Regulation of Nitric Oxide-Responsive Recombinant Soluble Guanylyl Cyclase by Calcium†

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ABSTRACT: Calcium (Ca^{2+}) and cyclic GMP (cGMP) subserve antagonistic functions that are reflected in their coordinated reciprocal regulation in physiological systems. However, molecular mechanisms by which Ca^{2+} regulates cGMP-dependent signaling remain incompletely defined. In this study, the inhibition of recombinant nitric oxide (NO)-stimulated soluble guanylyl cyclase (SGC) by Ca^{2+} was demonstrated. The α- and β-subunits of recombinant rat SGC were heterologously coexpressed in HEK 293 cells which do not express NO synthase, whose Ca^{2+}-stimulated activity can confound the effects of that cation on SGC. Ca^{2+} inhibited basal and NO-stimulated SGC in a concentration- and guanine nucleotide-dependent fashion. This cation inhibited SGC in crude cell extracts and immunopurified preparations. Ca^{2+} lowered both the V_{max} and K_m of SGC via an uncompetitive mechanism through direct interaction with the enzyme. In intact HEK 293 cells, increases in the intracellular Ca^{2+} concentration induced by ionomycin, a Ca^{2+} ionophore, and thapsigargin, which releases intracellular stores of that cation, inhibited NO-stimulated intracellular cGMP accumulation. Similarly, carbachol-induced elevation of the intracellular Ca^{2+} concentration inhibited NO-stimulated intracellular cGMP accumulation in HEK 293 cells. These data demonstrate that SGC behaves as a sensitive Ca^{2+} detector that may play a central role in coordinating the reciprocal regulation of Ca^{2+}- and cGMP-dependent signaling mechanisms.

Guanylyl cyclases (GCs)[1] and their product, cGMP, are essential components of signaling cascades which mediate important physiological processes, including vascular smooth muscle contractility, retinal phototransduction, and intestinal secretion (*1*, *2*). Signaling is initiated through interaction of specific ligands with the amino-terminal binding domains of these proteins, which activates the carboxyl-terminal catalytic apparatus, resulting in the production of cGMP. While the molecular mechanisms underlying activation have been established, those mediating inactivation of GCs, which are important in the dynamic control of the intracellular cGMP concentration, remain incompletely understood.

In a number of tissues, Ca^{2+} and cGMP appear to subserve antagonistic functions associated with reciprocal and coordinated regulation of their cellular concentrations. In vascular smooth muscle, increases in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) are required for contraction while increases in the intracellular cGMP concentration ($[cGMP]_i$) mediate relaxation (*3*−*10*). Similarly, in retinal photoreceptor cells, increases in $[Ca^{2+}]_i$ are associated with the resting dark state while increases in $[cGMP]_i$ mediate dark recovery and adaptation following exposure to light (*11*−*14*). Close coordination of $[cGMP]_i$ and $[Ca^{2+}]_i$ regulating opposing cellular processes suggests that cytosolic Ca^{2+} may directly interact with and modulate GCs. However, this hypothesis has been difficult to examine due, in part, to the confounding effects imposed by multiple Ca^{2+}-dependent regulatory mechanisms that coexist with GCs in cells. In this regard, defining GC regulation at the level of recombinant proteins is a necessary step in further evaluating mechanisms modulating this important signaling protein.

SGCs, one of the most abundant isotypes of this enzyme family found in almost all mammalian tissues, are essential components of the NO signaling mechanism (*1*, *2*). This enzyme is a heterodimer of α- and β-subunits that are products of separate genes, and coexpression of both subunits

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[1] Abbreviations: BSA, bovine serum albumin; $[Ca^{2+}]_i$, intracellular calcium concentration; cDNA, complementary DNA; cGMP, guanosine 3′,5′-cyclic monophosphate; $[cGMP]_i$, intracellular concentration of cGMP; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; *F*, intensity of fluorescence; F_{max}, maximal fluorescence intensity; F_{min}, minimal fluorescence intensity; GC, guanylyl cyclase; GCA, guanylyl cyclase A; GCAPs, guanylyl cyclase activating proteins; $[GTP]_i$, intracellular concentration of GTP; HEK 293, human embryonic kidney cells; HPLC, high-pressure liquid chromatography; IBMX, isobutylmethylxanthine; IC_{50}, concentration yielding 50% inhibition; K_d, dissociation constant; K_i, concentration yielding half-maximal inhibition; NO, nitric oxide; PMSF, phenylmethanesulfonyl fluoride; PP_i, pyrophosphate; SNP, sodium nitroprusside; SGC, soluble guanylyl cyclase; TCA, trichloroacetic acid; TD buffer, Tris/DTT/PMSF buffer; 293-sGC4, HEK 293 cells expressing recombinant NO-activated rat soluble guanylyl cyclase.

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is required for catalytic activity and NO regulation (*1*, *2*). NO binds to a heme prosthetic group associated with the amino-terminal domains of α- and β-subunits, activating the carboxyl-terminal catalytic domains and cGMP production (*1*, *2*). In this study, recombinant α- and β-subunits of rat SGC were stably coexpressed in HEK 293 human embryonic kidney cells so we could directly examine the effects of Ca^{2+} on that enzyme. HEK 293 cells represent a unique model for investigating the regulation of recombinant SGC by Ca^{2+} since these cells do not express NO synthase, whose Ca^{2+}-dependent activity could confound studies of the direct regulation of SGC by that cation (*15*). This study demonstrates that Ca^{2+} directly inhibits recombinant SGC by a guanine nucleotide-dependent uncompetitive mechanism that regulates $[cGMP]_i$ under physiological conditions in intact cells. SGC itself appears to be a sensitive Ca^{2+} detector that may serve as one limb of a reciprocal feedback mechanism coordinating $[cGMP]_i$ and $[Ca^{2+}]_i$.

EXPERIMENTAL PROCEDURES

Expression of Recombinant Rat SGC in HEK 293 Cells. cDNAs for subunits α1 and β1 of rat SGC in the expression plasmid pcDL-SRa (*16*) were cotransfected into HEK 293 cells using lipofectamine (GIBCO) according to the manufacturer's instructions. Individual clones were selected using G418 and assayed for cGMP accumulation in response to sodium nitroprusside (SNP). Several clones were obtained, and the clone expressing the highest level of GC activity (293-sGC4) was employed.

Immunopurification of SGC. SGC was subjected to immunoaffinity chromatography employing a monoclonal antibody to the β-subunit of SGC so that the enzyme could be purified in a single step, as described previously (*17*, *18*). Briefly, confluent cultures of 293-sGC4 cells were washed, collected, and homogenized in ice-cold Tris buffer (50 mM, pH 7.5) containing 1 mM DTT and 1 mM PMSF (TD buffer) and supernates containing SGC prepared by centrifugation at 100000*g* for 60 min at 4 °C. Monoclonal antibody B4 (1:500) was incubated with the supernatant for 2−4 h at 4 °C, and protein G−Sepharose (50 μL/mL) was added and incubated for a further 60 min. Beads were collected and washed six times in TD buffer. SGC employed in studies described below remained attached to the immunoaffinity matrix since elution can dissociate the heme prosthetic group which is absolutely required for NO activation.

Cyclic GMP Accumulation in Intact Cells. Confluent cultures of 293-sGC4 cells were seeded in 48-well plates and permitted to reach confluence. Cells were washed twice with Krebs buffer containing 120 mM NaCl, 4.75 mM KCl, 1.44 mM $MgSO_4$, 11 mM glucose, 25 mM Hepes (pH 7.4), and 0.1% BSA. Cells were incubated in Krebs buffer containing 50 μM EGTA and 4 μM ionomycin/100 nM thapsigargin or solvent (DMSO/acetone) as indicated for 15 min. At this time, SNP (0.1 mM) and Ca^{2+} (from 100 μM to 20 mM) were added for 2 min. Cells were lysed with 6% TCA, and the level of cGMP accumulation was determined by RIA (*19*). In other experiments, the effect of carbachol (100 μM), an agonist that releases intracellular Ca^{2+}, was examined. Separate wells were treated with Krebs buffer containing 1 mM Ca^{2+}. After 15 min, SNP and carbachol were added. After 2 min, 6% TCA was added and cGMP levels were determined as previously described (*19*). IBMX was omitted from these assays to minimize cGMP accumulation prior to addition of Ca^{2+} agonists, to ensure that $[Ca^{2+}]_i$ flux was not inhibited by a cGMP-dependent mechanism.

Guanylyl Cyclase Assay. GC activity was determined essentially as previously described (*19*) with some modifications. Supernatants or immunopurified SGC was incubated at 37 °C for 10 min in 0.1 mL of a Tris buffer (50 mM, pH 7.5) containing 500 μM IBMX, 7.5 mM creatine phosphate/20 mM creatine phosphokinase, and the indicated concentrations of MgGTP, calcium, and SNP. In experiments in which unbuffered calcium solutions were employed, 250 μM EGTA was added to control reaction mixtures. Enzyme reactions were terminated by addition of 0.5 mL of sodium acetate (50 mM, pH 4.0) and the mixtures boiled for 5 min. Cyclic GMP was quantified by radioimmunoassay (*19*).

Epifluorescent Digital Imaging. Confluent 293-sGC4 cells were loaded with the esterified form of the fluorescent probe Fluo-3 (Fluo-3AM, dissolved in dimethyl sulfoxide and pluronic acid; Molecular Probes), excited at 488 nM and imaged by epifluorescent digital microscopy at 37 °C. The amount of fluorescence emitted at 520 nM was captured by an intensified charge-coupled device camera, and digitized using an imaging system (Attoflor RatioVision) coupled to an inverted microscope (Zeiss Axiovert-135). An estimate of the cytosolic Ca^{2+} concentration, as a function of Fluo-3AM fluorescence, was calculated according to the equation $[Ca^{2+}] = K_d(F - F_{min}/F_{max} - F)$, where F_{min} and F_{max} are the minimal and maximal fluorescence intensity, respectively, K_d is the dissociation constant of the Fluo-3AM−Ca^{2+} complex (422 nM), and F is the intensity of fluorescence (*20*−*23*). The same protocol used to determine cGMP in 293-sGC4 cells was also applied for cytosolic Ca^{2+} concentration measurements. Results are expressed as means ± the standard error of the mean. Significant differences between two means were determined with the Student's t test ($P < 0.05$ was considered significant).

Quantification of $[GTP]_i$. Intracellular GTP concentrations were quantified following perchloric acid extraction of 293-sGC4 cells (*24*). Neutralized extracts were examined by HPLC as described previously except mobile phase B contained 10% MeOH (*19*). $[GTP]_i$ determinations were adjusted for a recovery of 76 ± 8% determined using external standards processed in parallel with cell samples. Internal cell volume, calculated by measuring the differential levels of retention of ^3H_2O and [^{14}C]mannitol by 293-sGC4 cells, was 2.457 ± 0.179 μL/mg of protein or 1.229 ± 0.073 pL/cell.

Miscellaneous. Buffered Ca^{2+} concentrations were determined as described previously (*25*). All chemicals were from Sigma unless stated otherwise. Monoclonal antibody B4, which recognizes subunit β1 of SGC, was a generous gift from F. Murad (University of Texas, Houston, TX; *16*, *17*). Complementary DNAs encoding subunits α1 and β1 of rat SGC were generously provided by M. Nakane (Abbott Laboratories, Abbott Park, IL). Data analysis was performed using Prism (GraphPad Software Inc., San Diego, CA) unless otherwise indicated. Protein concentrations were determined by the method of Bradford with BSA as the standard (Bio-Rad, Hercules, CA).

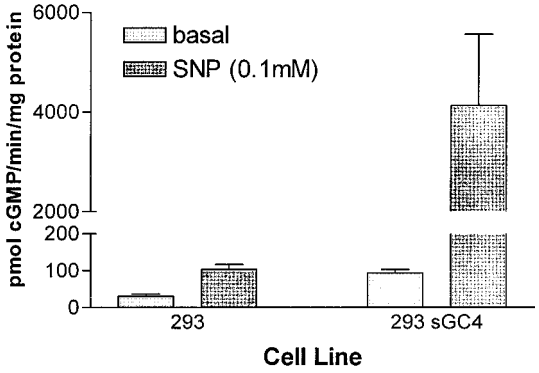

FIGURE 1: Heterologous expression of recombinant soluble guanylyl cyclase in HEK 293 cells. Supernatants containing soluble guanylyl cyclase activity were prepared from parent HEK 293 and recombinant 293-sGC4 cells, and then stimulated with 100 μM SNP or H_2O as described in Experimental Procedures. Results are the mean ± SEM of three determinations performed in duplicate with each sample analyzed in triplicate.

RESULTS

Expression of SGC in HEK 293 Cells and Its Inhibition by Ca^{2+}. Expression of subunits α1 and β1 of SGC in HEK 293 cells produced an increase in SNP-dependent GC activity from soluble cell extracts (Figure 1). HEK 293 cells express low levels of endogenous SGC. Inclusion of SNP increases the endogenous GC activity in HEK 293 cell extracts from 31.0 ± 6.3 to 103.9 ± 12.6 pmol cGMP min^{-1} (mg of protein)$^{-1}$. Extracts prepared from 293-sGC4 cells expressing subunits α1 and β1 of SGC demonstrate a dramatic increase in the activity compared with that of the HEK 293 parent cells. SNP increased the GC activity from 93.6 ± 9.7 to 4129.3 ± 1439.4 pmol of cGMP min^{-1} (mg of protein)$^{-1}$ in 293-sGC4 extracts, confirming the functional expression of recombinant SGC in these cells.

Ca^{2+} Inhibits SGC in 293-sGC4 Cell Extracts. Ca^{2+} can indirectly activate SGC by activating NOS. Since HEK 293 cells do not express NOS (*15*), extracts from 293-sGC4 cells are suitable for examining mechanisms by which Ca^{2+} regulates SGC. Ca^{2+} (250 μM) inhibits both basal and SNP-stimulated GC activity (Figure 2A). Ca^{2+} lowered the basal GC activity from 244.4 ± 19.9 to 117.0 ± 37.5 pmol of cGMP min^{-1} (mg of protein)$^{-1}$ and the SNP-stimulated GC activity from 11 698.2 ± 1173.0 to 2005.3 ± 129.2 pmol of cGMP min^{-1} (mg of protein)$^{-1}$ in 293-sGC4 extracts. Ca^{2+} lowered both the V_{max} and K_m of the SNP-stimulated enzyme ~80% in 293-sGC4 extracts (Figure 2B,C and Table 1).

Ca^{2+} Inhibits SGC by Directly Interacting with the Enzyme. The effects of Ca^{2+} on the kinetic properties of NO-activated SGC immunopurified from 293-sGC4 extracts were examined. Ca^{2+} lowered the SNP-stimulated GC activity from 4075.0 ± 605.7 to 366.4 ± 77.8 pmol of cGMP/min. Ca^{2+} lowered both the V_{max} and K_m of the immunopurified enzyme (Figure 3 and Table 1), in close agreement with results obtained with crude soluble extracts from those cells (compare with Figure 2 and Table 1). Decreases in both the V_{max} and K_m of immunopurified SGC suggest that Ca^{2+} directly regulates this enzyme.

Ca^{2+} Inhibition of SGC Is Guanine Nucleotide-Dependent. Although the above studies demonstrate that Ca^{2+} directly inhibits SGC, they were performed employing EGTA as a calcium chelator in control incubations. However, EGTA is only a selective, not specific, calcium chelator and also affects free Mg^{2+} concentrations, a substrate cofactor required by GCs. To minimize the effect of EGTA on the substrate cation cofactor concentration in control incubations, the concentration response of heterologously expressed recombinant SGC to Ca^{2+} was determined using buffered Ca^{2+}

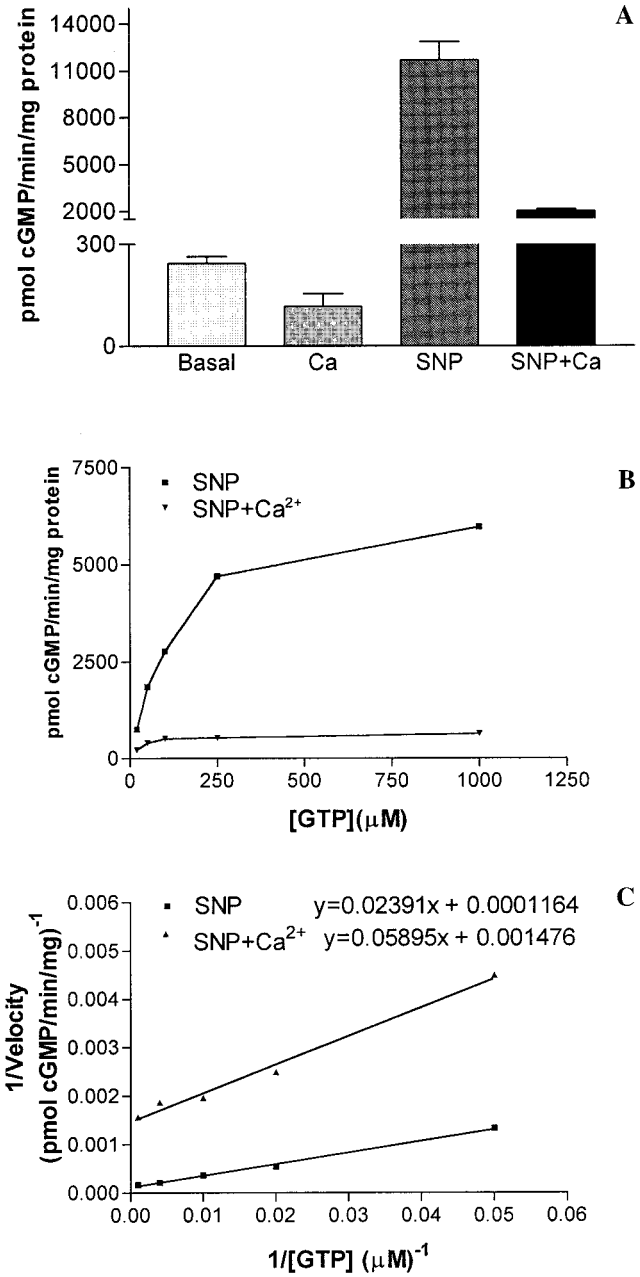

FIGURE 2: Ca^{2+} inhibition of recombinant soluble guanylyl cyclase. (A) Effect of Ca^{2+} on basal and SNP-stimulated recombinant soluble guanylyl cyclase activity in supernatants prepared from 293-sGC4 cells. Supernatants were prepared and guanylyl cyclase stimulated in the presence and absence of 250 μM Ca^{2+}, as described in the legend of Figure 1. Results are the mean ± SEM of three determinations performed in duplicate with each sample analyzed in triplicate. (B and C) Supernatants were stimulated with SNP (100 μM) in the presence of increasing concentrations of the substrate Mg^{2+}−GTP. (B) Michaelis−Menten analysis of SNP-stimulated soluble guanylyl cyclase in the presence of 250 μM Ca^{2+} (▼, SNP and Ca^{2+}) or 250 μM EGTA (■, SNP). (C) Lineweaver−Burke analysis of SNP-stimulated soluble guanylyl cyclase in the presence of Ca^{2+} (▲, SNP and Ca^{2+}) or EGTA (■, SNP). The results are representative of eight supernatants, which are summarized in Table 1.

Table 1: Effect of Calcium on the Kinetic Parameters of Crude and Immunopurified Soluble Guanylyl Cyclase[a]

	293-sGC4 extract		immunopurified sGC	
	EGTA (250 μM)	Ca^{2+} (250 μM)	EGTA (250 μM)	Ca^{2+} (250 μM)
V_{max}[b]	2727.0 ± 1083.2[c]	501.2 ± 111.3	4075.0 ± 605.7	366.4 ± 77.8
K_m (μM)	204.7 ± 80.6	32.3 ± 5.5	238.5 ± 71.0	121.3 ± 71.7

[a] Guanylyl cyclase was prepared and the kinetics analyzed in the presence of 100 μM SNP as described in Experimental Procedures. [b] Supernatant (picomoles of cGMP per minute per milligram of protein), immunopurified (picomoles of cGMP per minute). [c] Mean ± SEM of at least four experiments.

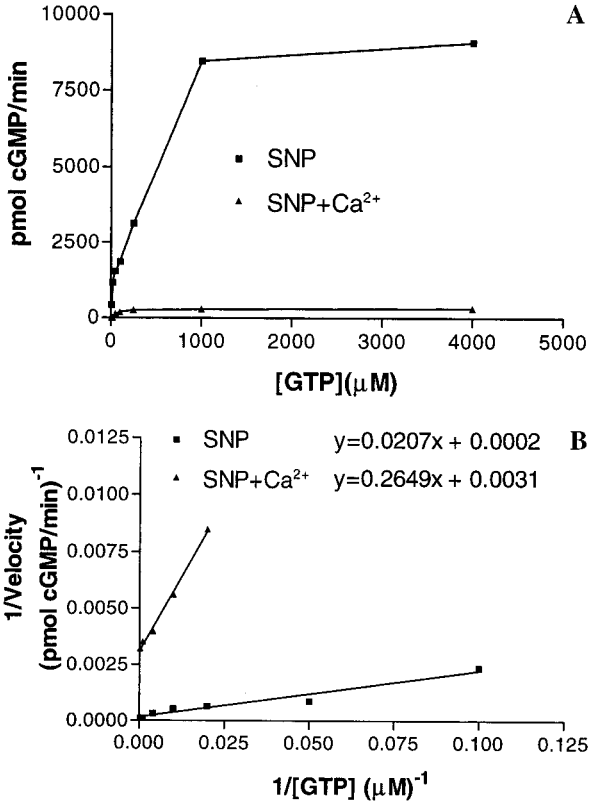

FIGURE 3: Kinetic analysis of Ca^{2+} inhibition of immunopurified recombinant soluble guanylyl cyclase. Immunopurified recombinant soluble guanylyl cyclase was prepared as described in Experimental Procedures. The immunopurified enzyme was stimulated with SNP (100 μM) in the presence of increasing concentrations of the substrate Mg^{2+}−GTP. (A) Michaelis−Menten analysis of SNP-stimulated soluble guanylyl cyclase activity in the presence of Ca^{2+} (▲, SNP and Ca^{2+}) or EGTA (■, SNP). (B) Lineweaver−Burke analysis of SNP-stimulated soluble guanylyl cyclase activity in the presence of Ca^{2+} (▲, SNP and Ca^{2+}) or EGTA (■, SNP). The results are representative of four immunopurified preparations, which are summarized in Table 1.

solutions. Ca^{2+} inhibited the SGC activity in a concentration-dependent fashion by a mechanism dependent on the guanine nucleotide concentration (Figure 4A). The inhibitory constant (K_i) for Ca^{2+} decreased in a linear fashion from 320.7 ± 126.8 to 70.8 ± 8.9 μM as the Mg^{2+}−GTP concentration was increased from 5 to 100 μM (Figure 4B). These data demonstrate that in the physiological range of intracellular GTP concentrations (~200 μM), Ca^{2+} inhibits SGC with a K_i of ~2.6 μM, within the physiological range of Ca^{2+} concentrations in vivo (see the equation in Figure 4B).

Ca^{2+} Inhibits SGC in an Uncompetitive Fashion. The effects of buffered Ca^{2+} concentrations on the kinetic

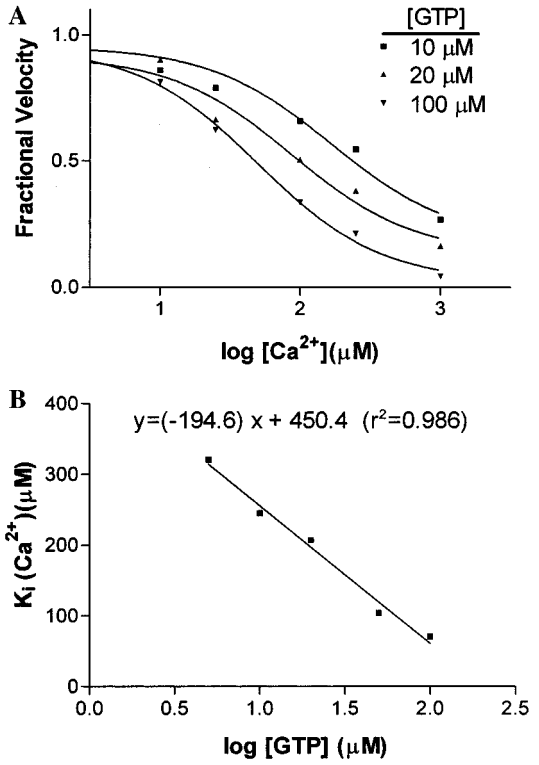

FIGURE 4: GTP dependence of Ca^{2+} inhibition of recombinant soluble guanylyl cyclase. SNP (100 μM)-stimulated soluble guanylyl cyclase activity was incubated with increasing buffered Ca^{2+} concentrations (0.126 μM to 1 mM) in the presence of multiple concentrations of GTP as indicated. (A) Dose response of Ca^{2+} inhibition of soluble guanylyl cyclase activity. The maximal velocity (fractional velocity = 1.0) was assigned from the velocity determined in the presence of 0.126 μM Ca^{2+} at a given GTP concentration [10 μM GTP, V_{max} = 1364.4 pmol of cGMP min^{-1} (mg of protein)$^{-1}$; 20 μM GTP, V_{max} = 2517 pmol of cGMP min^{-1} (mg of protein)$^{-1}$; 100 μM GTP, V_{max} = 7782.0 pmol of cGMP min^{-1} (mg of protein)$^{-1}$]. Curves were generated by nonlinear regression analysis. The results shown are representative of three experiments. (B) Relationship of Ca^{2+} K_i and substrate concentration. K_i values were from nonlinear regression analysis of dose responses to buffered Ca^{2+} concentrations determined as described for panel A. Individual data points are the means of three experiments. The equation shown in the panel B describes the linear regression analysis of the data points.

properties of GC were closely examined (Figure 5). Lineweaver−Burke analysis revealed uncompetitive inhibition of GC by Ca^{2+}, demonstrated by the parallel isotherms obtained with increasing concentrations of Ca^{2+}. Also, Ca^{2+} reduced both the V_{max} and K_m of SGC in a concentration-dependent manner, characteristic of an uncompetitive mechanism of inhibition (Table 2). Inhibition through an uncompetitive mechanism in conjunction with the observations that Ca^{2+} inhibits immunopurified SGC (Figure 3) and inhibition is guanine nucleotide-dependent (Figure 4) suggests that Ca^{2+} interacts with either the substrate (Mg^{2+}−GTP) or one of the products (cGMP or PP_i) to repress the activity at the catalytic site. These properties are similar to P-site inhibition of adenylyl cyclases where cAMP analogues irreversibly bind to the catalytic apparatus in the presence of the product Mg^{2+}−PP_i (*26*).

Elevating $[Ca^{2+}]_i$ Inhibits NO-Dependent $[cGMP]_i$ Accumulation in 293-sGC4 Cells. The linear relationship between the Ca^{2+} inhibitory constant (K_i) and guanine nucleotide concentration suggests that Ca^{2+} inhibits SGC in

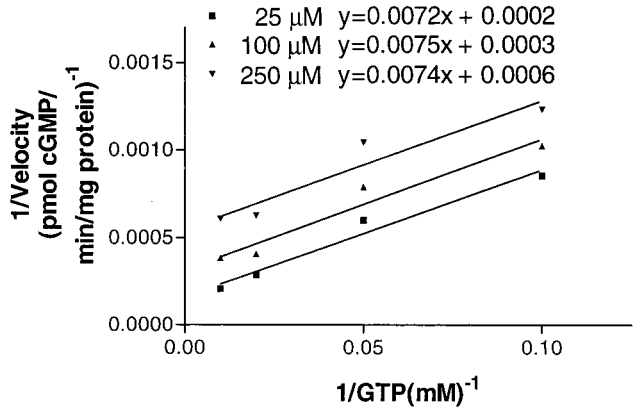

FIGURE 5: Lineweaver−Burke analysis of recombinant soluble guanylyl cyclase with different concentrations of buffered Ca^{2+}. SNP (100 μM)-stimulated soluble guanylyl cyclase activity was incubated with three buffered Ca^{2+} concentrations (25, 100, and 250 μM) in the presence of increasing concentrations of GTP as indicated. Michaelis−Menten kinetics were subjected to double-reciprocal plot analysis: (■) 25 μM Ca^{2+}, (▲) 100 μM Ca^{2+}, and (▼) 250 μM Ca^{2+}. Results shown are representative of three experiments which are summarized in Table 2. The equations describe the linear regression analysis of the data points.

Table 2: Kinetic Parameters of Soluble Guanylyl Cyclase with Buffered Calcium Concentrations[a]

	0.126 μM free Ca^{2+}	25 μM free Ca^{2+}	100 μM free Ca^{2+}	250 μM free Ca^{2+}
V_{max}[b]	8740.4 ± 1054.0[c]	5501.1 ± 715.4	3128.8 ± 283.1	1873.2 ± 308.4
K_m (μM)	53.2 ± 7.0	38.1 ± 6.2	23.2 ± 1.4	15.1 ± 3.8

[a] Guanylyl cyclase was prepared and the kinetics were analyzed in the presence of 100 μM SNP as described in Experimental Procedures. [b] Picomoles of cGMP per minute per milligram of protein. [c] Mean ± SEM of at least four experiments.

intact cells. This hypothesis was examined by investigating the effect of increasing $[Ca^{2+}]_i$ on SNP-dependent intracellular cGMP accumulation in 293-sGC4 cells. Addition of extracellular Ca^{2+} to cells treated with ionomycin, a Ca^{2+} ionophore, in the presence of thapsigargin, a Ca^{2+}-ATPase inhibitor, significantly increased $[Ca^{2+}]_i$ in a concentration-dependent manner (Figure 6A). This increase in $[Ca^{2+}]_i$ was associated with a concomitant reduction in the extent of SNP-induced cGMP accumulation (Figure 6B). Carbachol moderately increases $[Ca^{2+}]_i$ in intact HEK 293 cells (Figure 7, left and middle panels; *28*−*30*). Increases in $[Ca^{2+}]_i$ induced by carbachol (100 μM) were associated with significant reductions in the extent of SNP-induced cGMP accumulation (~50%, $p \leq 0.05$) in 293-sGC4 cells (Figure 7, right panel). In these experiments, the IC_{50} of $[Ca^{2+}]_i$, ranging from 400 nM (carbachol-treated cells; Figure 7, right panel) to 8 μM (extracellular Ca^{2+}-, ionomycin-, and thapsigargin-treated cells; Figure 6) predicts a [GTP] of 199 ± 7 μM (mean ± SEM, $n = 3$) in 293-sGC4 cells (see the equation in Figure 4B).

Concentration of GTP in 293-sGC4 Cells. The above studies, in which $[Ca^{2+}]_i$ and the extent of inhibition of SNP-induced cGMP accumulation were empirically determined, predict that [GTP] is 199 ± 7 μM (mean ± SEM, $n = 3$) in 293-sGC4 cells. Analysis of intracellular nucleotide concentrations revealed that the [GTP] in those cells was 211 ± 83 μM (mean ± SEM, $n = 3$), nearly identical to that predicted by the relationship between $[Ca^{2+}]_i$ and the extent of

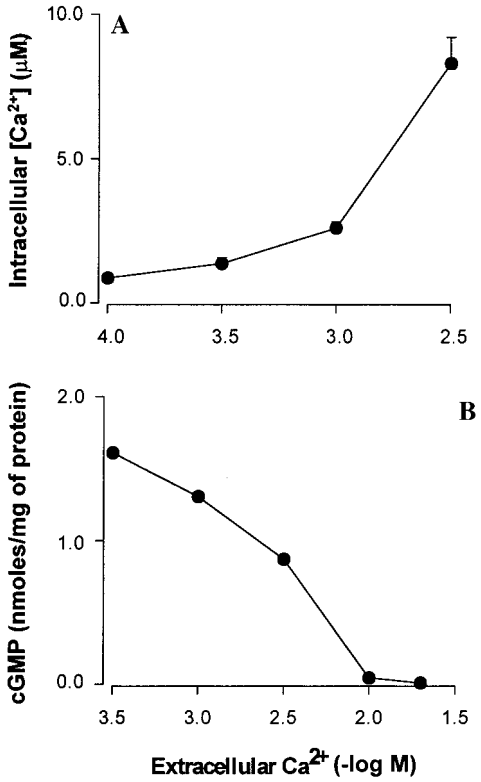

FIGURE 6: Concentration−response relationship between extracellular Ca^{2+} concentration and intracellular Ca^{2+} concentration or the extent of cGMP accumulation in 293-sGC4 cells treated with ionomycin and thapsigargin. Cells were treated with 4 μM ionomycin/100 nM thapsigargin in Krebs buffer for 15 min prior to exposure to 100 μM SNP and the indicated concentrations of extracellular Ca^{2+}. Either intracellular Ca^{2+} (A) or cGMP (B) levels were determined. Values are means ± SEM ($n = 6$−8).

inhibition of SNP-induced cGMP accumulation in those cells. These data confirm the linear relationship between the K_i for Ca^{2+} and log($[GTP]_i$) (see Figure 4) and support the suggestion that $[Ca^{2+}]_i$ inhibits SNP-activated SGC in cells in a guanine nucleotide-dependent fashion.

DISCUSSION

Cyclic GMP and Ca^{2+} mediate opposing mechanisms of central importance in a variety of physiological processes, reflected in coordinated and reciprocal regulation of their intracellular concentrations. However, mechanisms coupling regulation of $[cGMP]_i$ and $[Ca^{2+}]_i$ remain incompletely understood. In this study, we define one limb of this feedback mechanism wherein Ca^{2+} directly inhibits SGC by a guanine nucleotide-dependent uncompetitive mechanism. This mechanism coordinates intracellular concentrations of those messengers since physiological or pharmacological increases in $[Ca^{2+}]_i$ prevent NO-stimulated cGMP accumulation in intact cells. This is the first demonstration that $[Ca^{2+}]_i$ attenuates the cGMP signaling cascade by directly interacting with guanylyl cyclase.

Previous studies examined the ability of Ca^{2+} to support GC catalytic activity as the required cation cofactor in crude membrane and cytosolic extracts from rat lung and heart (*32*−*34*). Those studies concluded that Ca^{2+} was less efficacious than either Mn^{2+} or Mg^{2+} as the divalent cation required to form a complex with GTP and support catalysis (*32*−*34*). In this study, Ca^{2+} inhibited basal and NO-activated

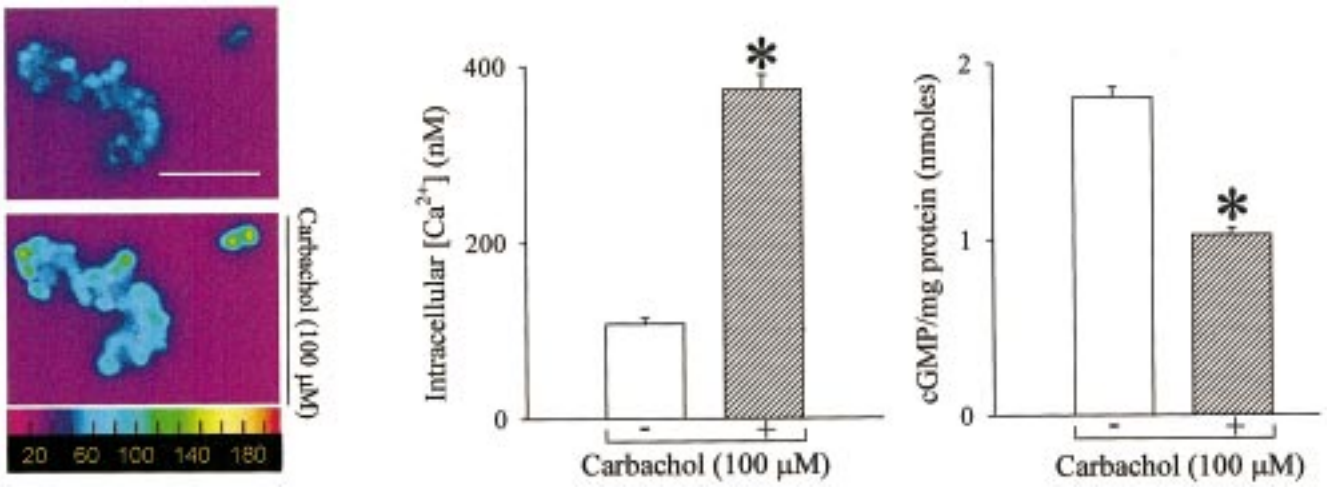

FIGURE 7: Carbachol-induced increases in $[Ca^{2+}]_i$ and inhibition of SNP-induced cGMP production in 293-sGC4 cells. Cells were treated with 100 μM carbachol in Krebs buffer for 15 min prior to exposure to 100 μM SNP and extracellular Ca^{2+}. Either intracellular Ca^{2+} (left and center panels) or cGMP (right panel) levels were determined. Intracellular Ca^{2+} values are means ± SEM ($n = 6-8$). Epifluorescent images of a population of Fluo-3-loaded 293-sGC4 cells (>100) prior to and following exposure to carbachol (left panel). The increase in the intensity of fluorescence, in the presence of carbachol, reflects increases in the cytosolic Ca^{2+} concentration in the population of imaged cells. The horizontal bar corresponds to 100 μM. The extracellular Ca^{2+} concentration was 1 mM throughout. Cyclic GMP determinations are representative of at least three experiments. Asterisks denote $p \leq 0.05$.

crude and immunopurified recombinant SGC in the presence of excess $Mg^{2+}-GTP$, the cation preferred for supporting ligand activation of GCs (*1*, *2*). Inhibition was associated with an increase in the affinity of the enzyme for substrate since Ca^{2+} decreased both the K_m and V_{max} of SGC. Also, inhibition was guanine nucleotide-dependent, and increasing concentrations of the substrate, $Mg^{2+}-GTP$, increased the ability of Ca^{2+} to inhibit SGC. Thus, inhibition of basal and NO-activated SGC does not simply reflect the inability of Ca^{2+} to support catalytic activity as the substrate cation cofactor.

Rather, Ca^{2+} inhibits SGC by directly interacting with that enzyme, regulating the activity by a guanine nucleotide-dependent uncompetitive mechanism. The kinetic characteristics of Ca^{2+} inhibition of SGC are identical to those of P-site inhibition of adenylyl cyclases (*26*). Thus, Lineweaver−Burke analysis of adenylyl cyclase activity revealed a series of parallel lines with increasing P-site inhibitor concentrations (*26*, *35*). Similarly, increasing the nucleotide substrate concentration enhanced the potency of P-site inhibitors (*35*). The identity between the characteristics of Ca^{2+} inhibition of SGC and P site inhibition of adenylyl cyclase suggests that these enzymes are regulated by a common catalytically based mechanism. P-site inhibitors combine with one of the products of catalysis, $Mg-PP_i$, to form a dead-end inhibitor at the catalytic site of adenylyl cyclase (*26*, *35*). Substrate-dependent uncompetitive inhibition of SGC by Ca^{2+} suggests that this cation stabilizes the substrate (GTP) or products (cGMP and PP_i) in the active site, preventing catalytic apparatus function.

Inhibition by Ca^{2+} appears to be a general characteristic of nucleotide cyclases. Thus, Ca^{2+} also inhibits adenylyl cyclases, particularly types V and VI (*36*, *37*). Although the kinetic mechanism remains undefined, the ability of Ca^{2+} to inhibit adenylyl cyclases is comparable to the ability of Ca^{2+} to inhibit SGC. In the presence of 100 μM ATP, 27 μM Ca^{2+} inhibited type V adenylyl cyclase expressed in Sf9 cells ~40% (*36*) while 25 μM Ca^{2+} inhibited SGC ~40% with 100 μM GTP (see Figure 4A). It is likely that the catalytic mechanisms of guanylyl and adenylyl cyclases are similar, on the basis of their primary sequence homology, substrate and cation cofactor requirements, and product formation, suggesting that Ca^{2+} may be a mechanistic inhibitor of both. Deletion of part of the C1 catalytic domain of adenylyl cyclase decreases the sensitivity to inhibition by Ca^{2+}, suggesting that Ca^{2+} inhibits this cyclase by interacting with the active site (*37*). Similarly, uncompetitive inhibition of SGC by Ca^{2+} suggests the involvement of the active site of guanylyl cyclase in mechanisms underlying inhibition. However, the Ca^{2+} binding site in the C2 domain of type V adenylyl cyclase proposed to mediate Ca^{2+} inhibition is not conserved in either the α- or β-subunits of SGC (*37*). Further investigation is required to determine whether a common mechanism mediates Ca^{2+} inhibition of adenylyl and guanylyl cyclases.

Studies of SGC inhibition by direct interaction with Ca^{2+} in cell-free and immunopurified preparations were extended to the ability of increases in $[Ca^{2+}]_i$ to prevent NO-induced intracellular cGMP accumulation in intact HEK 293 cells. Thapsigargin and ionomycin dramatically increased $[Ca^{2+}]_i$, inhibiting NO-induced intracellular cGMP accumulation in 293-sGC4 cells (see Figure 6). Carbachol elicited a more modest physiological increase in $[Ca^{2+}]_i$ that inhibited NO-induced intracellular cGMP accumulation in those cells (see Figure 7). The working hypothesis suggests that attenuation of NO-induced intracellular cGMP accumulation by increases in $[Ca^{2+}]_i$ reflects the uncompetitive inhibition of SGC by that cation that is guanine nucleotide-dependent, with a linear relationship between the K_i of Ca^{2+} and [GTP] (see Figure 4). Attenuation of intracellular cGMP accumulation by thapsigargin/ionomycin or carbachol was associated with an increased $[Ca^{2+}]_i$ which required specific intracellular GTP concentrations, if attenuation was mediated by noncompetitive inhibition of SGC. Empirical quantification of intracellular nucleotide concentrations demonstrated that 293-sGC4 cells contained intracellular GTP concentrations precisely required to support uncompetitive inhibition by increases in $[Ca^{2+}]_i$ produced by thapsigargin/ionomycin or carbachol.

These data strongly support the hypothesis that $[Ca^{2+}]_i$ coordinates the reciprocal regulation of $[cGMP]_i$ through direct uncompetitive inhibition of SGC in intact cells. It should be pointed out that these studies do not, however, exclude the possibility that increases in $[Ca^{2+}]_i$ may inhibit nitric oxide stimulation of $[cGMP]_i$ accumulation via mechanisms other than direct interaction with and inhibition of guanylyl cyclase.

Physiological systems in which coordinated reciprocal regulation of $[Ca^{2+}]_i$ and $[cGMP]_i$ mediate opposing processes include vascular smooth muscle and retinal photoreceptor cells. In vascular smooth muscle, increases in $[Ca^{2+}]_i$ are required for contraction whereas increases in $[cGMP]_i$ induce relaxation by decreasing $[Ca]_i$ (*5*−*10*, *38*). Cyclic GMP decreases $[Ca]_i$ by preventing influx through cell surface channels, inhibiting the production of inositol polyphosphates, increasing the uptake of Ca^{2+} into the sarcoplasmic reticulum, and/or stimulating efflux of Ca^{2+} out of the cell (*5*−*10*, *38*). This study suggests a previously unrecognized level of coordination between Ca^{2+}- and cGMP-dependent signaling cascades in which GC itself is a sensor for that cation. Regulation of SGC by Ca^{2+} may ensure that opposing cGMP-stimulated relaxation does not compromise a committed response of smooth muscle to contraction. Indeed, the instantaneous state of smooth muscle contractility likely reflects the balance between $[Ca^{2+}]_i$, $[GTP]_i$, and $[cGMP]_i$ and their integrated effects on SGC and mechanisms controlling $[Ca^{2+}]_i$.

Cyclic GMP and Ca^{2+} also regulate opposing limbs of the phototransduction cascade in retinal cells. Ca^{2+} flows into these cells through cGMP-gated cation channels that are open in the dark but closed in the light (*11*, *12*). In this signaling system, light activates a cGMP-specific phosphodiesterase and the resultant cyclic nucleotide hydrolysis reduces $[cGMP]_i$, closing cGMP-gated channels (*11*, *12*). Ca^{2+} is continuously extruded through light-independent mechanisms, and consequently, $[Ca^{2+}]_i$ decreases during periods of light when the cGMP-gated cation channels are closed (*12*). Decreases in $[Ca^{2+}]_i$ activate GCAPs, Ca^{2+}-binding proteins that stimulate retinal membrane-bound GC in the presence of low $[Ca^{2+}]_i$ (*13*, *14*). Accumulation of cGMP re-opens cGMP-dependent ion channels, an important step in light adaptation and dark recovery. GCAPs behave as Ca^{2+} switches for retinal membrane-bound GC and exhibit a K_i for Ca^{2+} in the high nanomolar range (*39*−*41*). Interestingly, this study suggests that SGC itself behaves as a sensor of $[Ca^{2+}]_i$. Uncompetitive inhibition involves direct interaction of Ca^{2+} with the catalytic apparatus, the domain with the greatest conservation of structure and function among all members of the GC family (*1*, *2*). Thus, direct uncompetitive inhibition by Ca^{2+} may be a generalized mechanism coordinating $[Ca^{2+}]_i$ and $[cGMP]_i$ in cells expressing different members of the GC family. In this study, Ca^{2+} inhibited NO-induced intracellular cGMP accumulation with a potency in the high nanomolar range (see Figure 7). These data suggest that in the retina, $[Ca^{2+}]_i$ may coordinately regulate $[cGMP]_i$ indirectly, through GCAPs, and/or directly, through uncompetitive inhibition, with potencies for both of these mechanisms near the high nanomolar range.

The possibility that Ca^{2+}-dependent uncompetitive inhibition may be a general characteristic of cytosolic and membrane-bound GCs is suggested by previous experimental observations. Increasing $[Ca^{2+}]_i$ with ionomycin prevented accumulation of cGMP induced by natriuretic peptide, which activates membrane-bound GCA in bovine chromaffin cells (*42*). Also, an increased $[Ca]_i$ prevented accumulation of cGMP in acinar cells, and increases in Ca^{2+} prevented activation of GC in cytosolic fractions of those cells (*43*). Significantly, increases in $[Ca^{2+}]$ upregulated NO synthase, which should stimulate SGC, yet $[cGMP]_i$ was decreased in acinar cells, supporting the suggestion that Ca^{2+} is a potent and efficacious inhibitor of SGC (*44*). Furthermore, Ca^{2+} inhibited GCA from human kidney cells (*43*, *45*), although this effect might be mediated by protein kinase C (*46*). These examples support the suggestion that $[Ca]_i$ may coordinate reciprocal regulation of $[cGMP]_i$ by directly interacting with and inhibiting cytosolic or membrane-bound GCs. Whether Ca^{2+}-dependent uncompetitive inhibition is a general characteristic of the family of GC enzymes is currently being investigating.

In summary, the data presented demonstrate that Ca^{2+} directly inhibits basal and NO-stimulated recombinant SGC through a guanine nucleotide-dependent uncompetitive mechanism, most likely involving interaction of that cation with the catalytic apparatus of the enzyme. Uncompetitive regulation of SGC by Ca^{2+} is reflected in the inhibition of NO-stimulated intracellular cGMP accumulation by pharmacological or physiological increases in $[Ca^{2+}]_i$. Taken together, these studies demonstrate that SGC is a sensitive Ca^{2+} detector. Coordinated reciprocal regulation of $[cGMP]_i$ by $[Ca^{2+}]_i$ likely involves a feedback mechanism in which the production of that cyclic nucleotide by GC is directly inhibited by Ca^{2+}. This mechanism may play an important role in cross talk between Ca^{2+}- and cGMP-dependent signaling mediating a variety of physiological processes in different tissues.

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