# Role of cyclic nucleotides in store-mediated external $Ca^{2+}$ entry in human platelets

Koji NAKAMURA, Masayuki KIMURA and Abraham AVIV*

Hypertension Research Center, University of Medicine and Dentistry of New Jersey, New Jersey Medical School, Newark, NJ 07103-2714, U.S.A.

This study explores the role of cyclic nucleotides (i.e. cyclic AMP and cyclic GMP) in store-regulated external $Ca^{2+}$ entry in human platelets. To stimulate store-regulated external $Ca^{2+}$ entry, thapsigargin was used to deplete $Ca^{2+}$ from the dense tubules, and sodium nitroprusside and iloprost respectively were used to stimulate endogenous cyclic GMP and cyclic AMP formation. Pretreatment with sodium nitroprusside and iloprost (a) attenuated the thapsigargin-evoked external $Ca^{2+}$ entry and (b) reduced the rate of $Ca^{2+}$ release from the dense tubules. The effects on external $Ca^{2+}$ entry and $Ca^{2+}$ release from the dense tubules were exerted independently and were apparently mediated through activation of the respective cyclic nucleotide-dependent protein kinases. Both sodium nitroprusside and iloprost reduced tyrosine kinase phosphorylation of a number of proteins, particularly a 72 kDa protein band. Both agents also attenuated the thapsigargin-evoked tyrosine kinase phosphorylation of the 72 kDa band. Intracellular $Ca^{2+}$ depletion resulted in a reduction in tyrosine kinase-mediated phosphorylation of a number of protein bands, including the 72 kDa band and the further attenuation of thapsigargin-mediated tyrosine phosphorylation of this band. The effects of the cyclic nucleotides on cellular $Ca^{2+}$ homoeostasis in thapsigargin-treated platelets were not exerted via acceleration of $Ca^{2+}$ extrusion or $Ca^{2+}$ sequestration into the mitochondria. We conclude that cyclic nucleotides participate in store-regulated control of external $Ca^{2+}$ entry by slowing down the rate of external $Ca^{2+}$ entry and $Ca^{2+}$ release from intracellular $Ca^{2+}$ stores. These effects are apparently mediated via cyclic nucleotide-dependent protein kinases and the attenuation of protein phosphorylation by tyrosine kinases.

## INTRODUCTION

Little is known about the mechanisms by which intracellular $Ca^{2+}$ stores regulate external $Ca^{2+}$ entry. Recent investigations have suggested that novel intracellular messengers are involved in transmitting the $Ca^{2+}$-filling status from intracellular organelles to the plasma membrane [1,2]. However, a substantial body of work indicates that in circulating platelets a variety of well-characterized intracellular signals may participate in the control of store-regulated external $Ca^{2+}$ entry, including second messengers such as inositol 1,4,5-trisphosphate [3,4], protein phosphorylation through families of $Ca^{2+}$-sensitive protein kinases (e.g. tyrosine kinase and protein kinase C) [5–8], cytosolic pH [9], the cytochrome *P*-450 system [10,11] and cyclic nucleotides [12–14]. Here we focus on the mechanisms by which cyclic nucleotides play a role in this process.

One approach to deciphering the roles of intracellular signals in store-regulated external $Ca^{2+}$ entry in platelets has been to monitor the effect of agonists that mobilize $Ca^{2+}$ from the dense tubules on specific cellular pathways that control $Ca^{2+}$ fluxes across the plasma membrane [7,8,10–12]. However, these $Ca^{2+}$-mobilizing agonists activate multiple cellular pathways, not all of which participate in the control of store-regulated external $Ca^{2+}$ entry. Another approach to the study of the relay mechanisms between the dense tubules and the plasma membrane has been to utilize agents that inhibit the dense-tubule $Ca^{2+}$-ATPase, e.g. thapsigargin and 2,5-di-(*t*-butyl)-1,4-benzohydroquinone [15]. By preventing $Ca^{2+}$ sequestration into the dense tubules, these agents produce a gradual emptying of $Ca^{2+}$ from these organelles and the transmission of cellular signals to open $Ca^{2+}$ channels on the plasma membrane. The use of these probes therefore circumvents the stimulation of multiple agonist-evoked cellular pathways that render it difficult to decipher the role of specific cellular perturbations in store-regulated external $Ca^{2+}$ entry. Using this approach, it became clear that in platelets, cyclic nucleotides [i.e. cyclic AMP (cAMP) and cyclic GMP (cGMP)] are involved in store-regulated external $Ca^{2+}$ entry [12]. Presumably, this effect is mediated via cAMP- and cGMP-dependent protein kinases [12,14] and perhaps by the inhibition of tyrosine kinases [6].

In the present work thapsigargin was used to address specific questions related to cyclic nucleotide-mediated control of store-regulated external $Ca^{2+}$ entry. First, we examined whether the effect of cyclic nucleotides is directly exerted by gating $Ca^{2+}$ channels on the plasma membrane, or indirectly by decelerating the rate of $Ca^{2+}$ release from intracellular organelles (presumably the dense tubules). Secondly, we re-examined whether the capacity of cyclic nucleotides to lower platelet cytosolic $Ca^{2+}$ is achieved by acceleration of $Ca^{2+}$ extrusion. Finally, we explored the role of mitochondria in the cyclic nucleotide-mediated effect on cytosolic free $Ca^{2+}$ $[Ca^{2+}]_i$. Our findings indicate that cyclic nucleotides slow down the rate of $Ca^{2+}$ release from the dense tubules and regulate external $Ca^{2+}$ entry by mechanisms that are independent of their effect on internal $Ca^{2+}$ stores. Moreover, we have found no evidence that in platelets these cellular messengers accelerate $Ca^{2+}$ extrusion across the plasma membrane or enhance $Ca^{2+}$ sequestration into the mitochondria.

Abbreviations used: cAMP, cyclic AMP; cGMP, cyclic GMP; $[Ca^{2+}]_i$, cytosolic free $Ca^{2+}$ concentration; SNP, sodium nitroprusside; BAPTA, bis-(*o*-aminophenoxy)ethane-*NNN'N'*-tetra-acetic acid; AM, acetoxymethyl ester; Sp-5,6-DCl-cBIMPS, adenosine 3′,5′-monophosphorothionate, Sp-isomer; 8-pCPT-cGMP, 8-(4-chlorophenylthio)guanisine 3′,5′-cyclic menophosphate; HBS, Hepes-buffered solution.

* To whom correspondence should be addressed.

## MATERIALS AND METHODS

### Materials

Thapsigargin (T-9033), sodium nitroprusside (SNP) (S-0501), ionomycin (I-0634), oligomycin B (O-5126), rotenone (R-8875), fatty acid-free BSA and Sepharose (CL-2B-300) were from Sigma, St. Louis, MO, U.S.A. Genistein (G-6055) and diadzien (D-2946) were obtained from LC Laboratories, Woburn, MA, U.S.A. Fura 2 acetoxymethyl ester (AM) and *bis*-(*o*-aminophenoxy)ethane-*NNN'N'*-tetra-acetic acid (BAPTA) AM came from Molecular Probes, Eugene, OR, U.S.A. Iloprost was a gift from Berlex Laboratories, Wayne, NJ, U.S.A. Adenosine 3',5'-cyclic monophosphorothionate, Sp-isomer (Sp-5,6-DCI-cBIMPS) (D-014) and 8-(4-chlorophenylthio)guanosine 3',5'-cyclic monophosphate (8-pCPT-cGMP) were purchased from Biolog, La Jolla, CA, U.S.A. Hyperfilm-ECL (RPN 2103) and enhanced chemiluminescence detecting agent (RPN 2106) were from Amersham Corp., Arlington, IL, U.S.A. Horseradish peroxidase-conjugated 4G10 anti-phosphotyrosine antibody (16-105) was obtained from UBI, Lake Placid, NY, U.S.A. $^{45}Ca^{2+}$ (specific radioactivity 12.7–18.7 mCi/mg of $Ca^{2+}$; NEZ-013) was from NEN–Dupont Biotechnology, Wilmington, DE, U.S.A.

### Platelet preparation

Blood (50 ml) was taken from the antecubital veins of healthy male volunteers, as approved by the institutional human investigation review board. It was taken into acid citrate/dextrose buffer (20:1, v/v) comprising (in mM): sodium citrate (14), citric acid (11.8) and dextrose (18) (final pH 6.5). Platelet-rich plasma was obtained by centrifugation at 200 *g* for 10 min (at room temperature). The platelet-rich plasma was centrifuged at 1000 *g* for 10 min and the pellet resuspended in $Ca^{2+}$-free buffer consisting of (in mM): NaCl (140), KCl (5), glucose (10), EGTA (0.3), Hepes (10) and 0.1 % BSA (pH 7.4). Aspirin (0.1 mM) was included in subsequent solutions (except for the solution used for the final fura 2 loading and $[Ca^{2+}]_i$ monitoring).

Platelets were isolated by filtration through Sepharose columns that were prewashed with 4 vol. (total 160 ml) of acetone followed by rinsing with 6 vol. of 0.9 % NaCl. Columns (2 cm × 8 cm) were supported by Spectra/Mesh macroporous nylon filters (53 $\mu$m pore size) in siliconized glass syringes. Each column was equilibrated with 150 ml of the $Ca^{2+}$-free Hepes buffer before use.

### Fura 2 loading and monitoring of $[Ca^{2+}]_i$ and external $Mn^{2+}$ entry

Gel-filtered platelets were incubated for 30 min in Hepes-buffered solution (HBS) comprising (in mM): NaCl (140), KCl (5), $CaCl_2$ (1), $MgCl_2$ (1), glucose (10), Hepes (10) plus 0.1 % BSA (pH 7.4) with 5 $\mu$M fura 2 AM. To remove the extracellular fura 2, 200 $\mu$l aliquots of platelets were centrifuged for 5 s (maximal centrifugation 3000 *g*) and resuspended in 3 ml of either 1 mM $Ca^{2+}$-HBS or $Ca^{2+}$-free HBS ($CaCl_2$ omitted and 0.3 mM EGTA added). $[Ca^{2+}]_i$ was monitored at 37 °C with constant stirring in SPEX Fluoromax (model DM 3000 F; SPEX Industries, Edison, NJ, U.S.A.). Excitation and emission wavelengths were respectively set at 340/380 and 505 nm. Fura 2 leakage from the platelets was assessed by observing the immediate fall in the fluorescence signal (at 358 nm, the isosbestic excitation wavelength) on the addition of 1 mM $MnCl_2$. $[Ca^{2+}]_i$ calibration was performed as described previously [16].

In most experiments in which the effects of pretreatment with iloprost or SNP on the thapsigargin-evoked $[Ca^{2+}]_i$ response were examined, platelets were preincubated with iloprost or SNP for 5 min in 200 $\mu$l aliquots during or after the fura 2 loading. After centrifugation to remove extracellular fura 2, platelets were injected into cuvettes containing 3 ml of HBS and subjected to experimental manipulations in the presence of iloprost, SNP or vehicles as described.

Genistein and daidzein quench fura 2 fluorescence [7]. However, genistein exerts no apparent effect on agonist-evoked $[Ca^{2+}]_i$ for several minutes [7]. Therefore, for experiments in which the effect of genistein on the $[Ca^{2+}]_i$ signal was examined, controls runs were performed with genistein added for 2 min of $[Ca^{2+}]_i$ monitoring after exposure to thapsigargin. These data are expressed as changes in the 340/380 nm fluorescence ratio.

$Mn^{2+}$ was used as a $Ca^{2+}$ surrogate to monitor the rate of $Ca^{2+}$ influx [17]. For these experiments, fura 2-loaded cells were treated as described in the various experimental protocols, and $MnCl_2$ (0.3–0.5 mM) was added. The excitation wavelength was set at 358 nm and the emission wavelength at 505 nm. Data are expressed as the percentage of the fluorescence intensity at the initiation of monitoring.

### $^{45}Ca^{2+}$ washout

Platelets were loaded with $^{45}Ca^{2+}$ for 120 min (at 37 °C) and subsequently gel-filtered on Sepharose columns eluted with HBS. Platelet suspensions in $Ca^{2+}$-HBS were incubated with or without the specific probes (i.e. thapsigargin, iloprost and SNP). Sequential 25 $\mu$l aliquots were then taken at 4 min intervals and rapidly filtered through HAWP filters (pore size 0.45 $\mu$m; Millipore) and rapidly washed with ice-cold $Ca^{2+}$-free HBS without glucose and BSA. Washout data were normalized and expressed as a percentage of the initial $^{45}Ca^{2+}$ content. There were no effects of the vehicle (0.01 % DMSO) on the washout data.

### Determination of tyrosine phosphorylation

Platelets subjected to the indicated experimental manipulations were mixed with an equal volume of Laemmli's buffer [18] and the mixture was boiled for 2 min. SDS/PAGE was performed using 7.5 % polyacrylamide gel in a Mini-Protean II electrophoresis cell (Bio-Rad, Hercules, CA, U.S.A.) at the 200 V setting (about 50 min). The proteins were transferred on to nitrocellulose transfer membranes (Hybond-ECL; Amersham Corp., Arlington Heights, IL, U.S.A.) using Mini Trans Blot Electrophoretic Transfer Cells (Bio-Rad) at 250 mA (75 min). The nitrocellulose membranes were washed three times (20 min each) with PBS containing 0.1 % BSA and 0.002 % Tween 20. The membranes were left in PBS/1 % BSA for 18 h and then incubated with horseradish peroxidase-conjugated 4G10 anti-phosphotyrosine antibody (0.75 $\mu$g/ml) in PBS for the next 20 h at 4 °C. The nitrocellulose membranes were washed three times (for 20 min each) with PBS, once with PBS/0.05 % Tween 20 (for 5 min) and rinsed with PBS (6 × 20 min). Enhanced chemiluminescence-detecting reagents were overlayed over the membrane for 1 min at room temperature. Blots were then exposed to Hyperfilm-ECL (Amersham Corp.).

Unless indicated, platelets from four subjects were used in the experimental protocols depicted in each Figure. Data, except for illustrations, are presented as means ± S.E.M. (vertical spikes or bars).

## RESULTS

To raise the endogenous cAMP and cGMP levels we used 10 nM iloprost and 100 $\mu$M SNP respectively. At these concentrations, the thapsigargin-evoked rise in $[Ca^{2+}]_i$ was maximally attenuated by both agents in both $Ca^{2+}$-HBS and $Ca^{2+}$-free HBS (not

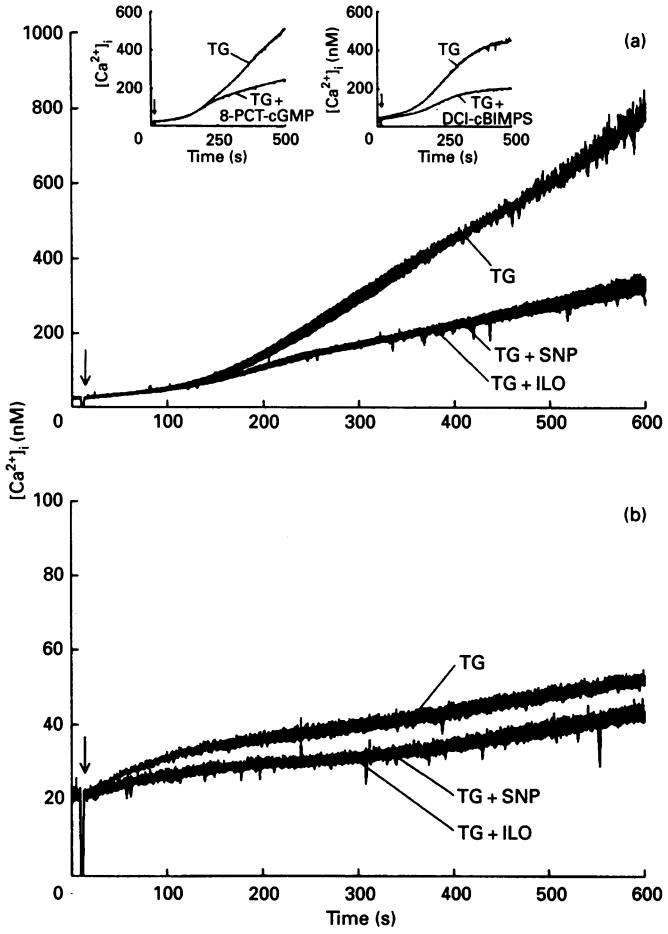


**Figure 1 Effects of 10 nM iloprost (ILO) and 100 $\mu$M SNP on the thapsigargin (TG)-evoked $[Ca^{2+}]_i$ response in $Ca^{2+}$-HBS (a) and $Ca^{2+}$-free HBS (b)**

Platelets were preincubated for 5 min with iloprost or SNP. Thapsigargin (250 nM) was added as indicated by the arrow. The inset in (**a**) illustrates the effects of 8-pCPT-cGMP (0.5 mM) and Sp-5,6-DCI-cBIMPS (0.2 mM) on the TG-evoked $[Ca^{2+}]_i$ response in $Ca^{2+}$-HBS. For these experiments, platelets were preincubated for 15 min with the cGMP-dependent protein kinase and the cAMP-dependent protein kinase activators. In both (**a**) and (**b**), the TG + SNP trace overlaps the TG + ILO trace. Spikes in the composites presented in (**a**) and (**b**) (except in the insets) denote S.E.M. The same applies for subsequent Figures depicting $[Ca^{2+}]_i$ signals.

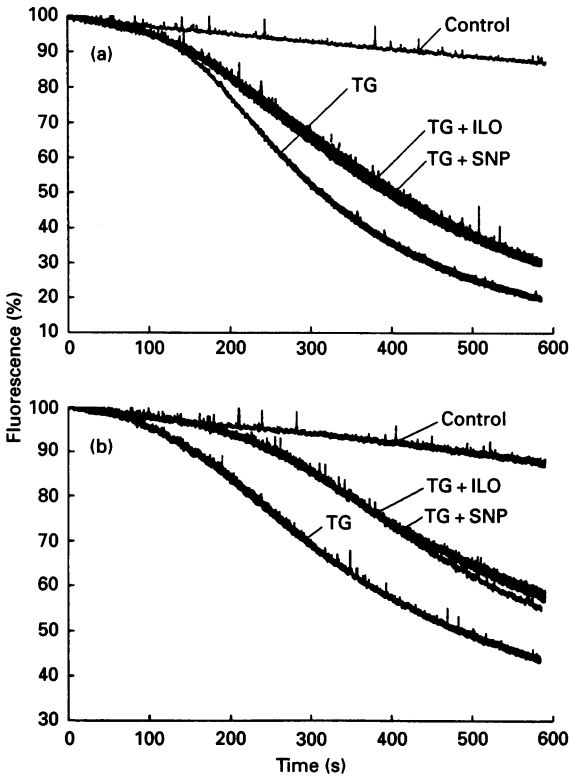


**Figure 2 Effects of iloprost (ILO) and SNP on the thapsigargin (TG)-evoked acceleration of $Mn^{2+}$ entry in $Ca^{2+}$-HBS (a) and $Ca^{2+}$-free HBS (b)**

Cells were preincubated for 5 min with 10 nM iloprost or 100 $\mu$M SNP, and thapsigargin (250 nM) was added at time = 0 s. Control platelets were treated with vehicle (DMSO). For monitoring of the rate of $Mn^{2+}$ entry, $MnCl_2$ (0.5 mM for **a** and 0. mM for **b**) was added at time = 0 s. In (**a**) the TG + SNP trace overlaps the TG + ILO trace. Data are expressed as percentage of the fluorescence at the initiation of monitoring.

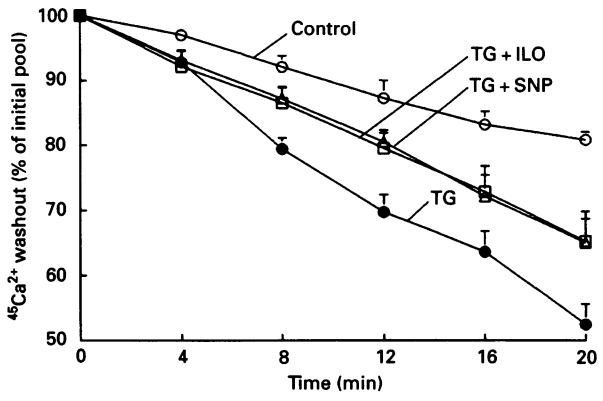


**Figure 3 Effects of thapsigargin (TG; 250 nM), iloprost (ILO; 10 nM), SNP (100 $\mu$M), and their combinations on $^{45}Ca^{2+}$ washout**

Platelets were loaded with $^{45}Ca^{2+}$ for 120 min and the various agonists or their combinations were added at time = 0 s. Control platelets were treated with DMSO.

shown), but the effects were more apparent in $Ca^{2+}$-HBS (Figures 1a and 1b). The specific cGMP-dependent protein kinase activator 8-pCPT-cGMP (0.5 mM) and cAMP-dependent protein kinase activator, Sp-5,6-DCI-cBIMPS (0.2 mM) attenuated the thapsigargin-evoked response in a manner indistinguishable from that of the respective cyclic nucleotides (inset, Figure 1a). As shown by monitoring $Mn^{2+}$ entry, iloprost and SNP exerted their effects by slowing down the rate of external $Ca^{2+}$ entry of thapsigargin-treated platelets in both $Ca^{2+}$-HBS and $Ca^{2+}$-free HBS (Figures 2a and 2b).

Figure 3 summarizes the effects of iloprost and SNP on the $^{45}Ca^{2+}$ extrusion in thapsigargin-treated platelets. Thapsigargin enhanced the rate of $^{45}Ca^{2+}$ extrusion, a process that was attenuated by either iloprost or SNP. As the overall effect of iloprost and SNP was to diminish the thapsigargin-evoked increase in $[Ca^{2+}]_i$ by inhibiting $Ca^{2+}$ release from intracellular organelles and blunting external $Ca^{2+}$ entry, it stands to reason that their effect on $Ca^{2+}$ extrusion was secondary in nature. In other words, by blunting the thapsigargin-evoked increase in $[Ca^{2+}]_i$, iloprost and SNP also diminished the rate of $Ca^{2+}$ extrusion. There was no effect of iloprost and SNP on $^{45}Ca^{2+}$ washout in platelets not treated with thapsigargin.

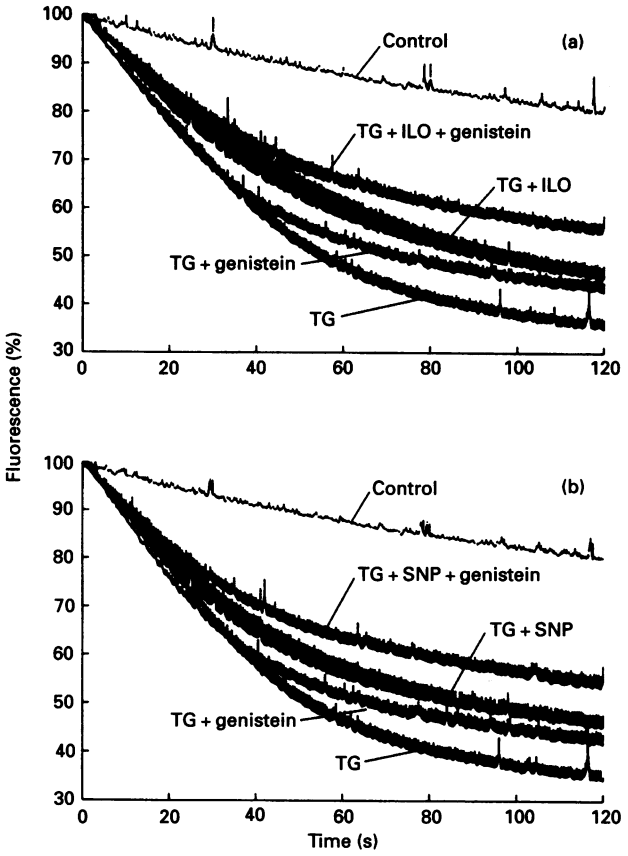


**Figure 4 Effects of thapsigargin (TG; 250 nM), iloprost (ILO; 10 nM), SNP (100 $\mu$M), genistein (100 $\mu$M) and their combinations on $Mn^{2+}$ entry in $Ca^{2+}$-HBS**

Platelets were preincubated with genistein for 30 min and with the other agents for 5 min. Thapsigargin was added at time = 0 s. Data are presented as percentage of the fluorescence at the initiation of monitoring. $MnCl_2$ (0.5 mM) was added at time = 0 s. Control cells were treated with DMSO.

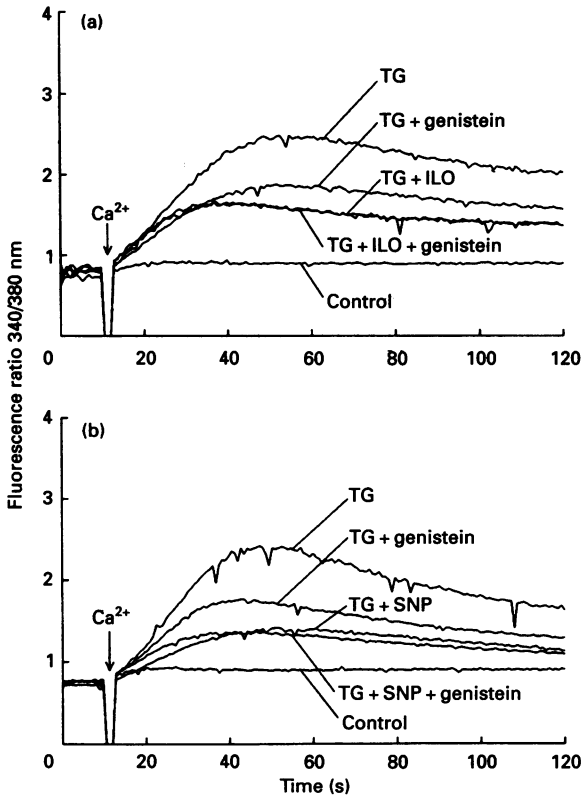


**Figure 5 Effects of iloprost (ILO; 10 nM), genistein (100 $\mu$M) and their combinations on the $[Ca^{2+}]_i$ response evoked by the addition of $Ca^{2+}$ to the external medium of thapsigargin (TG; 250 nM)-treated platelets**

Platelets were suspended in $Ca^{2+}$-free HBS and pretreated with genistein for 30 min and with the other agents for 5 min. $Ca^{2+}$ (final concentration 1 mM) was added as indicated by the arrow.

Recent studies have suggested that the stimulation of tyrosine kinases plays a role in agonist-evoked increase in external $Ca^{2+}$ entry [7,8]. We therefore examined the effect of genistein, an inhibitor of tyrosine kinase, on the thapsigargin-evoked increase in external $Ca^{2+}$ entry (as demonstrated by $Mn^{2+}$ entry) (Figures 4a and 4b). Genistein induced a decline in the thapsigargin-evoked increase in external $Ca^{2+}$ entry. Moreover, the effect of genistein was additive to those of iloprost and SNP. Monitoring the $[Ca^{2+}]_i$ profile in response to the introduction of $Ca^{2+}$ to the external medium in platelets pretreated with thapsigargin in $Ca^{2+}$-free HBS also showed that genistein attenuated the thapsigargin-evoked $[Ca^{2+}]_i$ response (Figures 5a and 5b). This effect of genistein was indistinguishable from that of iloprost or SNP. The difference between data in Figures 4 and 5 may be attributed to the fact that, whereas results presented in Figure 4 represent the effect of genistein on the rate of $Ca^{2+}$ entry, those in Figure 5 demonstrate the effect of genistein on the overall $[Ca^{2+}]_i$ which is a function of processes additional to $Ca^{2+}$ entry.

Several studies have demonstrated that cyclic nucleotides inhibit tyrosine phosphorylation [6,19], an observation confirmed in the present study. However, the profile of tyrosine phosphorylation is rather complex, as it depends on experimental conditions, including the type of agonists used [20] and duration of exposure of platelets to these agonists [5,6]. In the present work, thapsigargin treatment resulted in consistent tyrosine phosphorylation of a 72 kDa band. Pretreatment of platelets with iloprost resulted in diminished tyrosine phosphorylation and attenuation of tyrosine phosphorylation by thapsigargin of this specific band (Figures 6a–6c). Moreover, the effects of iloprost and genistein appeared to be additive. It is of interest to note that the thapsigargin effect was observed in both $Ca^{2+}$-HBS (Figure 6a) and $Ca^{2+}$-free HBS (Figure 6b). Further experiments, performed in platelets treated with 20 $\mu$M BAPTA (Figure 6c), showed that $[Ca^{2+}]_i$ plays a central role in tyrosine phosphorylation. By itself, BAPTA diminished tyrosine phosphorylation of a number of bands (e.g. 72, 110, 166 kDa; compare lanes 5–8 with lanes 1–4 in Figure 6c). More importantly, BAPTA attenuated the thapsigargin-evoked tyrosine phosphorylation of these protein bands. Platelets treated with SNP manifested the same profile of tyrosine phosphorylation as that of iloprost-treated platelets before and after treatment with thapsigargin (results not shown).

The above results suggest that cyclic nucleotides modify the thapsigargin-evoked $[Ca^{2+}]_i$ response, at least partially, by inhibiting tyrosine kinase-mediated mechanisms and that such an effect is exerted by slowing down $Ca^{2+}$ release from intracellular organelles and attenuating the rate of external $Ca^{2+}$ entry. Although cyclic nucleotides and genistein may inhibit a common pool of tyrosine kinases, other factors are involved, as

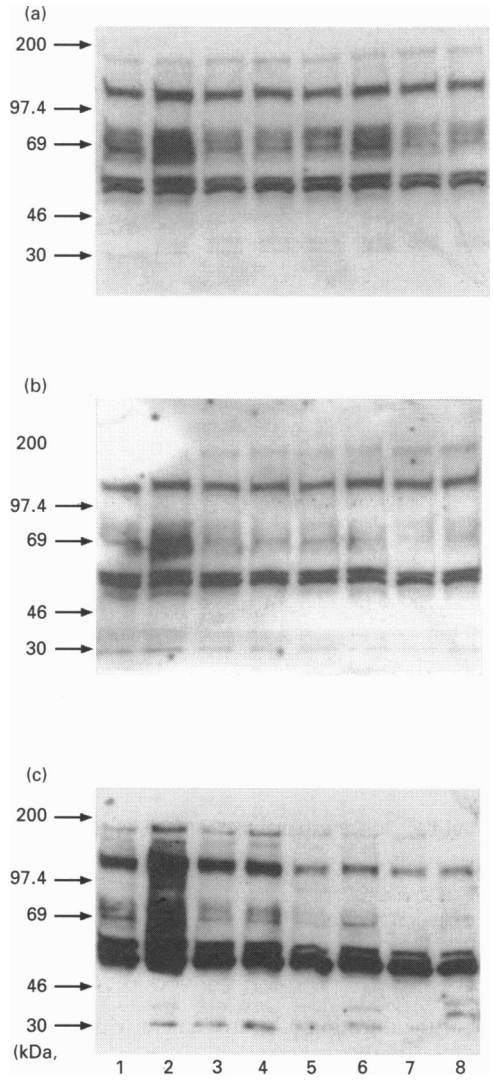


**Figure 6 Effects of iloprost (10 nM), genistein (100 μM) and their combination on thapsigargin-induced protein-tyrosine phosphorylation of platelets in $Ca^{2+}$-HBS (a), $Ca^{2+}$-free HBS (b) and in platelets that were further $Ca^{2+}$-depleted by treatment with BAPTA (c)**

Platelets were pretreated with genistein for 30 min and iloprost for 5 min and subsequently subjected to treatment with 250 nM thapsigargin for 5 min. Data are presented for immediately before (0 min) and 5 min after treatment with thapsigargin. For (**a**) and (**b**), lane 1, control platelets not exposed to thapsigargin; lane 2, control platelets after 5 min exposure to thapsigargin; lanes 3 and 4, genistein-pretreated platelets before and after 5 min exposure to thapsigargin respectively; lanes 5 and 6, iloprost-pretreated platelets before and after exposure to thapsigargin respectively; lanes 7 and 8, platelets pretreated with a combination of genistein and iloprost before and after 5 min exposure to thapsigargin respectively. Results in (**a**) and (**b**) demonstrate that the main target of thapsigargin-induced phosphorylation (at 5 min treatment) in $Ca^{2+}$-HBS and $Ca^{2+}$-free HBS is a 72 kDa protein. Iloprost and genistein diminished the steady-state phosphorylation of this protein band and attenuated its tyrosine phosphorylation by thapsigargin. For (**c**), lanes 1 and 2, control platelets in $Ca^{2+}$-HBS before and 5 min after exposure to thapsigargin respectively; lanes 3 and 4, platelets pretreated with iloprost before and 5 min after exposure to thapsigargin respectively; lanes 5 and 6, platelets pretreated with BAPTA in $Ca^{2+}$-free HBS before and 5 min after exposure to thapsigargin respectively; lanes 7 and 8, platelets pretreated with BAPTA and iloprost before and 5 min after exposure to thapsigargin respectively. These data show that pretreatment with BAPTA for 30 min in $Ca^{2+}$-free HBS resulted in a generalized reduction in protein-tyrosine phosphorylation, including the 72, 110 and 166 kDa bands and the attenuation of the thapsigargin-evoked tyrosine phosphorylation of the 72 kDa band. No effects of the vehicle (DMSO) or diadzien, an inactive analogue of genistein, were observed on resting or thapsigargin-evoked phosphorylation. Proteins were analysed by SDS/PAGE (7.5% gel) and subsequent immunoblotting with a monoclonal anti-phosphotyrosine antibody. Molecular markers (in kDa) are indicated on the left. Identical results were obtained in three additional experiments.

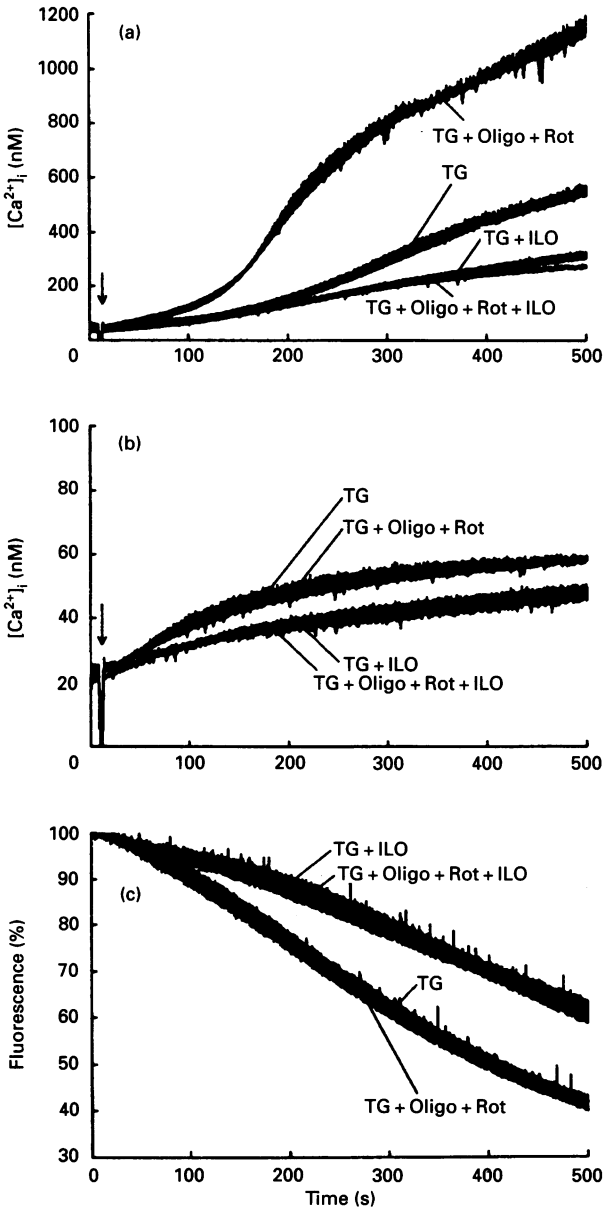


**Figure 7 Effects of oligomycin (Oligo; 3 μg/ml) plus rotenone (Rot; 2 μM) with or without iloprost (ILO; 10 nM) on the thapsigargin (TG)-evoked $Ca^{2+}$ response (a and b) and $Mn^{2+}$ entry (c) in $Ca^{2+}$-HBS (a and c) and $Ca^{2+}$-free HBS (b)**

Platelets were preincubated with iloprost for 5 min and exposed to thapsigargin. oligomycin and rotenone as indicated by the arrow for (**a**) and (**b**) and at time = 0 s for (**c**). For (**b**), the TG trace overlaps the TG + Oligo + Rot trace, and the TG + ILO trace overlaps the TG + Oligo + Rot + ILO trace. For (**c**) the TG + ILO trace overlaps the TG + Oligo + Rot + ILO trace, and the TG trace overlaps the TG + Oligo + Rot trace. Data for (**c**) are expressed as percentage fluorescence at the initiation of monitoring.

the effects of these agents on the role of $Mn^{2+}$ entry were additive (Figure 4). The effects of cAMP and cGMP may also be exerted by shifting $Ca^{2+}$ into intracellular compartments other than the dense tubules, e.g. mitochondria. This possibility was explored by using mitochondrial inhibitors, as depicted in Figure 7. In $Ca^{2+}$-HBS the mitochondria did play a role in the thapsigargin-evoked change in the $[Ca^{2+}]_i$ profile in that the increase in $[Ca^{2+}]_i$ was greater in the presence of the mitochondrial inhibitors

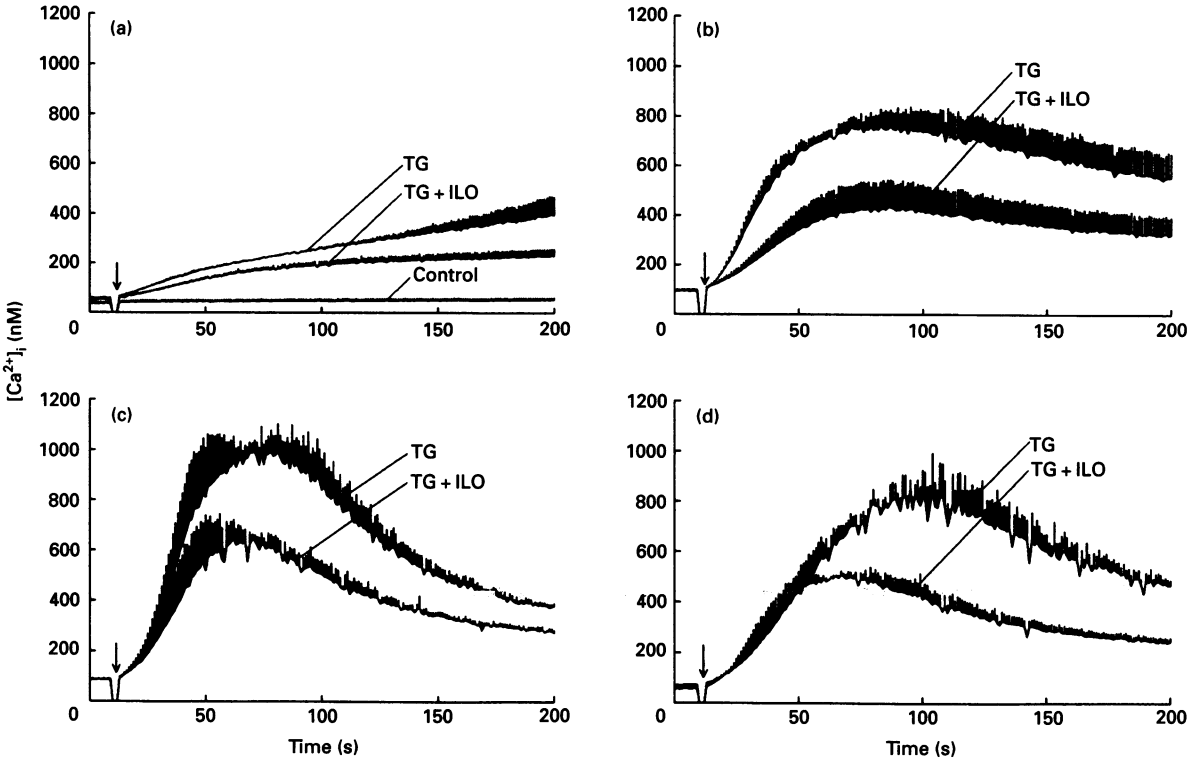


**Figure 8 Effect of $Ca^{2+}$ depletion on the thapsigargin (TG)-evoked response on the addition of $Ca^{2+}$ to the external medium and its relation to treatment with iloprost (ILO; 10 nM)**

Platelets were suspended in $Ca^{2+}$-free HBS with 250 nM thapsigargin for different time intervals: (**a**) 30 s; (**b**) 5 min; (**c**) 30 min; (**d**) 60 min. Iloprost (final concentration 10 nM) and $Ca^{2+}$ (final concentration 0.5 mM) were added to the medium as indicated by the arrow. The control in (**a**) was platelets not treated with thapsigargin.

oligomycin and rotenone than in their absence; this was not observed in $Ca^{2+}$-free HBS. The same results were obtained when the mitochondrial inhibitors were used in HBS minus glucose. These findings indicate that when $[Ca^{2+}]_i$ is sufficiently high, mitochondrial uptake of $Ca^{2+}$ occurs in thapsigargin-treated platelets. However, in $Ca^{2+}$-HBS, the attenuating effect of iloprost on the thapsigargin-evoked $[Ca^{2+}]_i$ response was exerted irrespective of the presence of the mitochondrial inhibitors. Inhibition of the mitochondria also did not alter the $Mn^{2+}$-entry response to iloprost in thapsigargin-treated platelets. Similar findings were obtained for SNP (results not shown). Thus cyclic nucleotides do not attenuate the thapsigargin-evoked rise in $[Ca^{2+}]_i$ by enhancing mitochondrial $Ca^{2+}$ uptake.

Together these findings indicate that the effect of cyclic nucleotides on the thapsigargin-evoked rise in $[Ca^{2+}]_i$ is exerted on two levels: they slow down $Ca^{2+}$ release from the dense tubules and they attenuate thapsigargin-evoked acceleration of external $Ca^{2+}$ entry. The question therefore arises whether the effect of cyclic nucleotides on external $Ca^{2+}$ entry is mediated by slowing down $Ca^{2+}$ release from the dense tubules. In other words, by decreasing the rate of $Ca^{2+}$ release in thapsigargin-treated platelets, cyclic nucleotides could promote a relatively greater retention of $Ca^{2+}$ in the dense tubules, resulting in attenuation of signals to open store-dependent $Ca^{2+}$ channels on the plasma membrane. The following experiment was performed to explore this possibility. Platelets were pretreated with thapsigargin in $Ca^{2+}$-free HBS (no iloprost) for periods ranging from 30 s to 60 min. $Ca^{2+}$ was then added to the medium (final concn. 0.5 mM) with or without iloprost. As shown (Figure 8), irrespective of the duration of pretreatment with thapsigargin in $Ca^{2+}$-free HBS, iloprost substantially attenuated the increase in $[Ca^{2+}]_i$ associated with the addition of $Ca^{2+}$ to the medium. Since progressive exposure to thapsigargin in $Ca^{2+}$-free HBS is expected to result in progressive depletion of $Ca^{2+}$ from the dense tubules, it is rather unlikely that, within the brief exposure to iloprost, the filling status of the dense tubules was sufficiently altered to modify significantly the signal relayed to the plasma membrane to enhance external $Ca^{2+}$ entry. Thus iloprost exerted its effect by a mechanism independent of its influence on $Ca^{2+}$ release from the dense tubles.

## DISCUSSION

The central finding of the present study is that the effect of cyclic nucleotides on store-regulated $Ca^{2+}$ entry is exerted on two levels. First, they slow down the rate of $Ca^{2+}$ release from intracellular organelles, and secondly, they act on the plasma membrane via unspecified mechanisms to decrease the rate of store-regulated external $Ca^{2+}$ entry. Both processes are presumably mediated via stimulation of cyclic nucleotide-dependent kinases. These effects may also be related to the ability of cyclic nucleotides to inhibit tyrosine kinases [6,19].

A number of studies have demonstrated that cyclic nucleotides antagonize agonist-evoked increases in platelet $[Ca^{2+}]_i$ [13,21]. Depending on the specific agonists and experimental conditions, this effect is exerted on different levels of the cellular signalling system which include: (a) decrease in receptor binding [22], (b) inhibition of G-protein-dependent stimulation of phospholipase

C [23]; (c) decrease in $Ca^{2+}$ mobilization [24], (d) decline in external $Ca^{2+}$ entry [12], and (e) stimulation of $Ca^{2+}$-ATPase on the plasma membrane [25,26]. The latest finding was demonstrated by monitoring the decay in the $[Ca^{2+}]_i$ signal of platelets treated with ionomycin (to raise $[Ca^{2+}]_i$) in the presence and absence of cyclic nucleotides. However, we [21] and others [27] have shown that, although cyclic nucleotides attenuate the agonist-evoked $[Ca^{2+}]_i$ response, they do not modify the ionomycin-induced $[Ca^{2+}]_i$ profile in human platelets. Moreover, in the present study, cyclic nucleotides reduced the rate of $^{45}Ca^{2+}$ extrusion in thapsigargin-treated platelets. The decrease in the rate of extrusion of $Ca^{2+}$ by these agents was probably a manifestation of the attenuation of the thapsigargin-evoked $[Ca^{2+}]_i$ signal. Thus, at present there is no clear consensus regarding a direct effect of cyclic nucleotides on $Ca^{2+}$-ATPase of the plasma membrane. It is still possible that cyclic nucleotides could stimulate the plasma-membrane $Ca^{2+}$-ATPase in concert with the reduction in $[Ca^{2+}]_i$ that arises from the blunting of $Ca^{2+}$ mobilization and/or attenuation of external $Ca^{2+}$ entry. The net result of their combined effect may not be expressed by enhanced $Ca^{2+}$ extrusion of thapsigargin-treated platelets.

Platelets are rich in tyrosine kinases [28]. Therefore the role of these enzymes in agonist-evoked platelet response and in cyclic nucleotide-dependent attenuation of this process has been previously explored. Both cAMP and cGMP can inhibit tyrosine phosphorylation in resting platelets and agonist-treated platelets [6,19]. There is also evidence showing that the stimulation of tyrosine kinases plays a role in agonist-evoked external $Ca^{2+}$ entry [7,8] and that tyrosine phosphorylation is involved in mediating store-regulated external $Ca^{2+}$ entry [29]. Furthermore, tyrosine kinase inhibitors attenuate thapsigargin-induced external $Ca^{2+}$ entry in platelets [12] and other cells [30]. Thus, although the tyrosine kinase system plays a role in store-regulated external $Ca^{2+}$ entry, the specific mechanisms by which this takes place are not fully understood. It is clear, nonetheless, that tyrosine kinase stimulation is a $Ca^{2+}$-dependent process, as shown in the present work and by others [5]. However, the $Ca^{2+}$-dependence of tyrosine kinase stimulation in intact cells was demonstrated only after intracellular $Ca^{2+}$ chelation, suggesting that a low threshold $[Ca^{2+}]$ concentration (not achieved by the acute removal of external $Ca^{2+}$) is sufficient for the expression of tyrosine kinase activity.

The effect of cyclic nucleotides on the $[Ca^{2+}]_i$ response may be exerted via mechanisms additional to that of tyrosine kinase inhibition, as the use of a combination of maximal concentrations of iloprost or SNP with genistein was additive with respect to the attenuation of thapsigargin-evoked external $Ca^{2+}$ ($Mn^{2+}$) entry. It is possible, however, that neither agent could totally inhibit the tyrosine kinase activity associated with store-regulated control of external $Ca^{2+}$. In this regard, the inhibitory effect of iloprost (and SNP) was additive to that of genistein with respect to the phosphorylation of the 72 kDa band. Whether this has any functional implication is uncertain.

Mitochondrial involvement in the attenuation by cyclic nucleotide of the thapsigargin-evoked response was ruled out in the present study. However, the mitochondria did play a central role in the final $[Ca^{2+}]_i$ profile, when platelets are exposed to thapsigargin in $Ca^{2+}$-HBS, as mitochondrial inhibition resulted in a dramatic increase in $[Ca^{2+}]_i$ to levels higher than those demonstrated in platelets with intact mitochondrial function. These organelles appear to play no apparent role in the thapsigargin-evoked changes in $[Ca^{2+}]_i$ in $Ca^{2+}$-free HBS, apparently because the $[Ca^{2+}]_i$ did not rise to a sufficiently high level to stimulate mitochondrial reaction.

Finally, the persistent effect of iloprost on the thapsigargin-evoked enhancement of external $Ca^{2+}$ entry, irrespective of the filling status of the intracellular $Ca^{2+}$ stores (Figure 8), suggests that cyclic nucleotides act on $Ca^{2+}$ release from intracellular $Ca^{2+}$ stores and external $Ca^{2+}$ entry via independent mechanisms. One cannot exclude the possibility, however, that cyclic nucleotides attenuate external $Ca^{2+}$ entry at various times after thapsigargin treatment by affecting different $Ca^{2+}$-entry and -extrusion pathways.

In conclusion, cyclic nucleotides play a role in store-regulated external $Ca^{2+}$ entry in platelets by at least two processes: they blunt $Ca^{2+}$ release from the dense tubules and attenuate external $Ca^{2+}$ entry. How these processes are related to each other and are integrated to other signals that convey the filling status of internal stores to the plasma membrane is not known.

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