3′:5′-Cyclic guanosine monophosphate (cGMP) potentiates the inositol 1,4,5-trisphosphate-evoked Ca^{2+} release in guinea-pig hepatocytes

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The effect of cGMP on noradrenaline-induced intracellular Ca^{2+} mobilization was investigated in whole-cell voltage-clamped guinea-pig hepatocytes. Treatment of the cells with 8-Br-cGMP (1–500 μM) resulted in an increase in the sensitivity of the cells to noradrenaline and to inositol 1,4,5-trisphosphate ($Ins P_3$) photoreleased from caged $Ins P_3$. The positive effect of 8-Br-cGMP on the Ca^{2+} release evoked by Ca^{2+}-mobilizing agonists or $Ins P_3$ was blocked by a protein kinase G (PKG; cGMP-dependent protein kinase) inhibitor, the *R*P-8-(4-chlorophenylthio)guanosine 3′:5′-monophosphorothioate. 8-Br-cGMP affected neither the basal $Ins P_3$ concentration nor the noradrenaline-induced production of $Ins P_3$. In permeabilized hepatocytes, the dose–response curve for $Ins P_3$-induced Ca^{2+} release was shifted to the left in the presence of 8-Br-cGMP. Furthermore, the treatment with 8-Br-cGMP did not affect the Ca^{2+} content of the $Ins P_3$-sensitive Ca^{2+} stores. These results indicate that intracellular cGMP potentiates the noradrenaline-induced Ca^{2+} response by enhancing Ca^{2+} release from the intracellular Ca^{2+} stores. We suggest that cGMP increases the apparent affinity of $Ins P_3$ receptors for $Ins P_3$ in guinea-pig hepatocytes probably by phosphorylation via the activation of PKG.

INTRODUCTION

Several studies indicate that cGMP is an intracellular second messenger involved in the regulation of the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) in vascular smooth muscle. Its association with a cGMP-dependent protein kinase (PKG) has been shown to mediate nitric oxide (NO)-induced or natriuretic peptide-induced vascular smooth muscle relaxation (reviewed in [1]). Several smooth muscle cell functions are affected by cGMP and their modification appears to account for the effect of cGMP on the vascular reactivity [2]. Among these, a cGMP-dependent inhibition of the inositol 1,4,5-trisphosphate ($Ins P_3$)-induced Ca^{2+} release was described [3] and was correlated with the phosphorylation of the $Ins P_3$ receptor induced by agents known to increase the intracellular cGMP concentration [4]. It is also known that cAMP-dependent phosphorylation of brain and liver $Ins P_3$ receptors have opposite effects and respectively inhibit or potentiate the $Ins P_3$-induced Ca^{2+} release [5–8]. For this reason, we have investigated the effects of cGMP on the release of Ca^{2+} from $Ins P_3$-sensitive stores in hepatocytes.

The variations of $[Ca^{2+}]_i$ were monitored in whole-cell voltage-clamped guinea-pig hepatocytes using either the well-characterized Ca^{2+}-dependent K^+ conductance [9] or furaptra, a Ca^{2+}-dependent fluorescent dye loaded from the patch pipette. We have studied the effect of cGMP on the release of Ca^{2+} from the $Ins P_3$-sensitive stores evoked by a Ca^{2+}-mobilizing agonist, i.e. noradrenaline (via the α_1-adrenoceptors), and $Ins P_3$ photoreleased from a caged precursor. Our results show cGMP pretreatment of the cells was able to potentiate the release of Ca^{2+} from $Ins P_3$-sensitive stores. Furthermore, we have shown that the cGMP-evoked effects were blocked by inhibitors of PKG but not by cAMP-dependent protein kinase (PKA) inhibitors and resulted from an increased sensitivity of $Ins P_3$ receptors for $Ins P_3$. In contrast to some other studies showing that cGMP decreases $[Ca^{2+}]_i$, our results show that this second messenger facilitates the mobilization of Ca^{2+} from the internal stores.

These data strongly indicate that cGMP, like cAMP, modulates the affinity of $Ins P_3$ receptors in guinea-pig hepatocytes to regulate the $Ins P_3$-induced Ca^{2+} release.

EXPERIMENTAL

Guinea-pig liver cells (male of the Hartley strain, 200–250 g) were dispersed after enzymic digestion as described previously [9,10]. Freshly dissociated hepatocytes (approx. 400000 cells) were plated in 35-mm-diam. Falcon dishes in 2 ml of William's E medium supplemented with fetal-calf serum (10%, v/v), penicillin (200000 units/ml) and streptomycin (100 mg/ml), and kept at 37 °C in an atmosphere of CO_2 (5%) in air.

Whole-cell recording

Standard tight-seal whole-cell recording techniques were used [11]. Patch pipette resistances were typically 5 MΩ. Whole-cell currents were measured with a Biologic RK300 patch-clamp amplifier, digitized by a CED 1401 interface (CED Ltd) and analysed by using the VCAN package supplied by J. Dempster (University of Strathclyde, U.K.). Experiments were made in

Abbreviations used: $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; $Ins P_3$, inositol 1,4,5-trisphosphate; Rp-8-pCPT-cGMPS, *R*P-8-(4-chlorophenylthio) guanosine 3′:5′-monophosphorothioate; Rp-8-pCPT-cAMPS, *R*P-8-(4-chlorophenylthio)adenosine 3′:5′-monophosphorothioate; PKG, cGMP-dependent protein kinase; PKA, cAMP-dependent protein kinase; NO, nitric oxide; iNOS, inducible NO synthase.

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chloride-free conditions with gluconate replacing chloride. The external solution contained (mM): sodium gluconate, 145; potassium gluconate, 5.6; $MgSO_4$, 1.2; $CaSO_4$, 5; NaH_2PO_4, 1; Hepes, 8 (pH 7.3). The medium used in Ca^{2+}-free experiments contained (mM): sodium gluconate, 149; potassium gluconate, 5.6; $MgSO_4$, 3; NaH_2PO_4, 1; Hepes, 8 (pH 7.3). The patch pipettes were filled with a solution containing (mM): potassium gluconate, 153; $ATPNa_2$, 3; $MgSO_4$, 3; Hepes, 8 (pH 7.3). The dish was thermostatically maintained at 34 °C and continuously perfused. All the solutions were filtered through a 0.22-μm-pore-size Millipore filter.

Fluorescence measurements and flash photolysis

We used the same experimental set-up as described previously [12,13]. Furaptra (potassium salt) and the caged compounds were introduced into the cell by diffusion from the patch pipette. A period of 5–6 min was required for equilibration of the furaptra fluorescence signal. $[Ca^{2+}]_i$ was derived from fluorescence signals using a method described previously [12,13]. Photolysis of caged InsP_3 [P-5 1-(2-nitrophenyl)ethyl ester InsP_3] was produced by a 1 ms pulse from a xenon arc flashlamp [14] focused through a UG11 filter (280–360 nm) from 4 cm above the cell to produce a 4–5 mm spot [15]. The method to assess the amount of InsP_3 released per flash has been described previously [13]. The optical artefact arising from the UV pulse was minimized (4–8 ms) by use of quartz coverslips and UV block oil between the coverslip and the objective [15,16].

Measurement of InsP_3

The cellular InsP_3 content was measured according to the method of Combettes et al. [17] with small modifications. Briefly, cells were incubated (3×10^6 cells/ml) for 90 min in the William's E medium containing [^{3}H]*myo*-inositol (30 μCi, 1 mCi/mmol). The cells were washed and resuspended (5×10^6 cells/ml) in a medium containing (mM): NaCl, 106; KCl, 5.6; $CaCl_2$, 1.8; $MgCl_2$, 1.2; LiCl, 10; $NaHCO_3$, 10. Aliquots (1 ml) were incubated at 37 °C under carbogene for 1 min, then treated with noradrenaline (10 μM, 2 min), or with 8-Br-cGMP (1 mM, 5 min), or with both 8-Br-cGMP (1 mM, 5 min) and noradrenaline (10 μM, 2 min). The reactions were stopped with 2 ml of perchloric acid (3.3 % final concentration, 4 °C, 30 min). The supernatants were neutralized with saturated $KHCO_3$ and diluted with 5 mM $Na_2B_4O_7$/0.5 mM EDTA. The ^{3}H-labelled inositol phosphates were separated by anion-exchange chromatography on a Dowex AG X8 column (0.5 cm × 3 cm; formate form 100–200 mesh; Bio-Rad).

Ca^{2+} release from saponin-treated cells

Hepatocytes were permeabilized by saponin as reported previously [18]. Briefly, cells were centrifuged and washed twice (50 ***g*** for 1 min) with an Eagle's medium lacking Ca^{2+}, then resuspended (3×10^6 cells/ml) in a cytosol-like medium containing: 100 mM KCl, 20 mM NaCl, 5 mM $MgCl_2$, 1 mM NaH_2PO_4, 25 mM Hepes buffer (pH 7.2 at room temperature), 1.5 mM Na_2ATP, 5 mM creatine phosphate, 5 units/ml creatine kinase and 20–40 μM fluorescent Ca^{2+} buffer quin2, in the presence of a contaminating Ca^{2+} concentration of a few micromolar plus endogenous Ca^{2+}. Cells were transferred to the spectrofluorimeter cuvette under continuous stirring. Saponin was added at a final concentration of 50 μg/ml to allow permeabilization of the plasma membrane but not of the internal Ca^{2+} stores, which retained their capacity for active accumulation of Ca^{2+}. After this treatment, about 90 % of the cells were permeable to Trypan Blue. The cell Ca^{2+} content was determined as previously published [18] and expressed as nmol of Ca^{2+}/mg of cell protein from the difference between the Ca^{2+} bound to quin2 before and after addition of the agent. Ionomycin (5 μM) was used to determined the size of the total Ca^{2+} compartment.

Chemicals

All the chemicals were from Sigma except InsP_3 (Amersham Corp.), quin2 and furaptra (Molecular Probes Inc.), Williams' E medium (Gibco) and 8-Br-cGMP, *R*P-8-(4-chlorophenylthio)-guanosine 3′:5′-monophosphorothioate (Rp-8-pCPT-cGMPS) and *R*P-8-(4-chlorophenylthio)adenosine -3′:5′-monophosphorothioate (Rp-8-pCPT-cAMPS) (Biolog, Germany). Caged-InsP_3 was generously given by Dr. D. R. Trentham.

RESULTS

The plasma membrane of guinea-pig hepatocytes possesses K^+ channels that are activated by an increase of intracellular Ca^{2+} upon stimulation by Ca^{2+}-mobilizing hormones or neurotransmitters [9,10]. It has been previously shown that this Ca^{2+}-activated K^+ conductance closely follows the responses obtained using Ca^{2+} fluorescent dyes and therefore can be used to monitor the variations in $[Ca^{2+}]_i$ in whole-cell voltage-clamped guinea-pig hepatocytes [12,13,15]. We sometimes used the Ca^{2+} dye furaptra to simultaneously monitor the increases in $[Ca^{2+}]_i$ and in the Ca^{2+}-dependent K^+ conductance. This confirmed that, in our study, the activation of the Ca^{2+}-dependent K^+ conductance reflected the changes in $[Ca^{2+}]_i$.

cGMP potentiates the noradrenaline-induced Ca^{2+} increases

Ca^{2+} mobilization by hormones or neurotransmitters is often characterized by a marked delay that shortens at increased concentrations of agonists [19]. This delay is mainly due to the time taken to accumulate a sufficient local concentration of the second messenger InsP_3 or Ca^{2+} to set off a regenerative response [20,21]. Figure 1 shows the Ca^{2+}-dependent K^+ conductance due

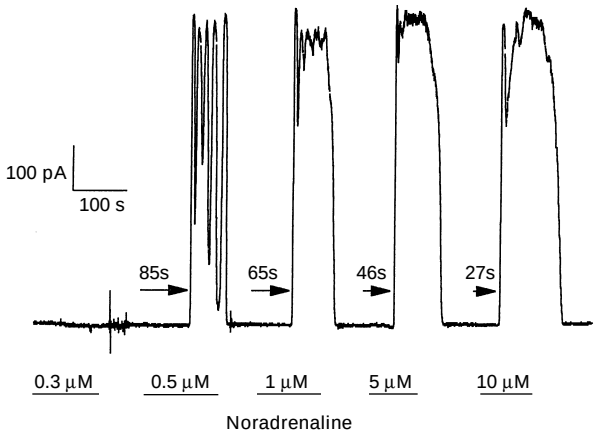

Figure 1 Activation of the Ca^{2+}-dependent K^+ current by increasing concentrations of noradrenaline in guinea-pig hepatocytes

The cell was sequentially perfused with increasing concentrations of noradrenaline (0.3 to 10 μM) applied along horizontal bars. The delay (horizontal arrows) is defined as the latency between the addition of noradrenaline and the activation of the Ca^{2+}-dependent K^+ conductance. Holding potential −40 mV.

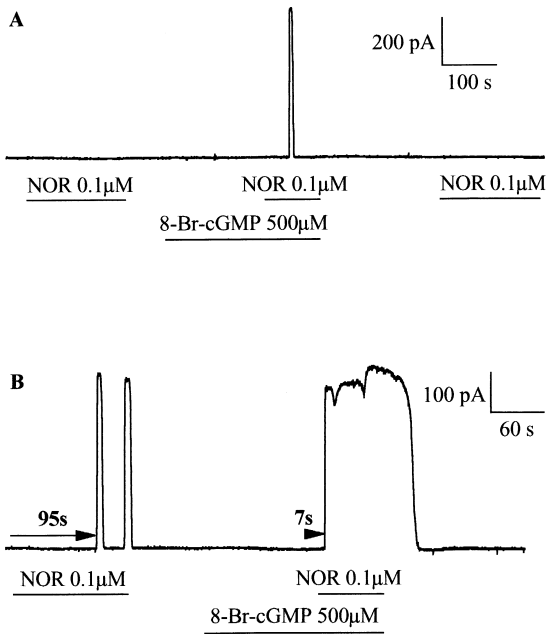

Figure 2 Effect of 8-Br-cGMP on the noradrenaline (NOR)-induced increase of Ca^{2+}-dependent K^+ current in guinea-pig hepatocytes

The traces A and B represent the effects of 8-Br-cGMP (500 μM) on the activation of the Ca^{2+}-dependent K^+ conductance induced by 0.1 μM noradrenaline (NOR) in two different guinea-pig hepatocytes. The delay (horizontal arrows) is defined as in Figure 1. Holding potential −40 mV.

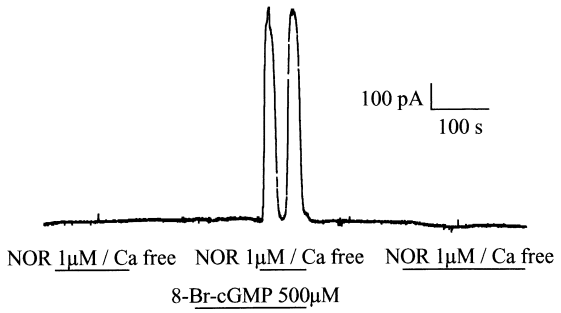

Figure 3 Potentiation by 500 μM 8-Br-cGMP of the response induced by noradrenaline does not require the presence of extracellular Ca^{2+}

The cell was treated as indicated in Figure 1 except that noradrenaline (NOR, 1 μM) was added in a Ca^{2+}-free medium. Holding potential −40 mV. This trace is representative of three cells tested.

to the variations of $[Ca^{2+}]_i$ of a single guinea-pig hepatocyte after sequential addition of five different concentrations of noradrenaline. The cell perfusion by a low concentration of noradrenaline (i.e. 0.3 μM) did not result in the activation of the Ca^{2+}-dependent K^+ conductance, at least not during the time of perfusion (2 min). Increasing the noradrenaline concentration to 0.5 μM promoted the activation of the K^+ current due to an increase in $[Ca^{2+}]_i$ after 65 s. Further increases in the noradrenaline concentration resulted in the decrease of the lag time between the onset of the agonist and the appearance of the response (65, 46 and 27 s at respectively 1, 5 and 10 μM noradrenaline). The sequence shown in Figure 1 was observed in four different cells, the main difference between these cells being the threshold concentration of agonist which varied from 0.1 to 0.5 μM. Thus a low concentration of noradrenaline is likely to trigger, if any, a response with a long delay and it was therefore interesting to investigate how cGMP would affect this response in guinea-pig hepatocytes. The results presented in Figure 2 illustrate the effect of cGMP on the response induced by 0.1 μM noradrenaline in two different cells both voltage-clamped at −40 mV. An application of 0.1 μM noradrenaline for 3 min was ineffective on the first cell (Figure 2A). The cell was washed for 2 min in the absence of neurotransmitter and then perfused for the next 3 min with 500 μM 8-Br-cGMP (a permeant analogue of cGMP that is poorly hydrolysable). The K^+ conductance remained inactive until the addition of 0.1 μM noradrenaline in the external medium. A transient increase of the K^+ current was observed 50 s after the reintroduction of the agonist in the external medium. This activation appeared to be related to the presence of 8-Br-cGMP, since a subsequent perfusion by noradrenaline alone later on the same cell did not result in a similar activation of the Ca^{2+}-dependent K^+ current. On a second cell (Figure 2B), 0.1 μM noradrenaline evoked a transient increase in the K^+ current with a delay of 95 s. In the presence of 500 μM 8-Br-cGMP added 2 min before the neurotransmitter, 0.1 μM noradrenaline also led to an increase in K^+ current, this time with a much shorter delay of 7 s. The presence of 8-Br-cGMP changed the pattern of the response from repetitive spikes to a sustained plateau, a change that was also observed when the noradrenaline concentration was increased from 0.1 to 5–10 μM in the absence of 8-Br-cGMP. The potentiation by cGMP of the noradrenaline-evoked responses was observed in six out of seven cells tested.

cGMP does not require external Ca^{2+} to potentiate the noradrenaline-induced response

The increase of intracellular Ca^{2+} induced by noradrenaline is supported by the release of Ca^{2+} from internal stores but also by an influx of Ca^{2+} from the external medium. It is possible that the potentiating effect of cGMP on the noradrenaline-evoked activation of the Ca^{2+}-dependent K^+ conductance may be due to an enhanced entry of Ca^{2+} into the cytosol independently or not of internal Ca^{2+} release. This increased Ca^{2+} entry may be due to an effect of cGMP on noradrenaline-activated Ca^{2+} influx or cGMP itself independently of noradrenaline [22–24]. We have examined the effect of 8-Br-cGMP on the response induced by submaximal concentrations of noradrenaline in the absence of external Ca^{2+}. The protocol was the same as in Figure 2 except that noradrenaline (1 μM) was added in a Ca^{2+}-free medium. 8-Br-cGMP (500 μM) was added 2 min before the agonist in a Ca^{2+}-containing medium to avoid a partial depletion of the internal Ca^{2+} stores. Then, 1 μM noradrenaline was perfused on the cell in a Ca^{2+}-free medium to block the Ca^{2+} influx but still in the presence of cGMP. Figure 3 shows that the potentiating effect of cGMP on the intracellular Ca^{2+} increase remains unaffected in the absence of external Ca^{2+} as this was observed in all three of the cells tested. This strongly suggested that the cGMP potentiation of the noradrenaline responses in guinea-pig hepatocytes probably reflect an enhanced Ca^{2+} release from the internal stores.

We have verified that the potentiation by 8-Br-cGMP of the noradrenaline-evoked responses was not due to an increase in the $InsP_3$ production in the presence of the cyclic nucleotide. This was done as indicated in the Experimental section and the results

Table 1 Effect of 8-Br-cGMP and noradrenaline on the InsP_3 content of guinea-pig hepatocytes

Experimental conditions are described in the Experimental section. Results are means ± S.E.M. ($n = 3$).

	InsP_3 content (%)
Control	100 ± 5 ($n = 3$)
1 mM 8-Br-cGMP	101 ± 7 ($n = 3$)
10 μM Noradrenaline	115 ± 3 ($n = 3$)
10 μM Noradrenaline + 1 mM 8-Br-cGMP	112 ± 7 ($n = 3$)

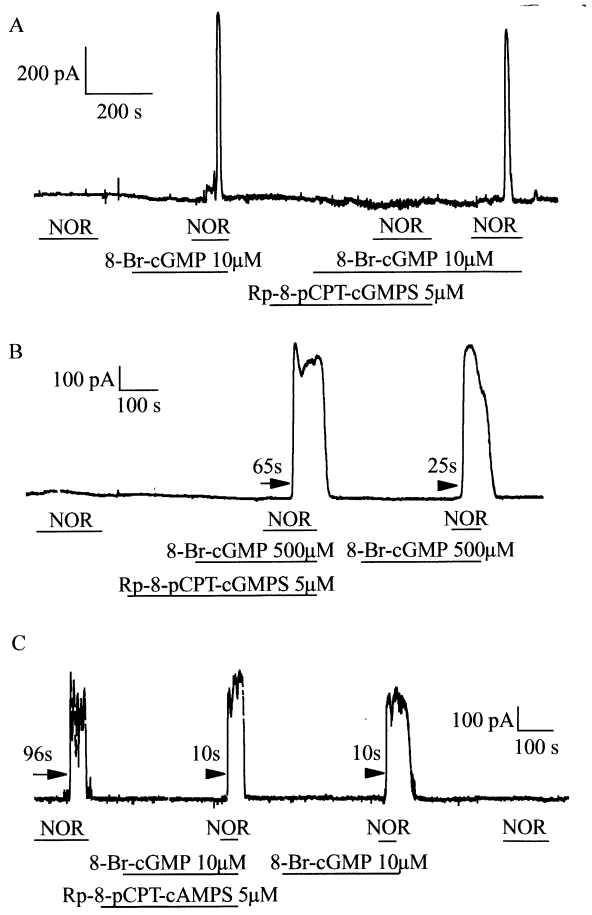

Figure 4 Stimulation by 8-Br-cGMP of the response induced by noradrenaline is mediated through the activation of a cGMP-dependent protein kinase

The three traces were obtained from three different cells (**A**, **B**, **C**) held at a holding potential of −40 mV. (**A**) Inhibitory effect of 5 μM Rp-8-pCPT-cGMPS on the potentiation by 10 μM 8-Br-cGMP of the response induced by 0.3 μM noradrenaline (NOR). The cell was first perfused with 0.3 μM NOR and 0.3 μM NOR in the presence of 10 μM 8-Br-cGMP then washed with external solution. Then 5 μM Rp-8-pCPT-cGMPS was added prior to 10 μM 8-Br-cGMP and 0.3 μM NOR to reversibly block the activation of the Ca^{2+}-dependent K^+ conductance. (**B**) The second cell shows the partial inhibition by 5 μM Rp-8-pCPT-cGMPS on the response induced by 0.2 μM NOR and stimulated by 500 μM 8-Br-cGMP. (**C**) Lack of effect of 5 μM Rp-8-pCPT-cAMPS on the response induced by 0.3 μM NOR and stimulated by 10 μM 8-Br-cGMP. Current (pA) and time (s) calibrations are indicated as vertical and horizontal bars in the Figure. The delays (horizontal arrows) are defined as in Figure 1. Holding potential −40 mV. Each sequence of traces is representative of three or four cells tested.

are presented in Table 1. The incubation of a guinea-pig liver cell suspension with 10 μM noradrenaline for 2 min significantly increased the InsP_3 content of the cells, whereas 1 mM 8-Br-cGMP for 5 min had no detectable effect. Furthermore, the preincubation of the cell suspension with 1 mM 8-Br-cGMP for 5 min had no effect on the InsP_3 production evoked by noradrenaline (10 μM for 2 min). These results indicated that the potentiation of the noradrenaline responses by cGMP is unlikely to reflect an activation of the phosphoinositidase C by the cyclic nucleotide.

cGMP stimulates the noradrenaline-induced rise of intracellular Ca^{2+} through the activation of a cGMP-dependent protein kinase

The results presented above indicated that cGMP might stimulate the release of Ca^{2+} from internal stores induced by noradrenaline. We have investigated the mechanism by which cGMP acts on this release. As reported in [1], cGMP can interact with several cGMP-binding proteins in order to mediate its intracellular effects. Among these, a cGMP-dependent protein kinase (PKG) has already been described in hepatocytes, although it is present in low amounts (for a review, see [25]). PKG may mediate the effect of cGMP on release of Ca^{2+} induced by noradrenaline. To test this hypothesis, we applied Rp-8-pCPT-cGMPS, which is a membrane-permeant and phosphodiesterase-resistant inhibitor of the PKG Ia ($K_i = 240$ nM, [26]). We compared the effect of 8-Br-cGMP on the noradrenaline-triggered Ca^{2+} release in the presence or absence of the inhibitor and the results are shown in Figure 4(A). First, as shown above, we observed that 0.3 μM noradrenaline evoked a Ca^{2+} release in the presence of 10 μM 8-Br-cGMP, whereas the cell remained quiescent in its absence. The two drugs were washed away for 3 min then we sequentially added the PKG inhibitor Rp-8-pCPT-cGMPS (5 μM) alone for 2 min, then together with the PKG activator 8-Br-cGMP (10 μM) for 3 min, and finally with 0.3 μM noradrenaline. In these conditions, the potentiation by cGMP of the agonist response was blocked, an effect directly linked to the presence of the PKG inhibitor since the noradrenaline response was recovered after its removal. We also investigated the efficiency of 5 μM Rp-8-pCPT-cGMPS to block the cGMP-dependent stimulation when the 8-Br-cGMP concentration was raised to 500 μM (Figure 4B). In that case, Rp-8-pCPT-cGMPS failed to totally block the potentiating effect of 8-Br-cGMP, but increased the delay of the noradrenaline-evoked response.

We have also investigated a role for cAMP-dependent protein kinase (PKA) in the stimulation by cGMP. Indeed, the results presented above could be explained if PKA was non-specifically activated by cGMP, since an enhancement of the Ca^{2+} release from intracellular stores by PKA has already been described [7,27]. This possibility was investigated by using a membrane-permeant inhibitor of PKA (Rp-8-pCPT-cAMPS). The results are illustrated in Figure 4(C). The cell was perfused as described in Figure 4(A), except that Rp-8-pCPT-cGMPS was replaced by Rp-8-pCPT-cAMPS. Under these conditions, the potentiation of the noradrenaline-induced response by 8-Br-cGMP was identical whether the PKA inhibitor was present or absent. Thus, our results indicate that the stimulation of the noradrenaline-induced response by cGMP is likely to be mediated through the activation of PKG.

cGMP stimulates the release of Ca^{2+} induced by the photolysis of caged-InsP_3

Komalavilas and Lincoln [4] have reported that the InsP_3 receptors of smooth muscle are phosphorylated by PKG and

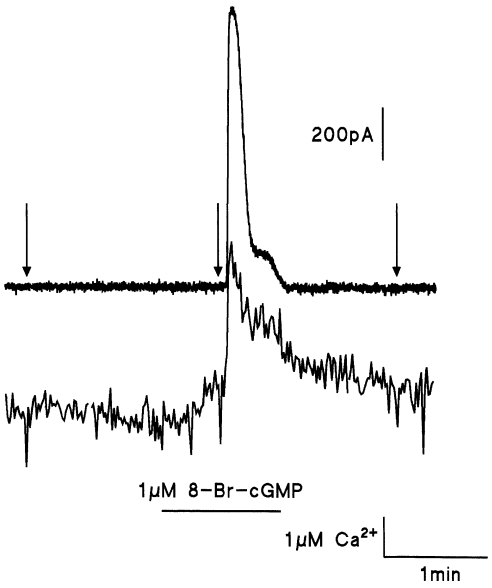

Figure 5 8-Br-cGMP stimulates the release of Ca^{2+} from InsP_3-sensitive stores

The cell was loaded via the patch pipette with 500 μM furaptra and 5 μM caged InsP_3 allowing 5 min for equilibration. Each flash released 300 nM InsP_3 inside the cell at the time indicated by the vertical arrows. The upper and lower traces respectively represent the activation of the Ca^{2+}-dependent K^+ conductance and the increase in $[Ca^{2+}]_i$. The release of Ca^{2+} and the activation in the K^+ conductance were only seen in the presence of 1 μM 8-Br-cGMP perfused on to the cell as indicated by the horizontal bar. Holding potential 0 mV.

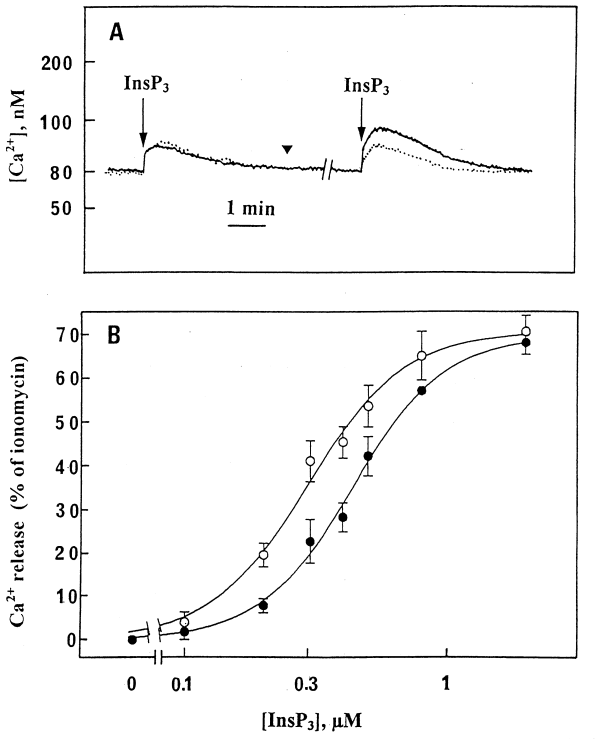

Figure 6 8-Br-cGMP potentiates the InsP_3-induced Ca^{2+} release in permeabilized hepatocytes

Hepatocytes were permeabilized by addition of 50 μg/ml saponin in cytosolic-like medium containing the Ca^{2+}-sensitive dye quin2 in the free acid form and an ATP-regenerating system. (**A**) Successive addition of 0.2 μM InsP_3, in the absence (dotted line), or after addition (continuous line) of 8-Br-cGMP. 8-Br-cGMP (1 mM) or the solvent (water) were added at the time indicated by the arrowhead 4 min before the second addition of InsP_3. To facilitate the comparison between the two effects, responses have been superimposed and showed that the amount of Ca^{2+} released by 0.2 μM InsP_3 was greater in the presence of 8-Br-cGMP. (**B**) Dose–response curves for InsP_3-induced Ca^{2+} release in saponin-treated hepatocytes in the absence (●) or presence (○) of 1 mM 8-Br-cGMP added to the cells 4 min prior to InsP_3. The amount of Ca^{2+} release by InsP_3 was expressed as a percentage of the ionomycin-sensitive compartment after addition of 5 μM ionomycin. The bars represent S.E.M. of between four and ten determinations.

proposed that this phosphorylation may modify the properties of the InsP_3 receptor. Although, the predominant isoforms of InsP_3 receptors present in the hepatocyte and in the smooth muscle are likely to be different [28], a cGMP-dependent phosphorylation of the InsP_3 receptor could represent the molecular basis of our observations. This led us to compare the Ca^{2+} release induced by InsP_3 in the presence or absence of 8-Br-cGMP. This was done by photoreleasing InsP_3 from 5 μM caged InsP_3 introduced into the cell via the patch-pipette together with 500 μM furaptra. The output energy of the flash lamp was adjusted to convert about 6% of the caged InsP_3 per flash [13]. The InsP_3-induced Ca^{2+} releases from the internal Ca^{2+} stores are characterized by a delay and a fast rise in guinea-pig hepatocytes, endothelial cells or cerebellar Purkinje neurons [12]. The response to three successive photoreleases of InsP_3 before, during and after the perfusion of 1 μM 8-Br-cGMP is shown in Figure 5. The photorelease of 300 nM InsP_3 into the cell had no effect on the Ca^{2+}-dependent K^+ conductance and the release of Ca^{2+} from the internal stores unless 1 μM 8-Br-cGMP was present. This potentiating effect of cGMP on the InsP_3-evoked Ca^{2+} release was observed in all the six cells tested. These results strongly suggested that a change in the InsP_3 receptor affinity was likely to be responsible for the potentiating effect of cGMP described here. These results also confirmed that the activation of the Ca^{2+}-dependent K^+ conductance mimicks the $[Ca^{2+}]_i$ increases and therefore can be used to monitor the variations in $[Ca^{2+}]_i$ in guinea-pig hepatocytes.

cGMP induced an increase in the InsP_3 receptor affinity for InsP_3

The experiments in intact cells suggested that, in the presence of cGMP, subthreshold concentrations of InsP_3 were able to increase $[Ca^{2+}]_i$. In order to investigate this phenomenon in more detail, we examined the effect of cGMP on Ca^{2+} release from the intracellular stores using saponin-treated hepatocytes. Addition of a submaximal InsP_3 concentration (0.2 μM) led to a rapid release of Ca^{2+} into the medium which was buffered by the cells within 3 min (Figure 6A). After the re-accumulation of Ca^{2+} to the resting value, addition of cGMP (1 mM) to the same cells did not induced any effect on steady-state buffering of Ca^{2+} by the cells. However, addition of 0.2 μM InsP_3 to these cells induced a larger release of Ca^{2+} (167 ± 18%, $n = 8$), compared with the first addition of the same InsP_3 concentration taken as reference (i.e. 100%)(Figure 6A). This effect resulted from the 8-Br-cGMP action since, using the same protocol, a second addition of InsP_3 (0.2 μM) in the absence of 8-Br-cGMP released the same amount of Ca^{2+} (96 ± 8%, $n = 5$). It was worth noting that the 8-Br-cGMP action was time- and concentration-dependent, the potentiation of the InsP_3-induced Ca^{2+} release in the presence of 1 mM 8-Br-cGMP was maximal after 4 min and no effect was observed for concentrations below 500 μM 8-Br-cGMP (results not shown).

Finally, the amount of Ca^{2+} release by 5 μM ionomycin was the same in the absence or in the presence of 8-Br-cGMP, being

respectively 0.85 ± 0.05 versus 0.93 ± 0.06 nmol of Ca^{2+}/mg of protein. Also, the size of the $InsP_3$-sensitive compartment was not affected in the presence of 8-Br-cGMP and corresponded to about 70% of the ionomycin-sensitive compartment (Figure 6B). The effect of 1 mM 8-Br-cGMP on the concentration–response relationship for $InsP_3$-induced Ca^{2+} release was also investigated. The addition of 8-Br-cGMP shifted the EC_{50} for $InsP_3$-induced Ca^{2+} release from 0.44 μM to 0.29 μM (Figure 6B).

DISCUSSION

The work described in this paper shows that cGMP potentiates the noradrenaline-induced Ca^{2+} mobilization in freshly isolated guinea-pig hepatocytes. Our results provide evidence that this effect results, via the action of PKG, from an increase in the sensitivity of the $InsP_3$ receptors to $InsP_3$.

cGMP potentiation of noradrenaline-induced $[Ca^{2+}]_i$ increases

The noradrenaline-evoked rise in $[Ca^{2+}]_i$ monitored by the guinea-pig Ca^{2+}-dependent K^+ conductance is deeply modified in the presence of cGMP. Such results could have resulted if the properties of Ca^{2+}-dependent K^+ channels were (directly or not) modified by cGMP [29]. Here, the experiments done with furaptra showed that cGMP stimulates the $InsP_3$-evoked Ca^{2+} release, resulting in a shorter delay in the activation of the K^+ conductance. The source of the Ca^{2+} for this increase in $[Ca^{2+}]_i$ is intracellular, since improved Ca^{2+} responses enhanced by cGMP were observed in the absence of extracellular Ca^{2+}. Several mechanisms, alone or together, could provide an explanation for the observed potentiation. The cGMP pretreatment could: (i) enhance the production of the putative Ca^{2+}-mobilizing second messenger $InsP_3$; (ii) increase the size of the agonist-releasable intracellular store(s); or (iii) increase the sensitivity of the Ca^{2+} stores to the Ca^{2+}-mobilizing effects of $InsP_3$. We can rule out the first hypothesis since, in the present work, the $InsP_3$ production was not affected by the prior treatment of cells with 8-Br-cGMP and furthermore it has been recently shown that 8-Br-cGMP slightly inhibited the agonist-induced $InsP_3$ production in rat hepatocytes [30]. Although it has been shown in other cells types that cGMP could activate the Ca^{2+}-ATPase [31], we can eliminate the second hypothesis since we have shown, on permeabilized guinea-pig cells, that the Ca^{2+} content of the ionomycin (or $InsP_3$)-sensitive Ca^{2+} store(s) was not changed by the cGMP pretreatment (see the Results section).

Our data are consistent with an increase in the sensitivity of the Ca^{2+} store(s) to the Ca^{2+}-mobilizing effect of $InsP_3$. Indeed, the apparent affinity of the release mechanism for $InsP_3$ was increased, as reflected by the shift to the left of the $InsP_3$ dose–response curve. The pronounced enhancement of the response to low concentrations of $InsP_3$ is likely to be responsible for the apparent increase in the sensitivity of cells to a subthreshold concentration either of noradrenaline or of photolytic release of $InsP_3$.

Implication of cGMP-dependent kinase

The $InsP_3$ receptors also are a major substrate for cGMP-dependent protein phosphorylation in smooth muscle and it was recently proposed that the phosphorylation could result in a diminished affinity for $InsP_3$ [4]. However, it is known that phosphorylation processes can trigger opposite effects depending on the observed tissue and therefore it is conceivable that the liver type of $InsP_3$ receptors may react differently after cyclic nucleotide-induced phosphorylation. The phosphorylation of $InsP_3$ receptors by PKA has been shown to have opposing effects, i.e. a decrease in the $InsP_3$ receptors' affinity to $InsP_3$ in brain [5,6] and, in contrast, an increase in hepatocytes [7,27]. Thus, although cGMP and cAMP probably cross-interact on the activation of PKA and PKG (for a review, see [32]), the specific action of PKG inhibitors on the cGMP effects confirmed the existence of a new pathway that could modulate the mobilization of intracellular Ca^{2+} by hormones and neurotransmitters in guinea-pig hepatocytes.

Role of cGMP-evoked regulation of $InsP_3$ receptors in the cellular physiology

$InsP_3$-induced variations of $[Ca^{2+}]_i$ represent a main signal encoding cytosolic and nuclear functions, probably through the differential activation of Ca^{2+}-binding proteins [33,34]. Our results suggest that the $InsP_3$-evoked Ca^{2+} release of hepatocytes is regulated by cGMP. Under these conditions, agents controlling the cGMP concentration may affect the $InsP_3$ pathway and thus affect the cellular response. In many cell types the concentration of cGMP is controlled by soluble and membrane guanylate cyclases which are known to be respectively regulated by NO and peptides. These different pathways for stimulation of the cGMP production are likely to exist in the hepatocyte. Atrial natriuretic peptide, pancreastatin and epidermal growth factor regulate cGMP production in hepatocytes and are known to affect glycolytic metabolism and DNA synthesis [35–37]. NO is a diffusible messenger that can be released from surrounding cells or directly produced within the hepatocytes by the inducible NO synthase (iNOS) (reviewed in [38]). The hepatic iNOS is induced by four factors acting simultaneously, lipopolysaccharide and the cytokines tumour necrosis factor, interleukin 1 and interferon γ [39], thus suggesting that NO appears mainly after liver immunological disorders. NO activates a soluble hepatic guanylate cyclase and triggers an enhancement of the cGMP level [40]. NO is also known to promote deep modifications of the hepatic energetics and metabolism [41–45]. It is not clearly established whether all these modifications are strictly mediated by cGMP and whether they are correlated with a cGMP-dependent alteration of the $InsP_3$ pathway. However, these observations, and the results shown here, strongly suggest that the cGMP-dependent modification of $InsP_3$ receptor properties has a role as part of the liver's physiological and physio-pathological responses.

We thank David Ogden for comments on the manuscript, Raymonde Leuillet for the excellent guinea-pig liver cell preparations and the Singer-Polignac Foundation for support (G.G.).

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Received 6 March 1996/22 May 1996; accepted 5 June 1996