Cyclic GMP Induces Oscillatory Calcium Signals in Rat Hepatocytes*

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The ability of guanosine-3',5'-cyclic monophosphate (cGMP) to induce increases in the intracellular free calcium ion concentration ([Ca\(^{2+}\)]\(_i\)) was studied at the single cell level in fura-2-loaded rat hepatocytes. Both 8-bromo-cGMP (Br-cGMP) and dibutyryl cGMP (db-cGMP) produced oscillatory [Ca\(^{2+}\)]\(_i\) increases in hepatocytes. In addition, Br-cGMP increased the frequency of agonist-induced spiking or converted [Ca\(^{2+}\)]\(_i\), oscillations into sustained nonoscillatory [Ca\(^{2+}\)]\(_i\) responses. Addition of the nitric oxide donor sodium nitroprusside also produced oscillatory [Ca\(^{2+}\)]\(_i\), increases similar to those generated by cGMP analogues. In the absence of extracellular Ca\(^{2+}\), cGMP-induced [Ca\(^{2+}\)]\(_i\) responses were significantly reduced and mainly appeared as single transient [Ca\(^{2+}\)]\(_i\) increases. The effects of cGMP analogues do not appear to be mediated by a secondary increase in cAMP or activation of cAMP-dependent protein kinase (PKA), since [Ca\(^{2+}\)]\(_i\) responses to cGMP analogues were inhibited by the G-kinase inhibitor 8-bromo-cGMP-[\(5\)-sulfamoyl-(R)-N6-cyclopentyladenosine-3',5'-cyclic monophosphorothioate (Rp-Br-cGMP[S]). Both Br-cGMP and db-cGMP also increased [Ca\(^{2+}\)]\(_i\) in the presence of the PKA inhibitor 8-bromoadenosine-3',5'-cyclic monophosphorothioate (Rp-Br-cAMP[S]) and when the cGMP-inhibitable cAMP phosphodiesterase activity was inhibited by pretreatment with siguazodan. Br-cGMP stimulated the Mn\(^{2+}\)-induced quench of compartmentalized fura-2 in intact hepatocytes, indicating a site of action at the level of the Ca\(^{2+}\) stores. This locus was further supported by the finding that pretreatment of hepatocytes with Br-cGMP potentiated submaximal inositol 1,4,5-trisphosphate (InsP\(_3\))-induced Mn\(^{2+}\) quench in subsequently permeabilized hepatocytes. db-cGMP also decreased PKA-mediated back phosphorylation of the hepatic type 1 InsP\(_3\) receptor, indicating that G-kinase phosphorylates the InsP\(_3\) receptor at sites targeted by PKA. These data indicate that phosphorylation of the hepatic InsP\(_3\) receptor by G-kinase increases the sensitivity to InsP\(_3\) for [Ca\(^{2+}\)]\(_i\) release and is associated with the production of [Ca\(^{2+}\)]\(_i\) oscillations in single rat hepatocytes.

An increase in intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) is the key sig-

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**The abbreviations used are: [Ca\(^{2+}\)]\(_i\), intracellular free calcium ion concentration; InsP\(_3\), inositol 1,4,5-trisphosphate; PKA, cAMP-dependent protein kinase; G-kinase, cGMP-dependent protein kinase; NO, nitric oxide; NOS, nitric oxide synthase; AM, acetoxymethyl ester; Br-cGMP, 8-bromo-cGMP; db-cGMP, dibutyryl cGMP; cAMP, cyclic AMP, dibutyryl cAMP; Rp-Br-cGMP[S], 8-bromoadenosine-3',5'-cyclic monophosphorothioate; SNP, sodium nitroprusside; SIN-1, 3-morpholinosydnonimine-hydrochloride; PDE, phosphodiesterase.

19817
Cyclic GMP Increases \([\text{Ca}^{2+}]\), in Hepatocytes

Cyclic GMP Increases \([\text{Ca}^{2+}]\), in Single Hepatocytes—The effects of cGMP analogues on \([\text{Ca}^{2+}]\), were examined at the single cell level in fura-2-loaded freshly isolated hepatocytes. Fig. 1 shows typical \([\text{Ca}^{2+}]\) responses in single hepatocytes exposed to Br-cGMP (200 \(\mu M\)) or db-cGMP (200 \(\mu M\)). In the present study we have used Br-cGMP and db-cGMP interchangeably since the \([\text{Ca}^{2+}]\) responses to both compounds are essentially equivalent and observed over similar concentration ranges.\(^2\) \([\text{Ca}^{2+}]\) responses produced by cGMP appeared as a series of base-line-separated \([\text{Ca}^{2+}]\) spikes with similar kinetics and amplitude to those previously obtained with inositol phospholipid-linked agonists in this preparation (36). The only significant differences were that cGMP-induced \([\text{Ca}^{2+}]\) responses frequently displayed secondary spiking activity during the falling phase of each \([\text{Ca}^{2+}]\) oscillation. As shown previously with receptor-linked agonists (36), individual hepatocytes displayed significant variation in the sensitivity to cGMP analogues, with cells already responding to low doses of cGMP producing sustained \([\text{Ca}^{2+}]\) responses at higher doses (data not shown). The \([\text{Ca}^{2+}]\) responses induced by cGMP could be mediated by activation of G-kinase or be due to activation of PKA and/or increases in cAMP resulting from an inhibition of cAMP-PDE activity. However, in all cases \([\text{Ca}^{2+}]\) responses produced by cGMP appeared as a series of base-line-separated \([\text{Ca}^{2+}]\) spikes with similar kinetics and amplitude to those previously obtained with inositol phospholipid-linked agonists in this preparation (36).
inhibitable cAMP phosphodiesterase) with siguazodan (30 μM) failed to increase [Ca^{2+}]_{i} and did not inhibit cGMP-induced [Ca^{2+}]_{i} signals (Fig. 3). Inhibition of PKA by pretreatment with Rp-Br-cAMP[S] (200 μM) also failed to prevent [Ca^{2+}]_{i} responses to cGMP in hepatocytes (data not shown).

Addition of the NO donor SNP also generated [Ca^{2+}]_{i} increases in hepatocytes. This is shown in Fig. 4 where addition of SNP produced oscillatory [Ca^{2+}]_{i} increases, comparable with those elicited by cGMP. The SNP-induced [Ca^{2+}]_{i} oscillations appeared after a well-defined latent period. Most cells responded with [Ca^{2+}]_{i} oscillations in the range of 1–10 μM SNP. Increasing doses of SNP increased the proportion of cells responding but did not produce a clear enhancement of [Ca^{2+}]_{i} oscillation frequency in individual hepatocytes already responding to SNP. We have observed similar [Ca^{2+}]_{i} responses with SIN-1 in hepatocytes (data not shown).

Role of Extracellular Ca^{2+}—Previous studies in liver, and other cell types, have shown that [Ca^{2+}]_{i} oscillations persist in the absence of extracellular Ca^{2+}, although they tend to have a reduced frequency followed by eventual run down and stopping (36, 41). To assess the contribution of extracellular Ca^{2+} influx to the initiation and maintenance of cGMP-induced [Ca^{2+}]_{i} oscillations, the ability of Br-cGMP to elicit [Ca^{2+}]_{i} responses in the absence of extracellular Ca^{2+} was examined. As shown in Fig. 5, removal of extracellular Ca^{2+} did not significantly affect the amplitude (368 ± 26 nm in the presence and 345 ± 23 nm in the absence of extracellular Ca^{2+}) of Br-cGMP-induced [Ca^{2+}]_{i} responses but significantly reduced their frequency or more often converted them to single transient [Ca^{2+}]_{i} increases. Removal of extracellular Ca^{2+} also had no effect on the time to peak Ca^{2+}. We have observed similar effects of Ca^{2+} depletion on vasopressin-induced [Ca^{2+}]_{i} responses in primary cultured hepatocytes (36). This indicates that Ca^{2+} influx is not required for the generation of Br-cGMP-induced [Ca^{2+}]_{i} responses but plays an important role in sustaining the [Ca^{2+}]_{i} oscillations by replenishing intracellular Ca^{2+} stores.

Cyclic GMP Potentiates Agonist-induced [Ca^{2+}]_{i} Responses—The data described above demonstrate that cGMP-induced [Ca^{2+}]_{i} responses have a similar temporal organization and Ca^{2+} dependence to those generated by receptor agonists. It was therefore of interest to establish the nature of any interaction between these two stimuli. For these experiments Br-cGMP was added to single hepatocytes during continuous exposure to phenylephrine. Br-cGMP potentiated the [Ca^{2+}]_{i} response to phenylephrine in all cells, but the extent of this enhancement was determined by the magnitude of the phenylephrine-induced [Ca^{2+}]_{i} increase (Fig. 6). Addition of Br-
Cyclic GMP Increases $[\text{Ca}^{2+}]$, in Hepatocytes

**FIG. 3.** cGMP-induced $[\text{Ca}^{2+}]$, responses are not dependent on inhibition of PDE III. Representative examples of $[\text{Ca}^{2+}]$, responses produced in single hepatocytes exposed to the PDE III inhibitor siguazodan (30 μM) (first arrow) followed by addition of Br-cGMP or db-cGMP (second arrow) in the continued presence of siguazodan. Similar responses were observed in a further 28 cells.

Cyclic GMP was also able to generate $[\text{Ca}^{2+}]$, responses produced in single hepatocytes exposed to the PDE III inhibitor siguazodan (30 μM) (first arrow) followed by addition of Br-cGMP or db-cGMP (second arrow) in the continued presence of siguazodan. Similar responses were observed in a further 28 cells.

Br-cGMP also potentiated the ability of phenylephrine to stimulate $[\text{Mn}^{2+}]$, quench of compartmentalized fura-2 fluorescence reflects the periodic opening and closing of the $[\text{Ca}^{2+}]$, channels within intracellular stores. Although it is not possible to monitor $[\text{Ca}^{2+}]$, changes simultaneously with the $[\text{Mn}^{2+}]$, quench of compartmentalized fura-2, the latency to the first quench step, the duration of the rapid phase of each step, and the frequency of the steps were all consistent with the temporal pattern of $[\text{Ca}^{2+}]$, oscillations observed under the same conditions.

Br-cGMP also potentiated the ability of phenylephrine to stimulate $[\text{Mn}^{2+}]$, quench of compartmentalized fura-2 in a manner that paralleled its effects on agonist-stimulated $[\text{Ca}^{2+}]$, signals. This is shown in Fig. 8 where the capacity of Br-cGMP to enhance phenylephrine-induced $[\text{Mn}^{2+}]$, quench was limited by the extent of the agonist response. In fact Br-cGMP had no effect in cells where the phenylephrine-induced $[\text{Mn}^{2+}]$, quench had already gone to completion (Fig. 8A). These results indicate that Br-cGMP mimics the effects of hormones that give $[\text{Ca}^{2+}]$, oscillations, by stimulating the sequential opening and closing of the intracellular $[\text{Ca}^{2+}]$, channels whose permeability to $[\text{Mn}^{2+}]$, is registered by the periodic quench of compartmentalized $[\text{Ca}^{2+}]$, channels.

3 Under these conditions $[\text{Mn}^{2+}]$, quench does not reflect activity of plasma membrane channels since cytosolic dye is already fully quenched with $[\text{Mn}^{2+}]$, and previous studies have shown that these steps continue after removal of extracellular $[\text{Mn}^{2+}]$ (44, 45).
fura-2. In addition, Br-cGMP and phenylephrine appear to stimulate Mn^{2+} entry into the same intracellular compartment, and this constitutes almost the entire ionomycin-sensitive Ca^{2+} store.

Cyclic GMP Phosphorylates the Type-1 InsP_{3} Receptor at a PKA-sensitive Site in Intact Hepatocytes—The type-1 InsP_{3} receptor has been shown to be phosphorylated by PKA in intact rat hepatocytes (8). This phosphorylation leads to an increased sensitivity of the hepatic InsP_{3} receptor to both Ca^{2+} and InsP_{3} (8, 46, 47) and has been suggested to underlie the stimulatory effects of cAMP on [Ca^{2+}]_{i} signals in hepatocytes (47, 48). Since the [Ca^{2+}]_{i} increases generated by cGMP in this study appear to be mediated by G-kinase activation, and the type-1 InsP_{3} receptor is known to be phosphorylated by G-kinase on a site also phosphorylated by PKA (22), we investigated whether cGMP phosphorylated the InsP_{3} receptor in intact hepatocytes. The phosphorylation of the type-1 InsP_{3} receptor was examined using a back phosphorylation assay, whereby solubilized extracts from control and cGMP-treated hepatocytes were immunoprecipitated with a type-1 InsP_{3} receptor antibody before being phosphorylated in vitro by incubation with [32P]ATP and the catalytic subunit of PKA. Accordingly, an increased phosphorylation of the InsP_{3} receptor in intact hepatocytes should be reflected by a decreased incorporation of [32P] during the in vitro phosphorylation assay. Fig. 9 demonstrates that both db-cAMP and db-cGMP treatment of intact hepatocytes decreased 32P incorporation into the immunoprecipitated InsP_{3} receptor. The results summarized in Table I demonstrate that exposure to db-cGMP reduced 32P incorporation by about 55% of that produced by db-cAMP. However, when both agents were added in combination their effects on 32P incorporation were completely nonadditive. This suggests that db-cAMP and db-cGMP phosphorylate common sites on the InsP_{3} receptor.

Cyclic GMP Potentiates InsP_{3}-induced Mn^{2+} Quench in Permeabilized Hepatocytes—As noted above, previous studies have reported that phosphorylation of the InsP_{3} receptor by PKA potentiates InsP_{3}-induced [Ca^{2+}]_{i} release in hepatocytes (46–48). To ascertain whether cGMP-mediated InsP_{3} receptor phosphorylation elicited a similar sensitizing action, the effect of Br-cGMP on InsP_{3}-induced Mn^{2+} quench of compartmentalized fura-2 was examined in permeabilized hepatocytes, as described previously (42, 44, 46). For these experiments fura-2. In addition, Br-cGMP and phenylephrine appear to stimulate Mn^{2+} entry into the same intracellular compartment, and this constitutes almost the entire ionomycin-sensitive Ca^{2+} store.

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2-loaded intact hepatocytes were preincubated at 37°C for 5 min with Br-cGMP in Ca^{2+}-free HEPES buffer before permeabilization in cytosolic buffer containing thapsigargin. The addition of thapsigargin depletes the intracellular Ca^{2+} stores and enables the effects of cGMP on the Mn^{2+}-induced quench elicited by submaximal concentrations of InsP_{3} receptor/channel to be monitored independent of changes in luminal Ca^{2+}. Pretreatment of intact hepatocytes with Br-cGMP (1 mM) resulted in a 2-fold increase in the rate and a 26% increase in the magnitude of the Mn^{2+}-induced quench elicted by a submaximal dose of InsP_{3} (150 nM) in permeabilized cells (Fig. 10 and Table II). Under identical experimental conditions Br-cGMP did not significantly affect the rate or magnitude of the Mn^{2+} quench produced by a maximal dose of InsP_{3} (5 μM). This indicates that pretreatment with cGMP increases the number of InsP_{3}-sensitive Ca^{2+} channels that can be activated in the presence of submaximal concentrations of InsP_{3} without altering the size of the InsP_{3}-sensitive Ca^{2+} pool, as measured by the Mn^{2+} quench response to a maximal dose of InsP_{3}.

**DISCUSSION**

In the present study we have shown that cGMP increases [Ca^{2+}], and potentiates the Ca^{2+}-mobilizing action of receptor agonists in rat hepatocytes. The effects of cGMP on hepatocyte Ca^{2+} homeostasis are mediated by activation of G-kinase and mimicked by the spontaneous release of NO through treatment with SNP. The [Ca^{2+}]_{i} responses induced by cGMP display a similar temporal organization to those obtained with phenylephrine and other InsP_{3}-linked agonists and are observed in the absence of extracellular Ca^{2+}. These findings suggest that alterations in plasma membrane Ca^{2+} fluxes are not integral to the basic mechanism of cGMP-induced [Ca^{2+}]_{i} increases and indicate a site of action at the level of intracellular Ca^{2+} stores. This is further supported by results obtained with the Mn^{2+}...
quench protocol to directly monitor the permeability of intracellular Ca^{2+} channels in intact hepatocytes. These experiments utilize Mn^{2+} to permeate intracellular Ca^{2+} channels and quench the fluorescence of compartmentalized fura-2. The high affinity of fura-2 for Mn^{2+} permits the rate and magnitude of Mn^{2+} quenching to be used as indices of net channel permeability and the size of the accessible intracellular stores, respectively (42, 44). The data obtained with this experimental paradigm show that cGMP increases the rate of Mn^{2+} quench of compartmentalized fura-2 in intact hepatocytes by initiating a series of quench steps that comprise essentially all of the ionomycin-sensitive intracellular Ca^{2+} store. Similar steps of Mn^{2+} quench have been described previously with receptor agonists and have been interpreted to reflect the sequential opening and closing of the intracellular channels responsible for the generation of [Ca^{2+}] oscillations (44).

The ability of both cGMP and receptor agonists to promote the Mn^{2+} quench of the entire ionomycin-sensitive store suggests that both agents stimulate [Ca^{2+}], release from intracellular stores that are luminally continuous (44). This is also supported by the finding that cGMP fails to produce additional quench when the response to phenylephrine has gone to completion. The effects of cGMP and phenylephrine on the Mn^{2+} quench of compartmentalized dye could result from 1) both stimuli activating identical intracellular Ca^{2+} channels, or 2) be due to activation of distinct Ca^{2+} channels that have access to luminally connected intracellular compartments. For inositol phospholipid-specific phospholipase C-linked agonists such as phenylephrine, [Ca^{2+}], mobilization has clearly been shown to be mediated by the binding of InsP_{3} to an intracellular receptor that functions as a release channel for luminal Ca^{2+} (6). The effects of cGMP on InsP_{3}-induced Mn^{2+} quench in permeabilized hepatocytes, and the similar kinetics and amplitude of cGMP-induced [Ca^{2+}], responses to those of hormones in intact hepatocytes, suggest that cGMP and phenylephrine release [Ca^{2+}], through common intracellular Ca^{2+} channels.

The data obtained with the back phosphorylation assay further indicate that cGMP targets the InsP_{3} receptor Ca^{2+} channel by phosphorylating PKA-sensitive sites on the type-1 InsP_{3} receptor. It is known that PKA phosphorylates two sites on the type-1 InsP_{3} receptor at serine 1755 and 1589 (49) and that
G-kinase only catalyzes the phosphorylation of one site at serine 1755 (22). The reduction in 32P incorporation into the immunoprecipitated InsP3 receptor by PKA after treatment of intact hepatocytes with cGMP was about 55% of that obtained with cAMP. This is similar to the results of a previous study where maximal 32P incorporation into the purified cerebellar InsP3 receptor (exclusively type-1) in the presence of G-kinase was shown to be 49–65% of that generated by PKA (22). Our finding that the InsP3 receptor is not fully phosphorylated in the presence of db-cAMP and that the combined effects of cGMP and cAMP on InsP3 receptor phosphorylation are non-additive is consistent with the fact that serine 1755 is preferentially phosphorylated by PKA (49). Thus, extensive phosphorylation of this residue by PKA could preclude further phosphorylation in the presence of activators of both PKA and G-kinase. Overall, the combined effects of cGMP and cAMP on InsP3 receptor phosphorylation in intact hepatocytes are consistent with the observed composite action of G-kinase and PKA on in vitro InsP3 receptor phosphorylation (22).

The functional effects of InsP3 receptor phosphorylation on InsP3-induced \([\text{Ca}^{2+}]\) release vary between different cell types and have even been shown to be inconsistent in studies on the same cell type (7). For example, phosphorylation by PKA in cerebellum (50) and platelets (51) inhibits InsP3-induced Ca2+ release in microsomal membranes. By contrast, other reports have established that PKA phosphorylation of a homotetrameric type-1 cerebellar InsP3 receptor in lipid vesicles increases InsP3-induced Ca2+ release (52). Treatment of permeabilized hepatocytes with PKA catalytic subunit also potentiates InsP3-induced \([\text{Ca}^{2+}]\), release (46, 47), and PKA activation in intact hepatocytes phosphorylates the type-1 InsP3 receptor and increases its sensitivity to Ca2+ and InsP3 measured subsequent to permeabilization (8). In addition, activation of protein kinase C in isolated liver nuclei also appears to stimulate InsP3-induced \([\text{Ca}^{2+}]\), release (53). Our data indicate that phosphorylation of PKA-sensitive sites on the InsP3 receptor in intact hepatocytes by cGMP-mediated G-kinase activation potentiates submaximal InsP3-induced \([\text{Ca}^{2+}]\), release in subsequently permeabilized hepatocytes. We propose that this sensitizing action underlies the generation of \([\text{Ca}^{2+}]\) oscillations by cGMP in single hepatocytes and its ability to potentiate \([\text{Ca}^{2+}]\) responses to receptor agonists.

Our data differ from results recently obtained with NO donors and cGMP analogues in hepatocyte couplets (33, 34).

Thus, Dufour et al. (33) have shown that pretreatment with SIN-1 and cGMP inhibits agonist-induced \([\text{Ca}^{2+}]\) increases and bile canaliculal contraction in rat hepatocyte doublets. By contrast, Burgstahler and Nathanson (34) have shown that pretreatment with SNP potentiates, and cGMP analogues have no effect on the rate of vasopressin-induced canaliculal contraction in rat hepatocyte couplets. SNP produced a small \([\text{Ca}^{2+}]\), increase in this study, but neither SNP nor cGMP analogues had any effect on agonist-induced \([\text{Ca}^{2+}]\), responses. The reasons for the contradictory findings of these two reports and the differences between the results of the present study are unknown. It is possible that an elevation in the levels of constitutive PKA phosphorylation may have precluded or altered the effects of cGMP in the earlier studies. It is also possible that the InsP3-Sensitive Ca2+ stores could have been depleted during the 15-min cGMP pretreatment protocols used by Dufour et al. (33). In addition, Burgstahler and Nathanson (34) have shown that high concentrations of SIN-1, in the absence of superoxide dismutase, can be toxic to hepatocytes due to peroxynitrite formation.

The results of the present study also differ from those obtained in smooth muscle where phosphorylation of the InsP3 receptor by G-kinase (22) is associated with an inhibition of InsP3-induced Ca2+ release (54). The reason for these differences between cell types is unclear, although they could reflect the presence or absence of regulatory factors that mediate InsP3 receptor phosphorylation or by the distribution of different isoforms of the InsP3 receptor. The contribution of membrane environment to the functional effects of InsP3 receptor phosphorylation has been documented in hepatocytes, where the ability of PKA to stimulate InsP3 receptor binding in permeabilized cells is lost when the InsP3 receptor is detergent-solubilized (8). The expression of different receptor subtypes could also influence the degree to which cyclic nucleotides can phosphorylate the InsP3 receptor since the PKA phosphorylation sites present on the type-1 InsP3 receptor are not conserved in the type-2 or type-3 InsP3 receptors (55). This diversity in InsP3 receptor function could be extended further by the propensity of different InsP3 receptor subunits to form homo- or heterotetrameric assemblies (56, 57).

An additional finding of the present study was that cGMP-induced \([\text{Ca}^{2+}]\) responses in hepatocytes are not dependent on stimulated Ca2+ entry. This excludes the possibility that \([\text{Ca}^{2+}]\) responses to cGMP are mediated by a direct action of cGMP on plasma membrane Ca2+ channels. This is also supported by the finding that cGMP still potentiates agonist-induced \([\text{Ca}^{2+}]\), responses in the absence of extracellular Ca2+ (data not shown). Our data also indicate that Ca2+ entry does not contribute significantly to the elevation of \([\text{Ca}^{2+}]\), as the amplitude and time to peak of cGMP-induced \([\text{Ca}^{2+}]\), responses are unaffected by removal of extracellular Ca2+.

In conclusion, the data presented here demonstrate that the effects of cGMP on cellular Ca2+ homeostasis in rat hepatocytes are mediated by activation of G-kinase. Phosphorylation of the InsP3 receptor by cGMP increases the sensitivity to InsP3 for \([\text{Ca}^{2+}]\), oscillations at the single cell level. These findings describe a new functional role for NO and cGMP in hepatocytes and reveal an additional regulatory mechanism whereby intracellular messengers modulate Ca2+ signaling in liver by regulating InsP3 receptor function.

### TABLE II

| Effect of cGMP on InsP3-Induced Mn2+ quench of compartmentalized fura-2 in permeabilized hepatocytes | Initial rate | Pool size |
|---|---|---|
| 150 nM InsP3 | 560 ± 63 | 31.1 ± 1.4 |
| 150 nM InsP5 + 1 mM Br-cGMP | 1037 ± 130x | 39.2 ± 1.5x |
| 5 μM InsP3 | 5095 ± 524 | 50.9 ± 0.9 |
| 5 μM InsP5 + 1 mM Br-cGMP | 6012 ± 451 | 51.9 ± 0.8 |

*Statistically different from Mn2+ quench responses to 150 nM InsP3 obtained in the absence of Br-cGMP, calculated by Student’s t test (p < 0.05).
Cyclic GMP Increases \([\text{Ca}^{2+}]\), in Hepatocytes