2+}]_i$, followed by BSA to terminate the action of the Ca^{2+} ionophore. Using this technique, we reached the following conclusions.

1. Upon addition of BSA, the decrease of $[Ca^{2+}]_i$ previously elevated by ionomycin is very rapid, such that also at 20 °C it is completed in less than 20 s.

2. The activities of the endomembrane and plasma membrane Ca^{2+}-ATPases responsible for the decay of $[Ca^{2+}]_i$ can be readily discriminated by including Tg that specifically inhibits the endomembrane Ca^{2+}-ATPases. These experiments show that pumping into the deposits prevails strongly over pumping across the plasma membrane.

3. Including PGI_2 or SNP is without visible effect on the BSA-induced decay of $[Ca^{2+}]_i$ both in the absence and presence of Tg. This shows that cAMP and cGMP do not interfere with the activity of either the endomembrane or the plasma membrane Ca^{2+}-ATPases.

4. In the absence of Tg, the BSA-activated decay of $[Ca^{2+}]_i$ is strongly decreased if thrombin is supplemented together with BSA. This shows that the thrombin-activated production of $InsP_3$, by inducing a (BSA-insensitive) Ca^{2+} efflux from the stores, counteracts the action of the endomembrane Ca^{2+} pumps, thus decreasing the $[Ca^{2+}]_i$ decrease rate. Adding PGI_2 or SNP along the $[Ca^{2+}]_i$ decay trace (slowed down by thrombin) promptly accelerates the decay of $[Ca^{2+}]_i$. However, PGI_2 or SNP do not promote the decay of $InsP_3$ elevated by thrombin.

It is concluded that the cyclic nucleotides do not modulate the Ca^{2+} pumps and do not promote the disappearance of $InsP_3$; rather, they prevent $InsP_3$ from releasing Ca^{2+} from the stores. Such an action of the cyclic nucleotides is unaffected by the specific PKC inhibitor GF 109 203X, but it is prevented by staurosporine, which inhibits both PKA and PKG.

The inhibition by the cyclic nucleotides of the Ca^{2+}-releasing action of $InsP_3$ is observable directly in saponin-permeabilized platelets. The effect of cAMP and cGMP are best evident at low $InsP_3$ concentrations and disappear progressively with increasing $InsP_3$. These results are similar to those reported for cAMP in cerebellum-derived microsomes (52).

The inhibition by the cyclic nucleotides is accompanied by the phosphorylation, operated by both PGI_2 and SNP, of the $InsP_3$ receptor. The latter, as evidenced by its reactivity with a specific antibody, appears as two distinct bands in transblots from SDS-polyacrylamide gel electrophoresis separations of human platelets. Both bands are phosphorylated by PGI_2 and SNP, although the phosphorylation is more evident on the heavier band. The presence of two bands may indicate the occurrence in platelets of two different isoforms of the receptor, as already described in several organs (53, 54). Alternatively, it may depend on variable degrees of glycosylation (55) or it was the result of a partial proteolysis at the NH_2-terminal region of the receptor.

Both PKA (54, 56–58) and PKG (59) are known to phosphorylate (at the same site) the $InsP_3$ receptor, which is also a substrate for PKC. In our experiments, a phosphorylation by thrombin, sensitive to the specific PKC inhibitor GF 109 203X is also observed; its onset is, however, remarkably slower than that promoted by PGI_2 and SNP (see also Ref. 54). The latter is fast enough to be compatible with its intervention in the inhibition of the receptor function. The slower PKC-dependent phosphorylation of the $InsP_3$ receptor may also be involved in the control of its sensitivity to $InsP_3$. Indeed, we observed that staurosporine decreases Ca^{2+} reuptake into the stores after its release by thrombin (19 and see also Ref. 60), and a decrease of Ca^{2+} reuptake was observed also in this study with the more specific PKC inhibitor GF 109 203X.

Different and frequently contrasting effects of the cAMP-dependent phosphorylation of the $InsP_3$ receptor are reported in the recent literature. After the initial observation by Supattapone *et al.* (52) that PKA inhibits the $InsP_3$-induced Ca^{2+} release in a rat cerebellar microsomal fraction preloaded with Ca^{2+}, a cAMP-dependent phosphorylation was reported to potentiate the Ca^{2+}-releasing effect of $InsP_3$ in vesicles reconstituted with a homotetrameric type I $InsP_3$ receptor (61). In platelet membranes, data consistent with those of Supattapone *et al.* (52) were reported in Ref. 44, but the effect of cAMP was not appreciated in isolated membranes (36). This variability of data may relate to the presence of regulatory factors in microsomes that regulate the $InsP_3$-dependent Ca^{2+} release. Besides, PKA (PKG) may exert different effects on various subtypes of $InsP_3$ receptors that differ in their functional consequences. Indeed, in some cells, such as the hepatocytes, the Ca^{2+}-releasing action of agonists is potentiated rather than inhibited by cAMP, which is therefore not expected to favor Ca^{2+} reuptake in the stores (62, 63).

In this study, we used intact human platelets to study the mechanism by which PGI_2 and SNP so powerfully depress the $[Ca^{2+}]_i$ previously increased by thrombin. By dissecting and studying separately each of the intervening components, we could demonstrate that the action of cAMP and cGMP is to inhibit the Ca^{2+}-releasing action of $InsP_3$. The effect is accompanied by the rapid phosphorylation of the receptor. It seems inevitable to conclude that in the intact system the cyclic nucleotides promote a receptor phosphorylation that prevents $InsP_3$ from releasing the store-associated Ca^{2+}.

It may be concluded that both PGI_2 and SNP, besides depressing the agonist-induced activation of phospholipase C if they are presented to the platelets prior to the agonist, as extensively demonstrated in the literature, also oppose the effect of previously produced $InsP_3$, by specifically inhibiting its Ca^{2+}-releasing property.

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Prostacyclin and Sodium Nitroprusside Inhibit the Activity of the Platelet Inositol 1,4,5-Trisphosphate Receptor and Promote Its Phosphorylation

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