Cyclic Nucleotides Modulate Store-mediated Calcium Entry through the Activation of Protein-tyrosine Phosphatases and Altered Actin Polymerization in Human Platelets*

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Agonists elevate the cytosolic calcium concentration in human platelets via a receptor-operated mechanism, involving both Ca2+ release from intracellular stores and subsequent Ca2+ entry, which can be inhibited by platelet inhibitors, such as prostaglandin E1 and nitroprusside which elevate cAMP and cGMP, respectively. In the present study we investigated the mechanisms by which cAMP and cGMP modulate store-mediated Ca2+ entry. Both prostaglandin E1 and sodium nitroprusside inhibited thapsigargin-evoked store-mediated Ca2+ entry and actin polymerization. However, addition of these agents after induction of store-mediated Ca2+ entry did not affect either Ca2+ entry or actin polymerization. Furthermore, prostaglandin E1 and sodium nitroprusside dramatically inhibited the tyrosine phosphorylation induced by depletion of the internal Ca2+ stores or agonist stimulation without affecting the activation of Ras or the Ras-activated phosphatidylinositol 3-kinase or extracellular signal-related kinase (ERK) pathways. Inhibition of cyclic nucleotide-dependent protein kinases prevented inhibition of agonist-evoked Ca2+ release but it did not have any effect on the inhibition of Ca2+ entry or actin polymerization. Phenytoin, phenylarsine oxide and vanadate, inhibitors of protein-tyrosine phosphatases prevented the inhibitory effects of the cGMP and cAMP elevating agents on Ca2+ entry and actin polymerization. These results suggest that Ca2+ entry in human platelets is directly down-regulated by cGMP and cAMP by a mechanism involving the inhibition of cytoskeletal reorganization via the activation of protein tyrosine phosphatases.

In platelets and other nonexcitable cells, receptor stimulation promotes the entry of extracellular Ca2+ through the plasma membrane (1). A major mechanism for Ca2+ influx is store-mediated Ca2+ entry (SMCE),1 in which the filling state

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1 The abbreviations used are: SMCE, store-mediated calcium entry;
findings also indicate that PGE\(_1\) and SNP inhibit both SMCE and actin polymerization by a mechanism independent of PKA and PKG and which requires activation of protein-tyrosine phosphatases. These results indicate that the inhibition of agonist-evoked elevations in \([\text{Ca}^{2+}]_i\) by cAMP- or cGMP-elevating agents might have two components: a reduction in \(\text{Ca}^{2+}\) store depletion mediated by PKA or PKG, with a consequent reduction in SMCE, and a direct effect on \(\text{Ca}^{2+}\) entry mediated by protein-tyrosine phosphatases and inhibition of actin polymerization.

**EXPERIMENTAL PROCEDURES**

**Materials**—Fura-2 acetoxymethyl ester (fura-2/AM) was from Texas Fluorescence (Austin, TX). Apyrase (grade V), aspirin, bovine serum albumin, paraformaldehyde, Nonidet P-40, fluorescein isothiocyanate-labeled phallolidin, thrombin, ADP, sodium nitroprusside, sodium vanadate, PGE\(_1\), and thapsigargin (TG) were from Sigma (Poole, Dorset, United Kingdom). KT5823, KT5720, ionomycin, and phenylarsine oxide (PAO) were from Calbiochem (Nottingham, UK). Anti-VASP Ser239 monoclonal antibody was from Alexis Corp. (Nottingham, UK). Pan-Ras (Ab-3) monoclonal antibody was from Oncogene Science (Cambridge, MA). Phospho-p44/42 extracellular signal-regulated kinases (ERK) monoclonal antibody (E10) was from New England Biolabs (Beverly, MA). Anti-phosphotyrosine monoclonal antibody (4G10), anti-phospho-Akt/PKB\(_{\alpha}\) (Ser473) monoclonal antibody, and horseradish peroxidase-conjugated rabbit anti-sheep IgG antibody were from Upstate Biotechnology (Lake Placid, NY). Horseradish peroxidase-conjugated ovine anti-mouse IgG antibody (NA931) was from Amersham Pharmacia Biotech (Little Chalfont, Bucks., UK). Dimethyl-bis(o-aminophenoxo)-ethane-N,N,N',N'-tetracetic acid (BAPTA) acetoxymethyl ester was from Molecular Probes (Leiden, The Netherlands). All other reagents were of analytical grade.

**Platelet Preparation**—Fura-2-loaded platelets were prepared as described previously (5). Briefly, blood was obtained from healthy volunteers and mixed with one-sixth volume of acid/citrate dextrose anticoagulant containing (in mM): 85 sodium citrate, 78 citric acid, and 111 D-glucose. Platelet-rich plasma was then prepared by centrifugation for 5 min at 700 \(\times g\) and aspirin (100 \(\mu M\)) and apyrase (40 \(\mu g/ml\) added. Platelet-rich plasma was incubated at 37 \(^\circ\)C with 2 \(\mu M\) fura-2/AM for 45 min. For loading with dimethyl-BAPTA, cells were incubated for 30 min at 37 \(^\circ\)C with 10 \(\mu M\) dimethyl-BAPTA AM. Cells were then collected by centrifugation at 350 \(\times g\) for 20 min and resuspended in HEPES-buffered saline containing (in mM): 145 NaCl, 10 HEPES, 10 \(\mu M\) glucose, 5 KCl, 1 MgSO\(_4\), pH 7.45, and supplemented with 0.1% (w/v) bovine serum albumin and 40 \(\mu g/ml\) apyrase.

**Measurement of Intracellular Free Calcium Concentration ([Ca\(^{2+}\)]\(_i\))**—Fluorescence was recorded from 1.5-ml aliquots of magnetically stirred platelet suspension (10\(^6\) cells/ml) at 37 \(^\circ\)C using a Cairn Research Spectrophotometer (Cairn Research Ltd., Sittingbourne, Kent, UK) with excitation wavelengths of 340 and 380 nm and emission at 500 nm.

**FIG. 1. Effects of PGE\(_1\) or SNP on thrombin- or ADP-induced elevations in [Ca\(^{2+}\)]\(_i\) in platelets.** Fura-2-loaded human platelets were incubated at 37 \(^\circ\)C for 1 min in the presence of 5 \(\mu M\) PGE\(_1\), 100 \(\mu M\) SNP, or the vehicles (Control). At the time of the experiment 1 mM Ca\(^{2+}\) or 100 \(\mu M\) EGTA was added as indicated. Cells were then stimulated with 0.1 unit/ml thrombin (A-D) or 40 \(\mu M\) ADP (E-F). Elevations in [Ca\(^{2+}\)]\(_i\) were monitored using the 340/380 nm ratio as described under “Experimental Procedures.” Traces shown are representative of 6 to 11 independent experiments.

**FIG. 2. Effects of PGE\(_1\) or SNP on TG-induced SMCE.** Fura-2-loaded human platelets were incubated at 37 \(^\circ\)C in the presence of 5 \(\mu M\) PGE\(_1\) (A), 100 \(\mu M\) SNP (B), or the vehicles (Control). At the time of the experiment 100 \(\mu M\) EGTA was added. Cells were then stimulated with TG (200 \(\mu M\)) and 3 min later CaCl\(_2\) (final concentration, 300 \(\mu M\)) was added to the medium to initiate Ca\(^{2+}\) entry. Elevations in [Ca\(^{2+}\)]\(_i\) were monitored using the 340/380 nm ratio as described under “Experimental Procedures.” Traces are representative of six to nine independent experiments.
Changes in \([\mathrm{Ca}^{2+}]\), were monitored using the fura-2 340/380 fluorescence ratio and calibrated according to the method of Grynkiewicz et al. (20).

**Determination of \([\mathrm{Ca}^{2+}]\) Entry**—\([\mathrm{Ca}^{2+}]\) influx in platelets that had been store depleted using TG was estimated using the integral of the rise in \([\mathrm{Ca}^{2+}]\), for 2½ min after addition of \(\mathrm{CaCl}_2\) (5). When platelets were preincubated with inhibitors, \([\mathrm{Ca}^{2+}]\) entry was corrected by subtraction of the rise in \([\mathrm{Ca}^{2+}]\), due to leakage of the indicator. TG-induced \([\mathrm{Ca}^{2+}]\) release was estimated using the integral of the rise in \([\mathrm{Ca}^{2+}]\), for 3 min after its addition. Thrombin-evoked \([\mathrm{Ca}^{2+}]\) elevation or release was measured as the integral of the rise in \([\mathrm{Ca}^{2+}]\), above basal for 90 s after the addition of the agonist in the presence of external \([\mathrm{Ca}^{2+}]\) or in a \([\mathrm{Ca}^{2+}]-\)free medium (with 100 \(\mu\)M EGTA), respectively.

**Measurement of F-actin Content**—The F-actin content of resting and activated platelets was determined according to a previously published procedure (5). Briefly, washed platelets (2 \(\times\) 10\(^6\) cells/ml) were activated in HEPES-buffered saline. Samples of platelet suspension (200 \(\mu\)l) were transferred to 200 \(\mu\)l of ice-cold 3% (w/v) formaldehyde in phosphate-buffered saline (PBS) for 10 min. Fixed platelets were permeabilized by incubation for 10 min with 0.025% (v/v) Nonidet P-40 detergent dissolved in PBS. Platelets were then incubated for 30 min with fluorescein isothiocyanate-labeled phalloidin (1 \(\mu\)M) in PBS supplemented with 0.5% (w/v) bovine serum albumin. After incubation the platelets were centrifuged and resuspended in an MSE Micro-Centaur Centrifuge (MSE Scientific Instruments, Crawley, Sussex, UK) for 60 s at 3000 \(\times\) g and resuspended in PBS. Staining of 2 \(\times\) 10\(^7\) cells/ml was measured using a PerkinElmer Fluorescence Spectrophotometer (PerkinElmer Life Sciences, Norwalk, CT). Samples were excited at 496 nm and emission was at 516 nm.

**Protein Tyrosine Phosphorylation**—Protein tyrosine phosphorylation was detected by gel electrophoresis and Western blotting (6). Platelets stimulation was terminated by mixing with an equal volume of 2 \(\times\) Laemmli’s buffer (21) with 10% dithiothreitol followed by heating for 5 min at 95 °C. One-dimensional SDS electrophoresis was performed with 10% polyacrylamide minigels and separated proteins were electrophoretically transferred, for 2 h at 0.8 mA/cm\(^2\), in a semi-dry blotter ( Hoefer Scientific, Newcastle, Staffs., UK) onto nitrocellulose for subsequent probing. Blots were incubated overnight with 10% (w/v) bovine serum albumin to block residual protein-binding sites. Immunodetection of tyrosine phosphorylation was achieved using the anti-phosphotyrosine antibody 4G10 diluted 1:2500 in TBST for 1 h. The primary antibody was removed and blots washed six times for 5 min each with TBST. To detect the primary antibody, blots were incubated with horseradish peroxidase-conjugated ovine anti-mouse IgG antibody diluted 1:10000 in TBST, washed six times in TBST, and exposed to enhanced chemiluminescence reagents for 1 min. Blots were then exposed to preflashed photographic film. Densitometric measurements were made using a Quantimet 500 densitometer (Leica, Milton Keynes, UK).

**Quantitative analysis of VASP Ser\(^{239}\) Phosphorylation**—Phosphorylation of the residue Ser\(^{239}\) of VASP, a phosphorylation site preferred by PKG but also used by PKA (22), was measured by SDS-PAGE and Western blotting as described above but using a specific anti-VASP Ser\(^{239}\) monoclonal antibody (16C2) diluted 1:1000 in TBST as previously described (22).

**Analysis of Akt/PKBa Ser\(^{473}\) Phosphorylation**—Phosphorylation of Akt/PKBa on the residue Ser\(^{473}\) was assessed by SDS-PAGE and Western blotting as described above but using a specific anti-phospho-Akt/PKBa (Ser\(^{473}\)) monoclonal antibody diluted 1:1500 in TBST as previously described (23), followed by incubation with horseradish peroxidase-conjugated rabbit anti-sheep IgG antibody diluted 1:10000 in TBST.

**Analysis of Diposphorylated p42/p44 ERK**—Analysis of diposphorylated p42/p44 ERK was performed by SDS-PAGE and Western blotting as described above but using a specific phospho-p42/44 MAP kinase monoclonal antibody (E10) diluted 1:1500 in TBST as previously described (24).
Cyclic Nucleotides and Ca$^{2+}$ Entry in Human Platelets

Subcellular Fractionation—Human platelet fractionation was carried out according to a previously published procedure (5). Briefly, platelets were pelleted in a microcentrifuge and the pellets were quickly resuspended in 0.5 ml of ice-cold Tris-HCl buffer containing: 10 mM Tris-HCl (pH 7.2), 158 mM NaCl, 1 mM EDTA, 50 μM leupeptin, 5 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, and 1 mM Na$_3$VO$_4$. The suspensions were sonicated and intact platelets were removed by centrifugation at 1,500 x g. The platelet lysate was centrifuged at 100,000 x g for 4°C for 60 min to obtain membrane and cytosolic fractions. Membranes were washed with PBS with 1 mM Na$_3$VO$_4$ at 4°C and resuspended in Tris-HCl buffer containing: 10 mM Tris-HCl, pH 7.2, 158 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% sodium deoxycholate, 1% Triton X-100, 50 μg/ml leupeptin, 5 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, and 1 mM Na$_3$VO$_4$. Lysates were centrifuged at 16,000 x g for 5 min to remove insoluble substances. Lysates of the subcellular fractions or total cell lysates (50 μg/well) were analyzed by Western blotting with pan-Ras (Ab1) monoclonal antibody diluted 1:500 in TBST. To detect the primary antibody, blots were incubated with horseradish peroxidase-conjugated anti-mouse IgG antibody diluted 1:10,000 in TBST and exposed to enhanced chemiluminescence reagents for 1 min as described above. Blots were then exposed to preflashed photographic film.

Statistical Analysis—Analysis of statistical significance was performed using Student’s t test. For multiple comparisons, one-way analysis of variance combined with the Dunnett test was used.

RESULTS

Regulation of Agonist-induced Ca$^{2+}$ Mobilization by PGE$_1$ and SNP—Treatment of fura-2-loaded human platelets with the physiological agonists thrombin (0.1 unit/ml) or ADP (40 μM) evoked rises in [Ca$^{2+}$]i in both a medium containing 1 mM Ca$^{2+}$ and in the absence of external Ca$^{2+}$ (100 μM EDTA was added). In agreement with previous studies (18, 19) thrombin- and ADP-induced Ca$^{2+}$ elevations in human platelets were significantly inhibited when the cells were preincubated for 1 min with 5 μM PGE$_1$ or 100 μM SNP (Fig. 1; p < 0.01; n = 6–11). In contrast, PGE$_1$ and SNP had no effect on resting [Ca$^{2+}$]i (Fig. 1).

Effect of PGE$_1$ and SNP on Store-mediated Ca$^{2+}$ Entry in Platelets—In a Ca$^{2+}$-free medium, TG, a specific inhibitor of the endomembrane Ca$^{2+}$-ATPase (SERCA, Ref. 25), evoked a sustained elevation in [Ca$^{2+}$]i, due to the release of Ca$^{2+}$ from internal stores. The subsequent addition of Ca$^{2+}$ (final concentration 300 μM) to the external medium induced a rapid increase in [Ca$^{2+}$]i, indicative of SMCE (Fig. 2). Treatment of platelets for 1 min with 5 μM PGE$_1$ or 100 μM SNP decreased Ca$^{2+}$ entry to 56.2 ± 4 and 52.5 ± 7.2% of control (vehicle added; Fig. 2; p < 0.001; n = 6–9). Platelets preincubated for 1 min with 5 μM PGE$_1$ or 100 μM SNP showed an identical release of Ca$^{2+}$ from the intracellular stores after treatment with TG in comparison with untreated cells (99.04 ± 1.9 and 98.8 ± 2.6% of control after treatment with PGE$_1$ or SNP, respectively).

Regulation of Actin Polymization by PGE$_1$, or SNP in Human Platelets—Recently a role for the actin cytoskeleton in the activation of SMCE has been suggested in several cells types (11, 12, 26). As we have previously shown (17), TG induces actin polymerization in the absence of a detectable rise in [Ca$^{2+}$]i, in platelets loaded with the Ca$^{2+}$-chelator dimethyl-BAPTA. After dimethyl-BAPTA loading, treatment of platelets with 1 μM TG in a Ca$^{2+}$-free medium raised the F-actin content by 29.7 ± 6.4% compared with control unstimulated cells. Treatment with various concentrations of PGE$_1$, or SNP did not significantly modify the F-actin content of resting platelets; however, TG-induced actin polymerization was inhibited in a concentration-dependent manner, with IC$_{50}$ values of 53.0 ± 2.9 nM and 1.7 ± 0.4 μM, respectively, and complete inhibition at 5 and 100 μM, respectively (Fig. 3; p < 0.05; n = 6).

Stimulation of dimethyl-BAPTA-loaded human platelets with thrombin (0.1 unit/ml) or ADP (40 μM) for 3 min increased F-actin content by 32.1 ± 3.2% and 23.6 ± 2.1% of control, respectively. Consistent with the above, treatment of platelets with 5 μM PGE$_1$ or 100 μM SNP abolished both thrombin- and ADP-induced actin polymerization (data not shown).

Effect of PGE$_1$ and SNP on the Maintenance of Store-mediated Ca$^{2+}$ Entry—We have previously reported that the integrity of the actin cytoskeleton is essential for both the activation and maintenance of SMCE in platelets (9). To investigate the effects of cAMP and cGMP on the maintenance of SMCE we examined the effect of PGE$_1$ and SNP, respectively, on Ca$^{2+}$ entry in platelets after SMCE had been previously stimulated using TG.

Fig. 4 shows the effect of adding PGE$_1$ or SNP to store-depleted human platelets. 5 μM PGE$_1$ (Fig. 4A), 100 μM SNP (Fig. 4B), or the vehicles were added 3 min after TG and cells were then incubated for a further 1 min before the addition of Ca$^{2+}$ to the medium (final concentration 300 μM) to initiate Ca$^{2+}$ entry. At the time when PGE$_1$ and SNP were added Ca$^{2+}$ entry was already stimulated (data not shown, but see Fig. 2). Addition of PGE$_1$ or SNP after Ca$^{2+}$ store depletion did not significantly modify subsequent Ca$^{2+}$ entry (Fig. 4; p = 0.87; n = 6). Consistent with these results, treatment of human platelets for 1 min with 5 μM PGE$_1$ or 100 μM SNP after SMCE had been activated by the addition of TG did not alter TG-evoked increase in the F-actin content (Table I; n = 6). These findings show that cAMP and cGMP inhibit the activation but not the maintenance of SMCE. In addition, these observations indicate that PGE$_1$ and SNP do not act either as Ca$^{2+}$ channel blockers or Ca$^{2+}$ chelators or as nonspecific inhibitors of actin polymerization.

Role of PKA and PKG in the Inhibitory Effects of PGE$_1$ and SNP on Actin Polymerization—To investigate the possible roles of PKA and PKG in the responses observed after treatment with PGE$_1$ and SNP we used KT5720 and KT5823, highly specific, cell-permeant inhibitors of PKA and PKG activity, respectively (27). Since VASP has been reported to be a substrate for both PKA and PKG (22, 28) and the residue Ser$^{239}$ is the site preferred by PKG is also used by PKA in platelets (29, 30), we monitored PKA and PKG activity by testing the effect of the inhibitors on PGE$_1$- and SNP-induced phosphorylation of this residue. As shown in Fig. 5, treatment of platelets for 30 min with KT5720 or KT5823 inhibited phosphorylation of VASP at residue Ser$^{239}$ in a concentration-dependent manner, with IC$_{50}$ values of 520.0 ± 17.4 and 168.8 ± 14.1 mM, respectively, and complete inhibition at 3 and 1 μM, respectively (Fig. 5; p < 0.001; n = 4).

Treatment of human platelets for 30 min with 3 μM KT5720 or 1 μM KT5823 did not alter the F-actin content of unstimulated platelets. In addition, these treatments did not reverse the inhibitory effects of PGE$_1$ or SNP, respectively.
polymerization (Table II; n = 6). These results indicate that the effects of PGE₆ and SNP are independent of PKA and PKG, respectively.

**PGE₆ and SNP Prevent Protein Tyrosine Phosphorylation in Human Platelets**—Agonist- and store depletion-induced protein tyrosine phosphorylation was assessed by gel electrophoresis and Western blotting with a specific antiphosphotyrosine antibody. Platelets heavily loaded with the Ca²⁺ chelator dimethyl-BAPTA were used for this study so as to eliminate Ca²⁺-dependent tyrosine phosphorylation (31).

Dimethyl-BAPTA-loaded platelets were incubated for 1 min at 37 °C with 5 µM PGE₆ or 100 µM SNP or the vehicle and Ca²⁺ stores were depleted using TG (1 µM). Samples for protein phosphorylation analysis were taken from the spectrophotometer cuvette 10 s prior the addition of PGE₆, SNP, or the vehicle and 10 s before and 180 s after the addition of TG. As shown in Fig. 6, A and B, pretreatment with PGE₆ or SNP significantly reduced protein tyrosine phosphorylation relative to its control in store depleted cells (e.g. bands at 72 and 130 kDa; n = 4). Similar results were observed when cells were stimulated with the physiological agonists thrombin (0.1 unit/ml; Fig. 6C and D; n = 4) and ADP (40 µM; not shown). In addition, treatment with 5 µM PGE₆ or 100 µM SNP significantly reduced tyrosine phosphorylation in unstimulated platelets by 20 ± 5 and 25 ± 5%, respectively (p < 0.01; n = 4).

**Effect of Protein-tyrosine Phosphatases on PGE₆ and SNP-Induced Responses**—The possibility that the inhibitory effects observed after treatment with PGE₆ or SNP might be mediated by protein-tyrosine phosphatases was tested by examining the effect of the tyrosine phosphatase inhibitors PAO and vanadate. Under our conditions, treatment of dimethyl-BAPTA-loaded human platelets for 30 min with either 10 µM PAO or 100 µM vanadate increased the phosphotyrosine level of both resting and stimulated cells without impairing TG-induced increase in the phosphotyrosine level (Fig. 7; n = 4). Interestingly, preincubation of platelets with 10 µM PAO or 100 µM vanadate for 30 min completely reversed the inhibition of actin polymerization observed after treatment with PGE₆ or SNP (Table II; n = 6), while this treatment per se did not modify either the F-actin content of unstimulated cells or TG-induced actin polymerization (data not shown).

Next we tested whether the inhibition of SMCE could be mediated by the activation of tyrosine phosphatase activity. Cells treated with PAO or vanadate showed similar resting levels of [Ca²⁺]i to control platelets (data not shown). In addition, PAO or vanadate-treated cells retained their ability to respond to Ca²⁺ mobilizing agents, such as TG, which indicates that this treatment did not affect the ability of platelets to store Ca²⁺ in intracellular compartments as well as demonstrating that these agents per se do not mobilize Ca²⁺ from the stores in platelets (data not shown). Platelets previously preincubated for 30 min with 10 µM PAO or 100 µM vanadate were treated for

**TABLE II**

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<th>Filamentous actin</th>
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<td>ADP</td>
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</table>

**FIG. 5**

**Quantitative analysis of Ser²⁵⁹ phosphorylated VASP in platelets incubated with KT5720 or KT5823.** Human platelets were incubated with various concentrations of KT5720 (left panel), KT5823 (right panel), or the vehicles. Cells were stimulated for 1 min with 5 µM PGE₆, 100 µM SNP, or the vehicle and stimulated with either TG (200 µM), thrombin (0.1 unit/ml) or ADP (40 µM). Samples were removed 5 s before and 3 min after the addition of the agonists and F-actin content was determined as described under “Experimental Procedures.” Values given are the changes in F-actin content expressed as a percentage of the

**TABLE II**

<table>
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<tr>
<th>Stimulation</th>
<th>Filamentous actin</th>
<th>PGE₆-treated cells</th>
<th>SNP-treated cells</th>
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<td></td>
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<td>ADP</td>
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</tr>
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</table>

**FIG. 5**

**Quantitative analysis of Ser²⁵⁹ phosphorylated VASP in platelets incubated with KT5720 or KT5823.** Human platelets were incubated with various concentrations of KT5720 (left panel), KT5823 (right panel), or the vehicles. Cells were stimulated for 1 min with 5 µM PGE₆, 100 µM SNP, or the vehicle and stimulated with either TG (200 µM), thrombin (0.1 unit/ml) or ADP (40 µM). Samples were removed 5 s before and 3 min after the addition of the agonists and F-actin content was determined as described under “Experimental Procedures.” Values given are the changes in F-actin content expressed as a percentage of the increase evoked by 5 µM PGE₆ (A) or 100 µM SNP (B) above control unstimulated values.
1 min with PGE1, SNP, or the vehicles and then stimulated with TG (200 nM) in a Ca2+-free medium and 3 min later CaCl2 (final concentration 300 μM) was added. At the times indicated 100-μl aliquots were removed and the reaction terminated by mixing with an equal volume of 2 × Laemmli’s buffer with 10% dithiothreitol. Proteins were analyzed by SDS-PAGE and subsequent Western blotting with a specific antiphosphotyrosine antibody as described under “Experimental Procedures.” Molecular size is indicated on the right. The panels show results from an experiment representative of three others.

The effect of PGE1 and SNP on thrombin-evoked Ca2+ mobilization is shown in Table IV. The effects of either PGE1 (5 μM) or SNP (100 μM) on thrombin-evoked Ca2+ release were almost completely reversed by pretreatment for 30 min with 3 μM KT5720 or 1 μM KT5823, respectively, in contrast, 10 μM PAO or 100 μM vanadate had negligible effects (Table IV; n = 6). Pretreatment of platelets with KT5720 or KT5823 partially reversed the effect of PGE1 or SNP on thrombin-induced Ca2+ responses in a medium containing 1 mM Ca2+ (Table IV; p < 0.05; n = 6). More importantly, the combined effect of inhibitors of either PKA or PKG and of tyrosine phosphatases completely prevented the inhibition induced by PGE1 and SNP on thrombin-induced Ca2+ elevations in the presence of 1 mM external Ca2+ (Table IV; p < 0.001; n = 6).

Role of the Ras-dependent Pathways on PGE1- and SNP-induced Responses—The activity of Rac proteins has been shown to be required for the activation of SMCE in several cell types (32, 33) including platelets (5). To investigate whether PGE1 and SNP impair the activation of Ras we have examined their effects on the association of Ras with membranes, a process essential for Ras activation (34). In resting platelets, pan-
PGE1 or SNP did not modify either basal or TG-induced phosphorylation of Akt/PKB on Ser473, indicating that these agents do not affect PI3K activity. Similar results were obtained when the effect of these agents on TG-induced ERK activation was investigated. ERK activation was determined using an antibody that specifically recognizes diphosphorylated and thus activated p42/44 ERK as previously described (24).

### TABLE III

**Effects of PKA, PKG, or protein-tyrosine phosphatases on TG-stimulated store-mediated Ca\(^{2+}\) entry**

Fura-2-loaded human platelets were incubated for 30 min at 37 °C with 3 μM KT5720, 1 μM KT5823, 100 μM sodium vanadate, 10 μM PAO or the vehicles. At the time of the experiment 100 μM EGTA was added. Cells were then incubated for 1 min with 5 μM PGE1, 100 μM SNP or the vehicle and stimulated with TG (200 nM) and 3 min later CaCl₂ (final concentration 300 μM) was added to the medium to initiate Ca\(^{2+}\) entry. Elevations in [Ca\(^{2+}\)] were monitored using the 340/380 nm ratio as described under “Experimental Procedures.” Data indicate the percentage of Ca\(^{2+}\) entry relative to respective controls (vehicle was added). Ca\(^{2+}\) entry was estimated as described under “Experimental Procedures.” Values are mean ± S.E. of six to ten separate determinations.

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<th>Treatment</th>
<th>% of control</th>
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<td>PGE1 + KT5720</td>
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<td>PGE1 + sodium vanadate</td>
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<tr>
<td>PGE1 + PAO</td>
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### TABLE IV

**Effects of PKA, PKG, or protein-tyrosine phosphatases on thrombin-stimulated Ca\(^{2+}\) elevations in the presence or absence of external Ca\(^{2+}\)**

Fura-2-loaded human platelets were incubated for 30 min at 37 °C with 3 μM KT5720, 1 μM KT5823, 100 μM sodium vanadate, 10 μM PAO, a combination of KT5720 or KT5823 plus sodium vanadate or PAO or the vehicles. At the time of the experiment 1 mM Ca\(^{2+}\) or 100 μM EGTA was added. Cells were then incubated for 1 min with 5 μM PGE1 or 100 μM SNP and stimulated with thrombin (0.1 unit/ml). Elevations in [Ca\(^{2+}\)], were monitored using the 340/380 nm ratio and thrombin-induced Ca\(^{2+}\) elevations and release were estimated by integration as described under “Experimental Procedures.” Data indicate the percentage of Ca\(^{2+}\) elevation or release relative to controls (vehicle was added). Values are mean ± S.E. of six determinations.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% of control</th>
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<td>100.0 ± 0.0</td>
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<tr>
<td>PGE1</td>
<td>15.5 ± 2.8</td>
</tr>
<tr>
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<td>98.9 ± 11.9</td>
</tr>
<tr>
<td>PGE1 + sodium vanadate</td>
<td>22.9 ± 6.3</td>
</tr>
<tr>
<td>PGE1 + PAO</td>
<td>38.8 ± 5.8</td>
</tr>
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<td>PGE1 + KT5720 + vanadate</td>
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<tr>
<td>PGE1 + KT5720 + PAO</td>
<td>99.8 ± 7.1</td>
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<table>
<thead>
<tr>
<th>Treatment</th>
<th>% of control</th>
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<tr>
<td>PGE1</td>
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<td>PGE1 + PAO</td>
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<tr>
<td>PGE1 + KT5720 + vanadate</td>
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<td>105.7 ± 10.1</td>
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Ras immunoreactive proteins were detected in both cytosolic and membrane fractions (Fig. 8A). As reported previously (5), TG induces translocation of Ras to the membrane fraction, an effect that was unmodified when platelets were incubated for 1 min with either PGE1 or SNP (Fig. 8A, n = 3), treatments which also did not alter the distribution of Ras in resting cells (data not shown). We also investigated the effect of PGE1 and SNP on the activation of phosphatidylinositol 3-kinase (PI3K) and ERK, which have been proposed as candidates for the cytoskeleton-dependent and -independent branches of the Ras-dependent activation of SMCE in platelets, respectively (35).² PI3K activity was investigated by examining the phosphorylation of its substrate, Akt/PKBα on Ser473. It is well known that activation of PI3K results in phosphorylation of Akt/PKBα on both Ser473 and Thr308 (36). As shown in Fig. 8B, treatment of platelets with TG (200 nM) resulted in an increase in phosphorylation of Akt/PKBα on Ser473 by 4.7 ± 1.2-fold. This increase was abolished by preincubation for 30 min with 10 μM LY 294002, a concentration that selectively inhibits PI3K activity (35) (Fig. 8B; n = 3). In contrast, treatment for 1 min with PGE1 or SNP did not modify either basal or TG-induced phosphorylation of Akt/PKBα on Ser473, indicating that these agents do not affect PI3K activity. Similar results were obtained when the effect of these agents on TG-induced ERK activation was investigated. ERK activation was determined using an antibody that specifically recognizes diphosphorylated and thus activated p42/44 ERK as previously described (24).

2 J. A. Rosado and S. O. Sage, unpublished observations.
Fig. 8. Effects of PGE1 or SNP on TG-induced membrane association of Ras and the activation of PI 3-kinase and ERK. A, dimethyl-BAPTA-loaded human platelets were incubated for 1 min with 5 μM PGE1, 100 μM SNP, or the vehicles as indicated and then stimulated with TG (200 nM) in a Ca2+-free medium. Samples were taken 5 s before (lanes 1 and 2) and 3 min after adding TG (lanes 3–8). Cytosolic fractions (C) and membrane fractions (M) were isolated as described under “Experimental Procedures.” Lysates of the subcellular fractions were analyzed by Western blotting with the specific monoclonal antibody to Ser473 phosphorylated Akt/PKB a as described under “Experimental Procedures.” B, dimethyl-BAPTA-loaded human platelets were incubated in the absence or presence of either 10 μM LY294002 for 30 min or 5 μM PGE1, or 100 μM SNP for 1 min as indicated and then stimulated with TG (200 nM) in a Ca2+-free medium. Samples were taken 5 s before and 3 min after adding TG. Samples were subjected to SDS-PAGE and analyzed by Western blotting with the specific monoclonal antibody to Ser473 phosphorylated Akt/PKB a as described under “Experimental Procedures.” C, dimethyl-BAPTA-loaded human platelets were incubated for 1 min with 5 μM PGE1, 100 μM SNP, or the vehicles as indicated and then stimulated with TG (200 nM) in a Ca2+-free medium. Samples were taken 5 s before and 3 min after adding TG. Samples were subjected to SDS-PAGE and Western blotting with the specific phospho-p44/42 ERK monoclonal antibody (E10) as described under “Experimental Procedures.” The panels show results representative of three independent experiments.

in [Ca2+]i, due to release of Ca2+ from intracellular stores. Since under these conditions the exclusive mechanism for Ca2+ extrusion in human platelets is the PMCA (37), we estimated PMCA activity by examining the rate of decay (see Ref. 37). Fig. 9A shows the effect of preincubation for 1 min with 100 μM SNP on the response evoked by TG (1 μM) plus ionomycin (50 nM). Preincubation with SNP did not modify the release of Ca2+ from the intracellular stores stimulated by TG plus ionomycin, however, the rate of decay was enhanced by 135%. The decay constants were 0.0150 ± 0.0008 in SNP-treated platelets and 0.0114 ± 0.0009 in paired controls (n = 6; p < 0.01). The effect of SNP was essentially abolished by preincubation with the PKG inhibitor KT5823. The decay constants were 0.0101 ± 0.0006 in cells preincubated for 30 min with 1 μM KT5823 and then treated with SNP and 0.0105 ± 0.0006 in controls (Fig. 9B; n = 6; p = 0.30).

Discussion

Ca2+ mobilization in nonexcitable cells regulates such diverse processes as secretion, contraction, gene expression, and apoptosis. In human platelets, most agonists elevate [Ca2+]i, through receptor-dependent mechanisms, activating both the release of Ca2+ from the intracellular stores and Ca2+ entry across the plasma membrane. These responses are antagonized by cAMP- and cGMP-elevating agents. Agents such as PGE1, or iloprost stimulate cAMP formation. On the other hand, nitroprusside and nitrovasodilators, such as nitroprusside, are potent cGMP-elevating platelet inhibitors (1). Several studies in platelets have reported that the effects of cyclic nucleotides on the Ca2+ entry process are secondary to their inhibitory effects on intracellular Ca2+ release (18, 19, 38). In the present study we shed new light on the mechanism of action of cyclic nucleotides on SMCE. By reducing the preincubation time with the cAMP- and cGMP-elevating agents we were able to detect for the first time substantial inhibition of SMCE without the modification of TG-induced release. These findings strongly suggest that cyclic nucleotides exert a negative regulation of Ca2+ entry independently of the extent of release from intracellular Ca2+ stores.

Recently it has been reported that the integrity of the actin cytoskeleton is required for the activation and maintenance of SMCE in different cell types (11, 12), including platelets (5, 9). Hence, we have investigated whether cyclic nucleotides interfere with actin polymerization. Treatment of platelets with the cAMP- and cGMP-elevating agents, PGE1 and SNP, inhibited actin polymerization stimulated by TG or the physiological agonists ADP and thrombin. This inhibition of actin polymerization was observed in cells heavily loaded with the Ca2+ chelator dimethyl-BAPTA, and thus was independent of any effects of the cyclic nucleotides on [Ca2+]i. These results, together with the degree of inhibition of SMCE, are consistent with the effects of the inhibitors of actin polymerization cytochalasin D and latrunculin A on SMCE in human platelets (5, 9). These agents inhibited SMCE by 50% at concentrations that abolished actin polymerization in platelets. Therefore, these findings suggest that the effect of cyclic nucleotides on SMCE might be mediated by impairment of actin filament polymerization.

Previous studies have shown that the effects of cyclic nucleotides are not mediated by acceleration of Ca2+ removal from the cytosol (38). Since more recent studies have shown that
cAMP stimulates PMCA activity (31), we performed a series of studies to test the effects of cGMP on Ca\(^{2+}\) extrusion. We have reported that the main mechanism for Ca\(^{2+}\) extrusion in human platelets is the PMCA (37, 39). Our findings demonstrate that, as well as cAMP-elevating agents (31), SNP significantly increases Ca\(^{2+}\) extrusion in platelets, a process that is entirely mediated by the PKG, since inhibition of this kinase abolished the effect of SNP. To evaluate whether Ca\(^{2+}\) entry is directly down-regulated by the cyclic nucleotides or if their effects are mediated by acceleration of Ca\(^{2+}\) extrusion we added PGE\(_1\) or SNP to the platelet suspension once store-mediated Ca\(^{2+}\) entry had been preactivated using TG. Our results show that treatment with cAMP- and cGMP-elevating agents after the activation of SMCE has no effect on Ca\(^{2+}\) entry, while PMCA activity is increased. These observations indicate that, in agreement with previous studies (38), cyclic nucleotides do not inhibit elevations in [Ca\(^{2+}\)]\(_i\), by accelerating Ca\(^{2+}\) extrusion alone, but that cAMP and cGMP are also involved in regulating the activation but not the maintenance of SMCE in platelets. Consistent with this, neither PGE\(_1\) nor SNP impaired actin polymerization in preactivated platelets.

Most cellular functions exerted by cyclic nucleotides are mediated by the activation of cAMP-dependent and cGMP-dependent protein kinases. To determine whether PKA and PKG mediate these responses we used KT5720 and KT5823, selective cell-permeant inhibitors of PKA and PKG, respectively (27). By monitoring PKA and PKG activity we found that treatment of platelets for 1 min with 5 \(\mu\)M PGE\(_1\) or 100 \(\mu\)M SNP effectively activated PKA or PKG activity, respectively. Our present results and numerous previous studies with cAMP- and cGMP-elevating agents (for review, see Refs. 1, 18, and 19) suggest that inhibition of agonist evoked intracellular Ca\(^{2+}\) discharge is an effect mediated by both PKA and PKG. Although phosphorylation of VASP correlates with the inhibition of Ca\(^{2+}\) release, at present we do not have evidence for the involvement of VASP in this process. However, a recent report has shown that VASP is not required for the inhibition of Ca\(^{2+}\) mobilization in murine platelets mediated by cAMP- and cGMP-elevating agents (28). In contrast, the analysis of the F-actin content and SMCE clearly excluded PKA and PKG as candidate proteins involved in cyclic nucleotide-mediated regulation of actin polymerization and Ca\(^{2+}\) entry, since these processes were unaffected by KT5720 or KT5823. The partial recovery observed in thrombin-induced Ca\(^{2+}\) elevation in the presence of 1 \(\mu\)M external Ca\(^{2+}\) after incubation with KT5720 or KT5823 can be explained by the effect of these inhibitors on PGE\(_1\)- and SNP-induced reductions in Ca\(^{2+}\) release. Different results have been reported in vascular endothelial cells where cGMP inhibits SMCE via a PKG-dependent mechanism (40).

An alternative pathway, responsible for the inhibitory effects of cAMP- and cGMP-elevating agents on Ca\(^{2+}\) entry and actin polymerization, remains to be identified. Platelet activation is accompanied by a dramatic increase in tyrosine phosphorylation of many cellular proteins (13). A role for protein tyrosine phosphorylation in the regulation of SMCE and actin polymerization has been proposed on the basis of the correlation between increases in phosphotyrosine levels and the filling state of the Ca\(^{2+}\)-stores, as well as the effects of different tyrosine kinase inhibitors on agonist and thapsigargin-evoked Ca\(^{2+}\) entry and actin polymerization (14–17). In agreement with previous studies (38, 41), we have found that cAMP- and cGMP-elevating agents inhibit agonist- or TG-stimulated tyrosine phosphorylation in dimethyl-BAPTA-loaded platelets. Therefore, we investigated whether protein-tyrosine phosphatases might be involved in the inhibitory effects of cAMP- and cGMP-elevating agents. Treatment of platelets with the protein-tyrosine phosphatase inhibitors, PAO and vanadate, prevented the effect of both PGE\(_1\) and SNP on actin polymerization and SMCE. We have found that PAO and vanadate do not impair agonist-evoked increases in phosphotyrosine levels. In addition, this treatment per se did not alter either basal or stimulated F-actin content or SMCE. These data suggest that the inhibitory effects of cAMP- and cGMP-elevating agents on actin polymerization and SMCE are mediated by the activation of tyrosine phosphatases. These findings are in agreement with earlier reports showing a role for tyrosine kinases in actin reorganization (17) and SMCE (14–16). Consistent with a recent study providing evidence that suggests that tyrosine kinases and Ras proteins have independent effects in the activation of SMCE in human platelets (17), we have found that treatment with PGE\(_1\) or SNP did not interfere with Ras activation or the Ras-activated P3K or ERK pathways. The effect of tyrosine phosphatases on cAMP and cGMP-induced inhibition of agonist-evoked Ca\(^{2+}\) mobilization is more complex. Our results indicate that tyrosine phosphatases are not involved in the effects of cyclic nucleotides on Ca\(^{2+}\) release from the intracellular stores, which appear to be mediated entirely through PKA and PKG. However, the effects of cAMP and cGMP on agonist-evoked elevations in Ca\(^{2+}\) (as a whole) that involves Ca\(^{2+}\) release and entry) are dependent on both cyclic nucleotide-dependent kinases, which regulate Ca\(^{2+}\) release, and tyrosine phosphatases, which are involved in the inhibition of Ca\(^{2+}\) entry. Thus, the effects of cAMP- and cGMP-elevating agents,
which are partially impaired by the inhibition of either of these pathways, are abolished by the inhibition of both. In conclusion, we report here for the first time that SMCE in human platelets is directly down-regulated by cAMP and cGMP by a mechanism involving the activation of protein-tyrosine phosphatases. Therefore, two different cyclic nucleotide-dependent mechanisms operate during the regulation of agonist-induced Ca²⁺ elevation: a PKA-/PKG- dependent mechanism for the inhibition of Ca²⁺ elevation: a PKA-/PKG- dependent mechanism for the inhibition of Ca²⁺ release and a PKA-/PKG-independent tyrosine phosphatase-dependent mechanism for the direct inhibition of Ca²⁺ entry. The findings that cAMP and cGMP-elevating agents prevent the activation but not in the maintenance of SMCE (17).

REFERENCES
