cGMP inhibits IP_3-induced Ca^{2+} release in intact rat megakaryocytes via cGMP- and cAMP-dependent protein kinases

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1. Inhibition of inositol 1,4,5-trisphosphate (IP_3) receptor-mediated Ca^{2+} release by cGMP was examined in intact rat megakaryocytes, by using a combination of single cell fluorescence microscopy to monitor intracellular free calcium ($[Ca^{2+}]_i$) and flash photolysis of caged second messengers.

2. Sodium nitroprusside (SNP), a nitric oxide (NO) donor, and the hydrolysis-resistant cGMP analogue 8-(4-chlorophenylthio)guanosine 3′,5′-cyclic monophosphate (pCPT-cGMP) inhibited Ca^{2+} release induced by photolysis of caged IP_3. Neither of them affected the rate of Ca^{2+} removal from the cytoplasm following photolysis of caged Ca^{2+}.

3. Photolysis of the caged NO donor 3-morpholinosydnonimine (SIN-1) during agonist-induced $[Ca^{2+}]_i$ oscillations inhibited Ca^{2+} release without affecting the rate of Ca^{2+} uptake and/or extrusion.

4. We conclude that the inhibition of IP_3-induced Ca^{2+} release is the principal mechanism of NO–cGMP-dependent inhibition of $[Ca^{2+}]_i$ mobilization.

5. IPG, a specific peptide inhibitor of cGMP-dependent protein kinase (cGMP-PK), blocked the inhibitory effect of pCPT-cGMP, indicating that the inhibition of IP_3-induced Ca^{2+} release by pCPT-cGMP is mediated by cGMP-PK. However, the simultaneous application of both IPG and IP_{20}, a specific peptide inhibitor of cAMP-dependent protein kinase (cAMP-PK), was required to block the inhibitory effect of SNP. These data strongly suggest that NO–cGMP-dependent inhibition of $[Ca^{2+}]_i$ mobilization is mediated via the activation of both cGMP-PK and cAMP-PK.

Elevation of intracellular cGMP by endothelium-derived relaxing factor (EDRF) or nitric oxide (NO)-generating agents, as well as introduction of hydrolysis-resistant cGMP analogues, causes inhibition of platelet activation and relaxation of vascular smooth muscle. Although the precise mechanisms of cGMP-mediated inhibition are not well understood, it is clear that cGMP-dependent reduction of intracellular free calcium ($[Ca^{2+}]_i$) mobilization is a critical event involved in this inhibitory pathway (for review see Moncada *et al.* 1991; Walter *et al.* 1993; Lincoln *et al.* 1996; Clementi & Meldolesi, 1997).

Several mechanisms have been proposed to account for cGMP-dependent inhibition of $[Ca^{2+}]_i$ mobilization, including inhibition of IP_3 formation via inhibition of phospholipase C (PLC) or PLC–G-protein–receptor coupling (Deana *et al.* 1989; Hirata *et al.* 1990; Nguyen *et al.* 1991), stimulation of Ca^{2+} uptake and/or extrusion (Cornwell *et al.* 1991; Johansson & Haynes, 1992; Karczewski *et al.* 1992) and inhibition of Ca^{2+} entry (Geiger *et al.* 1992; Alioua *et al.* 1995). Recent studies point to the direct inhibition of Ca^{2+} release from the endoplasmic reticulum as a possible molecular mechanism that mediates the inhibitory effect of cyclic nucleotide-elevating agents on calcium mobilization (Cavallini *et al.* 1996; Komalavilas & Lincoln, 1996; Quinton *et al.* 1996). In this context the IP_3 receptor has been found to be phosphorylated by cyclic nucleotide-dependent protein kinases in platelets and smooth muscle cells and its phosphorylation was associated with the cAMP- and cGMP-mediated inhibition of $[Ca^{2+}]_i$ accumulation (Cavallini *et al.* 1996; El-Daher *et al.* 1996; Komalavilas & Lincoln, 1996). However, whether phosphorylation of the IP_3 receptor actually regulates its ability to release calcium in the living cell has not yet been established.

The present experiments were designed to investigate whether elevation of intracellular cGMP actually inhibits IP_3-induced Ca^{2+} release in an intact cell, and if so whether this inhibition requires activation of cyclic nucleotide-dependent protein kinases. In order to test this possibility we used flash photolysis of caged second messengers and selective inhibitors of cGMP- and cAMP-dependent protein

kinases (cGMP-PK and cAMP-PK). This approach has proved valuable in studying the process of intracellular signalling in other cells types (Adams & Tsien, 1993). Previously we have reported that in megakaryocytes, the progenitor of platelets, agonist-induced $[Ca^{2+}]_i$ oscillations are reversibly inhibited by agents that elevate intracellular cGMP and cAMP (Tertyshnikova & Fein, 1997). The present study provides direct evidence that elevation of cGMP inhibits IP_3-induced Ca^{2+} release in intact cells, but does not affect the rate of Ca^{2+} removal from the cytoplasm. Furthermore our results indicate that cGMP-dependent inhibition of IP_3-induced Ca^{2+} release is mediated by both cGMP-PK and cAMP-PK.

METHODS

Cell preparation and loading

Megakaryocytes were obtained from adult Wistar rats as previously described (Tertyshnikova & Fein, 1997). Briefly, bone marrow was obtained from rats anaesthetized by inhalation of an overdose of isoflurane and then killed by decapitation. This procedure is in accordance with University of Connecticut Health Centre guidelines. After filtration through a 75 μm nylon mesh to eliminate large masses of cells, the bone marrow suspension was spun and washed twice before incubation in standard external solution of composition (mM): 140 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 glucose, 10 Hepes, pH 7·4, supplemented by 0·1 % bovine serum albumin (BSA). Megakaryocytes were clearly distinguished from other bone marrow cells on the basis of their large size (25–50 μm) and multilobular nucleus. All experiments were done within 2–6 h after preparation at room temperature (23–25 °C).

'Cell permeant' AM esters of *o*-nitrophenyl EGTA (caged calcium) and Oregon Green 488 BAPTA-1 (OGB488) were dissolved in DMSO, stored at −20 °C and applied at 10–30 and 2·5–5 μM, respectively. For intracellular loading, megakaryocytes were resuspended in standard external solution containing caged calcium (the final concentration of DMSO was less then 0·1 %) and incubated for at least 2 h. Then cells were transferred onto glass coverslips and were additionally incubated with OGB488 AM for 30 min. The coverslips with adherent cells were then washed several times with the standard external solution and kept in the dark until use to avoid unwanted photolysis of the caged calcium.

Agonist application

Activators and inhibitors (ADP, pCPT-cGMP, carbacyclin, SNP, or the mixture of ADP with one of the others) were dissolved in the standard external solution and applied directly to single megakaryocytes using a DAD-6 computer controlled local superfusion system (ALA Scientific Instruments Inc., Westbury, NY, USA). The output tube of the micromanifold (100 μm inside diameter) was placed approximately within 200 μm of the cell, and the puff pressure was adjusted to achieve rapid agonist application while avoiding any mechanical disturbance of the cell. The time delay for arrival of agonists at the cell was measured and accounted for in the figures. For the experiments with caged SIN-1, the caged NO donor was added to the bath solution at a final concentration of 0·5 mM.

Caged IP_3, IPG, IP_{20} and OGB488 (hexapotassium salt, 'cell impermeant'), were included in the intrapipette solution at 100, 100, 100 and 200 μM, respectively, (solution composition, mM: 20 KCl, 120 potassium glutamate, 1 $MgCl_2$, 2 Na-GTP, 10 Hepes, pH 7·3). Standard whole-cell patch-clamp recording techniques were used to voltage clamp and internally dialyse single megakaryocytes. Membrane current was monitored using an Axopatch-1D patch clamp amplifier (Axon Instruments). Recording pipettes were pulled from 1·5 mm borosilicate glass (no. 7052; Garner Glass Inc., Claremont, CA, USA) using a two-stage Narishige PB-7 vertical puller and then fire-polished on a Narishige MF-9 microforge. Pipette resistance was between 1 and 5 MΩ and the holding potential was −45 mV. For most cells 5–8 min was required for the OGB488 fluorescence signal to equilibrate in the patch clamped cell. Because of variability in cell responses to IP_3, for every cell after equilibration of the intracellular and the pipette solution, the duration of the UV light pulse was adjusted to produce an IP_3-induced rise in $[Ca^{2+}]_i$ similar in magnitude and time course to that resulting from the agonist application. In control experiments we were able to use up to ten to fifteen IP_3-releasing UV flashes without any significant degradation of the $[Ca^{2+}]_i$ response.

Fluorescence measurement and flash photolysis

Megakaryocytes were viewed through a coverslip forming the bottom of the recording chamber using a Nikon Diaphot microscope equipped with a Nikon Fluor ×100, 1·3 NA oil-immersion lens. Single cell fluorometry was accomplished using an IonOptix photon counting fluorescence subsystem (designed by D. Tillotson, IonOptix, Milton, MA, USA) as previously described (Tertyshnikova & Fein, 1997), except that in these experiments we used OGB488 as the Ca^{2+} indicator instead of fura-2. For OGB488 excitation light was delivered from one channel of the chopper based dual excitation 75 W xenon arc light source via the light guide. Excitation and emission were centred on 485 nm and 535 nm, respectively. For caged SIN-1 and caged IP_3 photolysis, pulses of ultraviolet light (290–370 nm) were applied to the cell through the second channel of the dual excitation light source. Caged calcium photolysis was produced by a 1 ms flash from an XF-10 high intensity xenon flash lamp (Hi-Tech Scientific, Salisbury, UK), focused through a 280–360 nm wide band filter from about 2·5 cm above the cell to produce a 2–3 mm spot. The flash energy was regulated by controlling the charging voltage of the capacitor bank used to fire the flash lamp. Fluorescence intensity was measured on-line using the PIA program (IonOptix). The time resolution was set at 0·1 s by averaging three points to obtain a better signal-to-noise ratio.

Chemicals

Oregon Green 488 BAPTA-1 AM (hexapotassium salt), NVOC-caged SIN-1 (*N*-(((4,5-dimethoxy-2-nitrobenzyl)oxy)carbonyl)-3-morpholinosydnoneimine) and caged NO (potassium nitrosylpentachlororuthenate) were obtained from Molecular Probes Inc. Caged IP_3, *o*-nitrophenyl EGTA AM and caged cGMP (guanosine 3′,5′-cyclic monophosphate, P-1-(2-nitrophenyl)ethyl ester) were from Calbiochem. pCPT-cGMP (8-(4-chlorophenylthio)guanosine 3′,5′-cyclic monophosphate) was purchased from BioLog Life Science Institute (La Jolla, CA, USA). ODQ (1H-[1,2,4]oxadiazolo[4,3,-a]-quinoxalin-1-one) was obtained from Tocris Cookson Inc. (Ballwin, MO, USA). All other reagents were purchased from Sigma Chemical Co. Peptide inhibitor of cGMP-dependent protein kinase (IPG) (Leu-Arg-Arg-Arg-Arg-Phe-D-Ala-Phe-NH_2) (Yan *et al.* 1996) was synthesized in D. S. Lawrence's laboratory (The Albert Einstein College of Medicine, Bronx, NY, USA).

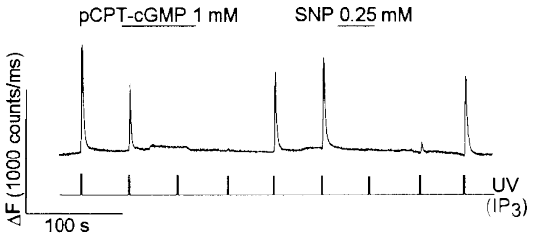

Figure 1. pCPT-cGMP and SNP inhibit the IP_3-induced rise in $[Ca^{2+}]_i$ in megakaryocytes

Caged IP_3 (100 μM) and OGB488 (200 μM) were included in the patch pipette solution. In this and all other figures changes in $[Ca^{2+}]_i$ were monitored by measuring OGB488 fluorescence intensity and expressed as ΔF(counts ms^{-1}). IP_3 was released by a pulse of UV light from the chopper based dual excitation light source, at the times indicated. SNP (0·25 mM) and pCPT-cGMP (1 mM) were applied to the cell via the local superfusion system as described in Methods.

RESULTS

Inhibition of IP_3-induced Ca^{2+} release by pCTP-cGMP and SNP

To determine whether an elevation of intracellular cGMP inhibits IP_3-induced Ca^{2+} release we first performed the experiment shown in Fig. 1. Caged IP_3 and OGB488 were included in the patch pipette (see Methods). $[Ca^{2+}]_i$ spikes produced by IP_3-releasing flashes in cells bathed at least 1 h in Ca^{2+}-free solution, containing 1 mM BAPTA, were similar to those obtained from megakaryocytes bathed in standard external solution, indicating that these IP_3-induced $[Ca^{2+}]_i$ spikes were the result of IP_3-induced Ca^{2+} release and not Ca^{2+} influx ($n = 4$ cells, data not shown).

To elevate intracellular cGMP we used either pCPT-cGMP, a membrane-permeant hydrolysis-resistant analogue of cGMP (Butt *et al.* 1992), or sodium nitroprusside (SNP), a NO donor that increases the intracellular level of cGMP through the activation of soluble guanylyl cyclase (for review see Buechler *et al.* 1994). Previous experiments have demonstrated that pCPT-cGMP and SNP reversibly inhibit agonist-induced $[Ca^{2+}]_i$ oscillations in megakaryocytes (Tertyshnikova & Fein, 1997). As shown in Fig. 1, application of either pCPT-cGMP or SNP reversibly inhibited the rise in $[Ca^{2+}]_i$ induced by photolysis of caged IP_3 ($n = 15$ cells).

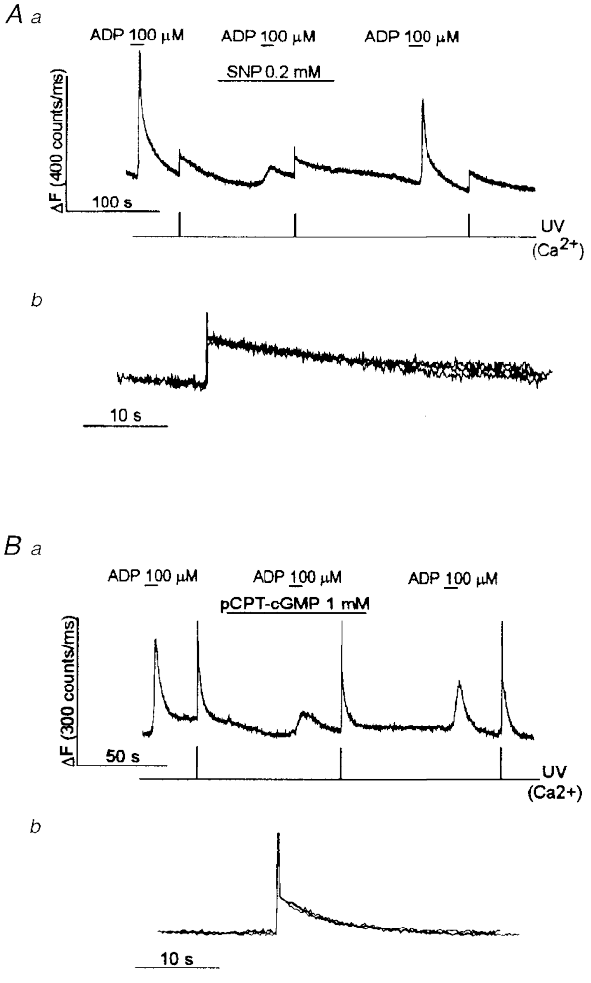

Figure 2. SNP and pCPT-cGMP reversibly inhibit Ca^{2+} mobilization by ADP without affecting the rate of Ca^{2+} removal from the cytoplasm

SNP (*A*) and pCPT-cGMP (*B*) reversibly inhibited ADP-induced Ca^{2+} mobilization, but had no effect on the time course of the fall in $[Ca^{2+}]_i$, following photolysis of caged Ca^{2+}. SNP (0·2 mM), pCPT-cGMP (1 mM) and ADP (100 μM) were applied as shown. In *Ab* and *Bb*, the $[Ca^{2+}]_i$ spikes resulting from photorelease of caged-Ca^{2+} in *Aa* and *Ba*, respectively, are shown superimposed on an expanded time scale. Ca^{2+} was released by a 1 ms flash of UV light from the xenon flash lamp at the times indicated (see Methods).

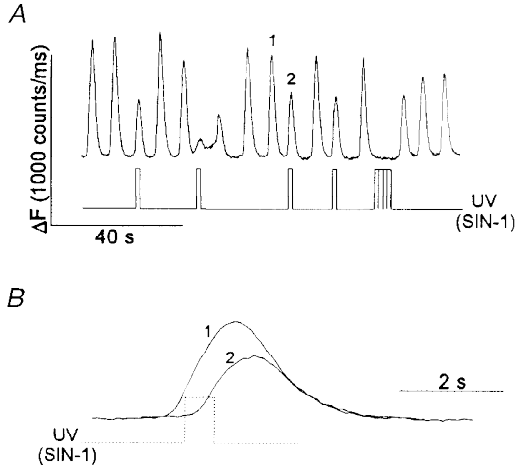

Figure 3. Photolysis of caged SIN-1 causes inhibition of ADP-induced $[Ca^{2+}]_i$ oscillations without affecting the kinetics of the falling phase of the individual $[Ca^{2+}]_i$ spikes
Megakaryocytes were loaded with OGB488 AM as described in Methods. Caged SIN-1 was added to the bath solution at a final concentration of 0·5 mM. *A*, $[Ca^{2+}]_i$ oscillations were induced by 100 μM ADP as shown. SIN-1 was released by a 0·5 s pulse of UV light at the times indicated. *B*, the $[Ca^{2+}]_i$ spikes labelled 1 and 2 in *A* are shown superimposed on an expanded time scale. The stepped line indicates the time of occurrence of the UV flash (SIN-1 release) during spike 2 (dotted line).

To investigate whether the inhibition of the IP_3-induced rise in $[Ca^{2+}]_i$ by pCPT-cGMP and SNP involves the stimulation of Ca^{2+} uptake and/or extrusion we used caged Ca^{2+} for the experiment in Fig. 2. The time course of the fall in $[Ca^{2+}]_i$, following the flash-induced rise in $[Ca^{2+}]_i$, reflects the activity of Ca^{2+} sequestration and/or extrusion mechanisms. Ca^{2+} was photoreleased following a short application of adenosine 5′-diphosphate (ADP) that served as a positive control for the inhibitory effect of SNP or pCPT-cGMP. ADP has been shown to mobilize Ca^{2+} from intracellular stores in platelets (MacKenzie *et al.* 1996) and megakaryocytes (Uneyama *et al.* 1993) presumably through formation of IP_3 as occurs in many other cell types. As can be seen in Fig. 2, both pCPT-cGMP ($n = 6$ cells) and SNP ($n = 7$ cells) reversibly inhibited the ADP-induced rise in $[Ca^{2+}]_i$ while photoreleased $[Ca^{2+}]_i$ declined with the same rate before, during and after application of SNP and pCPT-cGMP.

The experiment shown in Fig. 3 provides an additional test of whether or not elevation of cGMP stimulates Ca^{2+} removal from the cytoplasm. In these experiments we used SIN-1, a donor of NO, and a potent vasorelaxant and anti-thrombotic agent (for review see Feelisch & Stamler, 1996). Caged SIN-1 uncages in milliseconds after UV illumination to release SIN-1 which then spontaneously decomposes to release NO. This allows precise temporal control of the Ca^{2+} signal by SIN-1. We have previously reported that the falling phase of each $[Ca^{2+}]_i$ spike that makes up the agonist-induced $[Ca^{2+}]_i$ oscillation in megakaryocytes results from Ca^{2+} uptake and/or extrusion (Tertyshnikova & Fein, 1997). When SIN-1 was uncaged during the rising phase of a $[Ca^{2+}]_i$ spike, further Ca^{2+} release was inhibited and the $[Ca^{2+}]_i$ spike aborted (spike 2 in Fig. 3*A*) ($n = 4$ cells). However, photolysis of SIN-1 did not cause any change in the time course of the falling phase of the spike (Fig. 3*A*). In Fig. 3*B* the $[Ca^{2+}]_i$ spikes labelled 1 and 2 from Fig. 3*A* are shown superimposed on an expanded time scale. It can be seen that SIN-1 inhibits the development of the $[Ca^{2+}]_i$ spike without affecting its falling phase. Similar results were obtained using caged NO (potassium nitrosylpentachloro-ruthenate) ($n = 3$ cells) and caged cGMP ($n = 8$ cells) (data not shown).

Taken together, the results in Figs 1–3 indicate that the principal mechanism of NO–cGMP-dependent inhibition of Ca^{2+} mobilization in megakaryocytes is by inhibition of IP_3-induced Ca^{2+} release and not by stimulation of Ca^{2+} removal from the cytoplasm.

As mentioned above NO donors are thought to increase the intracellular level of cGMP via the activation of soluble guanylate cyclase (GC). However, in our experiments ODQ (1H-[1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one), a selective inhibitor of soluble guanylate cyclase (Moro *et al.* 1996), even at very high concentrations (10–100 μM), only partially blocked inhibition of agonist-induced $[Ca^{2+}]_i$ oscillations by SNP in 2 out of 7 cells (data not shown). This finding may be due to a insufficient inhibition of guanylate cyclase by ODQ.

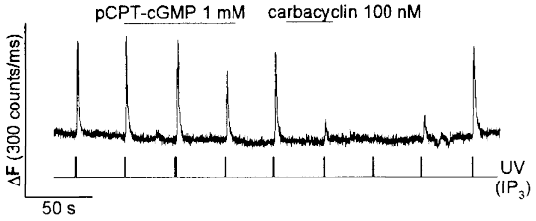

Figure 4. IPG prevents inhibition of IP_3-induced Ca^{2+} release by pCPT-cGMP, but not by carbacyclin
IPG (100 μM), caged IP_3 (100 μM) and OGB488 (200 μM) were included in the intrapipette solution. $IP_3$was released by UV flashes as indicated. Carbacyclin (100 nM) and pCPT-cGMP (1 mM) were applied to the cell as indicated.

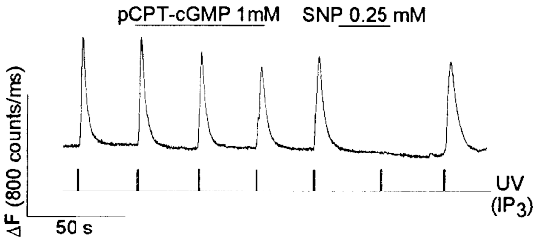

Figure 5. IPG prevents inhibition of IP_3-induced Ca^{2+} release by pCPT-cGMP, but not by SNP

IPG (100 μM), caged IP_3 (100 μM) and OGB488 (200 μM) were included in the intrapipette solution. $IP_3$was released by UV flashes as indicated. SNP (0·25 mM) and pCPT-cGMP (1 mM) were applied to the cell as indicated.

The inhibition of IP_3-induced Ca^{2+} release by pCPT-cGMP is mediated by cGMP-dependent protein kinase

If the inhibition of IP_3-induced Ca^{2+} release by cGMP is mediated by cGMP-PK, it should be possible to block the inhibitory effect of cGMP by inhibiting cGMP-PK. When 100 μM IPG, a specific peptide inhibitor of cGMP-PK (Yan *et al.* 1996), was added to the pipette solution together with caged IP_3 and OGB488, pCPT-cGMP failed to inhibit IP_3-induced Ca^{2+} release ($n = 10$ cells) (Fig. 4). As a control for the specificity of IPG action we used carbacyclin, a chemically stable analogue of prostacyclin (PGI_2) (Whittle *et al.* 1980), which is known to elevate intracellular cAMP through a G_s protein-dependent activation of adenylate cyclase (for review see Wise & Jones, 1996). Previous experiments have demonstrated that PGI_2 inhibits both agonist-induced $[Ca^{2+}]_i$ oscillations and IP_3-induced Ca^{2+} release in megakaryocytes (Tertyshnikova & Fein, 1997, 1998). Assuming, that the effect of cAMP is mediated by cAMP-dependent protein kinase, it would be expected that IPG would not prevent the inhibitory effect of carbacyclin. Indeed, as shown in Fig. 4, carbacyclin reversibly inhibited IP_3-induced Ca^{2+} release ($n = 4$ cells) (similar results with carbacyclin were seen in 11 other cells without IPG in the pipette). In summary, we conclude that inhibition of IP_3-induced Ca^{2+} release by pCPT-cGMP is mediated by cGMP-dependent protein kinase.

The inhibition of IP_3-induced Ca^{2+} release by SNP is mediated by both cGMP- and cAMP-dependent protein kinases

If the suppression of IP_3-induced Ca^{2+} release by SNP involves only cGMP-dependent stimulation of cGMP-PK, then inhibition of cGMP-PK should block the inhibitory effect of both pCPT-cGMP and SNP. However, when SNP was applied to a cell in which cGMP-PK had been inhibited by IPG and pCPT-cGMP no longer suppressed IP_3-induced Ca^{2+} release, SNP still reversibly inhibited IP_3-induced Ca^{2+} release (Fig. 5, $n = 7$ cells) (separate experiments showed that 0·2 mM SNP and 1 mM pCPT-cGMP were near the lowest concentrations which inhibited IP_3-induced Ca^{2+} release in megakaryocytes).

It has been reported that SNP can cause an increase in platelet cAMP even in the absence of adenylate cyclase activation due to the inhibition of cAMP degradation by cGMP-inhibited cAMP phosphodiesterase (PDE III) (Maurice & Haslam, 1990). On the other hand, we have previously shown that inhibition of cAMP-PK with IP_{20}, a specific peptide inhibitor of the catalytic subunit of cAMP-PK (Cheng *et al.* 1986), does not block the inhibitory effect of SNP (Tertyshnikova & Fein, 1998). In view of the above findings we tested the possibility that the inhibition of the IP_3-induced Ca^{2+} release by SNP involves the activation of both cGMP-PK and cAMP-PK. When both 100 μM IPG and 100 μM IP_{20} were added to the pipette solution together with caged IP_3 and OGB488, SNP failed to inhibit IP_3-induced Ca^{2+} release at the concentration range of 0·25–0·5 mM, and caused only partial inhibition at 1–5 mM ($n = 11$ cells) (Fig. 6). These data strongly suggest that SNP exerts its inhibitory effect via the activation of both cGMP-PK and cAMP-PK.

DISCUSSION

The data reported in this article provide the first direct demonstration that in the intact cell the NO–cGMP-dependent inhibition of Ca^{2+} release from IP_3-sensitive stores requires the activation of both cAMP-PK and cGMP-PK.

Although it is usually assumed that cGMP-mediated inhibition of $[Ca^{2+}]_i$ mobilization occurs predominantly via the activation of cGMP-PK (Butt *et al.* 1992; Geiger *et al.*

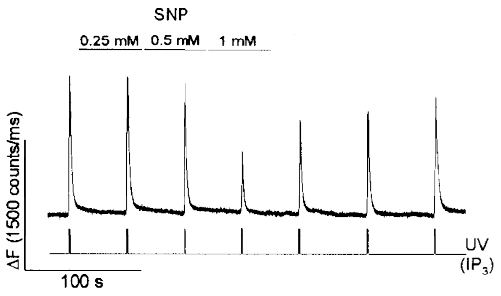

Figure 6. Simultaneous application of IP_{20} and IPG prevent inhibition of IP_3-induced Ca^{2+} release by SNP

IPG (100 μM), IP_{20} (100 μM), caged IP_3 (100 μM) and OGB488 (200 μM) were included in the intrapipette solution. $IP_3$was released by UV flashes as indicated. SNP (0·25, 0·5 and 1 mM) were applied to the cell as indicated.

1992; for review see Walter *et al.* 1993; Lincoln *et al.* 1996), it has also been reported that at least in part cGMP exerts its inhibitory effect via inhibition of cAMP breakdown due to inhibition of PDE III (Maurice & Haslam, 1990; for review see Beavo, 1995). Studies have shown that cGMP-elevating agents potentiate the elevation of cAMP by prostaglandins when both cAMP and cGMP elevating agents are present at low concentrations (Radomski *et al.* 1987; De Caterina *et al.* 1988; Maurice & Haslam, 1990; Bowen & Haslam, 1991). Furthermore, the nitrovasodilators (SNP and SIN-1) cause increases in platelet cAMP level even in the absence of adenylate cyclase activators and this effect is due to the inhibition of PDE III (Maurice & Haslam, 1990), the major phosphodiesterase isozyme present in platelets and vascular smooth muscles (for review see Beavo, 1995). We have previously reported that in rat megakaryocytes inhibition of cAMP breakdown by the non-specific phosphodiesterase inhibitor 3-isobutyl-1-methyl-xanthine (IBMX) leads to inhibition of ATP-induced $[Ca^{2+}]_i$ oscillations (Tertyshnikova & Fein, 1997). Therefore, we interpret our present results as evidence that NO donors exert their inhibitory effect via a direct elevation of cGMP and a secondary elevation of cAMP, mediated by the inhibition of PDE III.

Although pCPT-cGMP is a potent activator of cGMP-PK (Butt *et al.* 1992; Geiger *et al.* 1992), unlike cGMP or other hydrolysis-resistant cGMP analogues, such as 8-Br-cGMP, pCPT-cGMP has little or no effect on PDE III (Butt *et al.* 1992). This can explain our apparently contradictory findings that pCPT-cGMP inhibited Ca^{2+} release via the activation of cGMP-PK, whereas inhibition by SNP required the activation of both cGMP-PK and cAMP-PK (see Fig. 7).

Recently we have reported that in rat megakaryocytes elevation of cAMP by carbacyclin leads to the activation of cAMP-PK and thereby to the inhibition of IP_3-induced Ca^{2+} release (Tertyshnikova & Fein, 1998). Our present findings imply that the activation of both cGMP-PK and cAMP-PK is required for the inhibition of IP_3-induced Ca^{2+} release by NO-generating agents. These findings are summarized by the simplified diagram in Fig. 7. For simplicity we show cAMP-PK and cGMP-PK as directly inhibiting the IP_3 receptor (IP_3-R), although we have no evidence that the inhibition is not mediated via another protein that is phosphorylated by cyclic nucleotide-dependent protein kinases and that regulates IP_3-R function.

The role of cyclic nucleotide-dependent protein kinases in regulating Ca^{2+} release by the IP_3-R has been studied using the purified receptor. Both cGMP-PK and cAMP-PK catalyse the phosphorylation of serine 1755 on the IP_3-R, and in addition, cAMP-PK phosphorylates serine 1589 (Ferris *et al.* 1991; Komalavilas & Lincoln, 1994). However, whether phosphorylation of the purified IP_3-R in reconstituted systems potentiates or inhibits its ability to release Ca^{2+} remains controversial (Supattapone *et al.* 1988; Nakade *et al.* 1994).

The IP_3-R from platelets has also been found to be a substrate for both cGMP and cAMP-dependent protein

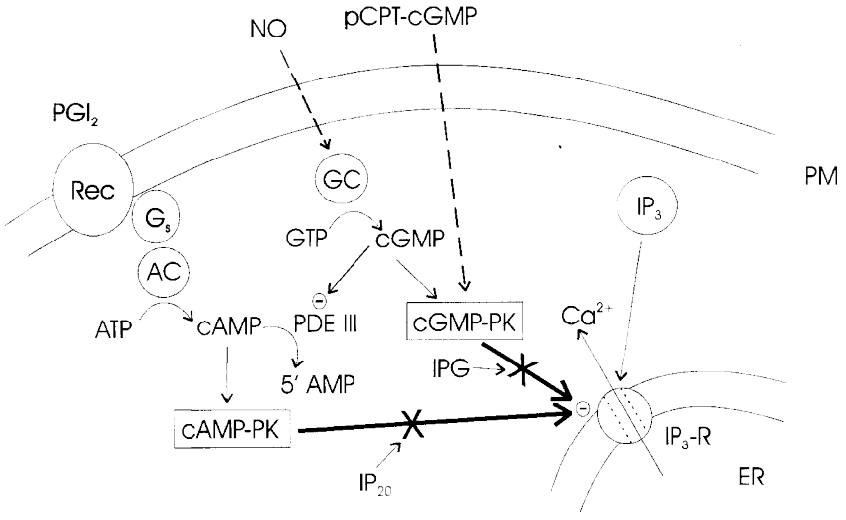

Figure 7. Simplified diagram illustrating inhibition of IP_3-mediated Ca^{2+} release by PGI_2 and NO

Prostacyclin (PGI_2) is shown as activating adenylate cyclase (AC) via the GTP-binding protein G_s and NO is shown as directly activating soluble guanylate cyclase (GC). Elevation of cAMP levels by PGI_2 inhibits IP_3-mediated Ca^{2+} release via cAMP-PK. The membrane permeable hydrolysis-resistant analogue of cGMP, pCPT-cGMP, inhibits Ca^{2+} mobilization via the direct activation of cGMP-PK. Elevation of cGMP levels by NO inhibits IP_3-induced Ca^{2+} release in two ways: by activating cGMP-PK and by inhibiting PDE III and thereby increasing cAMP, which inhibits IP_3-induced Ca^{2+} release by activating cAMP-PK. Not shown are other modes of action by which PGI_2 and NO might inhibit Ca^{2+} mobilization, including inhibition of IP_3 production and protein nitrosylation.

kinases (El-Daher *et al.* 1996). It was reported that PGI_2 and SNP promoted phosphorylation of the IP_3-R in platelets by activating cAMP- and cGMP-dependent protein kinases respectively, and that this phosphorylation was associated with the inhibition of IP_3-evoked Ca^{2+} release (Cavallini *et al.* 1996). It was also reported that the platelets' IP_3-R can be phosphorylated by cAMP-PK and endogenous membrane-bound kinases and that the additional phosphorylation by cAMP-PK inhibits the rate of the IP_3-mediated Ca^{2+} release (Quinton *et al.* 1996).

We did not find any evidence that elevated cGMP stimulates Ca^{2+} uptake and/or extrusion in intact megakaryocytes (see Fig. 2). Cyclic nucleotides have been reported to contribute to the reduction of $[Ca^{2+}]_i$ in aortic smooth muscle cells by phosphorylation of the regulatory protein phospholamban, thereby stimulating the activity of the sarcoplasmic reticulum Ca^{2+}-ATPase (Cornwell *et al.* 1991; Karczewski *et al.* 1992). However, at present there is no evidence that cyclic nucleotide-dependent protein kinases regulate the Ca^{2+}-ATPase by a similar mechanism in platelets. Although it has been suggested that cGMP and cAMP increase the rate of the calcium extrusion pump, and cAMP stimulates the dense tubular Ca^{2+} accumulation system (Johansson & Haynes, 1992), a more recent publication by Cavallini *et al.* (1996) failed to find any evidence for an effect of cyclic nucleotides either on platelet endomembrane or plasma membrane Ca^{2+}-ATPases (Cavallini *et al.* 1996). This discrepancy, as to whether the activity of platelet Ca^{2+} uptake and/or extrusion mechanisms is regulated by cyclic nucleotide-dependent protein kinases, probably results from differences in the experimental methods used in these studies.

Our data do not rule out NO-dependent inhibition of $[Ca^{2+}]_i$ mobilization at the sites upstream from the IP_3-R, such as the inhibition of IP_3 formation. The inhibition of IP_3 and 1,2-diacylglycerol (DAG) formation via the inhibition of phospholipase C (PLC) or PLC–G-protein–receptor coupling has been proposed to account for the cGMP-PK-dependent reduction of $[Ca^{2+}]_i$ in platelets (Deana *et al.* 1989; Nguyen *et al.* 1991) and smooth muscle tissues (Hirata *et al.* 1990).

Finally, some cGMP-independent but NO-dependent inhibition might contribute to the NO-mediated inhibition of $[Ca^{2+}]_i$ mobilization, especially at the high concentration of the NO donor used (see Fig. 6) (for review see Lincoln *et al.* 1996). It has been reported that production of NO leads to a number of downstream reactions in which NO, in one of its redox states, reacts with free thiols in proteins of diverse functions with broad regulatory consequences (for review see Stamler *et al.* 1992). For example, the inhibitory action of peroxynitrite (derived from the interaction of NO and superoxide) on platelet aggregation has been reported to be independent of cGMP, and to be due to protein nitrosylation (Yin *et al.* 1995). However, whether the IP_3-R undergoes *S*-nitrosylation by NO derivatives, and if so, how nitrosylation of the IP_3-R would regulate its physiological functions is not known.

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