Molecular Determinants of Gem Protein Inhibition of P/Q-type Ca\textsuperscript{2+} Channels*\textsuperscript{[S]}

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Background: Gem, an RGK protein, strongly inhibits P/Q-type Ca\textsuperscript{2+} channels. The molecular mechanisms of this inhibition are largely unclear. In Xenopus oocytes, Gem suppresses the activity of P/Q-type Ca\textsuperscript{2+} channels on the plasma membrane. This is presumed to occur through direct interactions of one or more Gem inhibitory sites and the pore-forming Ca\textsubscript{2,1} subunit in a manner dependent on the Ca\textsuperscript{2+} channel subunit \(\beta\) (Ca\textsubscript{1,\beta}). In this study we investigated the molecular determinants in Gem that are critical for this inhibition. Unlike other RGK proteins, Gem contains a conserved Ras-like core and extended N and C termini. A 12-amino acid fragment in the C terminus was found to be crucial for and sufficient to produce Ca\textsuperscript{2+}-dependent inhibition, suggesting that this region forms an inhibitory site. A three-amino acid motif in the core of Gem was also found to be critical, possibly forming another inhibitory site. Mutating either site individually did not hamper Gem inhibition, but mutating both sites together completely abolished Gem inhibition without affecting Gem protein expression level or disrupting Gem interaction with Ca\textsubscript{2,1} or Ca\textsubscript{1,\beta}. Mutating Gem residues that are crucial for interactions with previously demonstrated RGK modulators such as calmodulin, 14-3-3, and phosphatidylinositol lipids did not significantly affect Gem inhibition. These results suggest that Gem contains two candidate inhibitory sites, each capable of producing full inhibition of P/Q-type Ca\textsuperscript{2+} channels.

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Results: A 12-amino acid C-terminal fragment and a 3-amino acid motif in the core of Gem play an important role in this inhibition.

Conclusion: Gem possesses two candidate inhibitory sites.

Significance: This work identifies key molecular determinants of Ca\textsuperscript{2+} channel inhibition by RGK proteins, offering opportunities to modulate this inhibition.

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The RGK family of monomeric GTP-binding proteins potently inhibits high voltage-activated Ca\textsuperscript{2+} channels. The molecular mechanisms of this inhibition are largely unclear. In Xenopus oocytes, Gem suppresses the activity of P/Q-type Ca\textsuperscript{2+} channels on the plasma membrane. This is presumed to occur through direct interactions of one or more Gem inhibitory sites and the pore-forming Ca\textsubscript{2,1} subunit in a manner dependent on the Ca\textsuperscript{2+} channel subunit \(\beta\) (Ca\textsubscript{1,\beta}). In this study we investigated the molecular determinants in Gem that are critical for this inhibition. Unlike other RGK proteins, Gem contains a conserved Ras-like core and extended N and C termini. A 12-amino acid fragment in the C terminus was found to be crucial for and sufficient to produce Ca\textsuperscript{2+}-dependent inhibition, suggesting that this region forms an inhibitory site. A three-amino acid motif in the core of Gem was also found to be critical, possibly forming another inhibitory site. Mutating either site individually did not hamper Gem inhibition, but mutating both sites together completely abolished Gem inhibition without affecting Gem protein expression level or disrupting Gem interaction with Ca\textsubscript{2,1} or Ca\textsubscript{1,\beta}. Mutating Gem residues that are crucial for interactions with previously demonstrated RGK modulators such as calmodulin, 14-3-3, and phosphatidylinositol lipids did not significantly affect Gem inhibition. These results suggest that Gem contains two candidate inhibitory sites, each capable of producing full inhibition of P/Q-type Ca\textsuperscript{2+} channels.

HVA\textsuperscript{2} Ca\textsuperscript{2+} channels, including L-, N-, P/Q- and R-type channels, conduct Ca\textsuperscript{2+} into excitable cells and are important for diverse physiological processes such as neurotransmitter release, hormone secretion, muscle contraction, and gene transcription. They are typically composed of a pore-forming \(\alpha_1\) subunit (Ca\textsubscript{1,\alpha_1}) and auxiliary \(\alpha_2\delta\) and \(\beta\) (Ca\textsubscript{1,\beta}) subunits. The activity of HVA Ca\textsuperscript{2+} channels is regulated by protein kinases, trimeric G proteins, membrane lipids, intracellular Ca\textsuperscript{2+}, and various adaptor and signaling proteins (1, 2). Of the latter, the RGK family of Ras-related monomeric small GTPases, including Rad, Rem, Rem2, and Gem/Kir, has emerged as the most potent endogenous protein inhibitors of HVA Ca\textsuperscript{2+} channels (3–6).

Two modes of action have been proposed for the inhibition of HVA Ca\textsuperscript{2+} channels by RGK proteins; they are reducing the number of surface channels (7–14) or suppressing the activity of channels on the plasma membrane (13, 15–20). Both forms of inhibition require Ca\textsubscript{1,\beta} (14, 15, 21), which interacts directly with RGK proteins (7, 10, 16, 17, 19–24). This interaction has been generally assumed to be essential for RGK inhibition (15, 21). We recently showed, however, that Gem inhibition of surface P/Q-type Ca\textsuperscript{2+} channels expressed in Xenopus oocytes remained intact after the disruption of the Gem-Ca\textsubscript{1,\beta} interaction and that this inhibition appeared to be caused by direct interactions between Gem and Ca\textsubscript{1,\alpha_1} (18). We postulated that Gem and Ca\textsubscript{1,\alpha_1} interact via an anchoring site in a Ca\textsubscript{1,\beta}-independent manner, that Ca\textsubscript{1,\beta} induces the formation of an inhibitory site on Ca\textsubscript{1,\alpha_1}, and that an inhibitory site in Gem binds to this Ca\textsubscript{1,\alpha_1} site to cause inhibition. The regions involved in these interactions on both Ca\textsubscript{1,\alpha_1} and Gem remain to be identified.

The four RGK proteins contain a highly conserved Ras-like core and extended variable N and C termini that are absent in Ras (3, 4). The core contains binding sites for Ca\textsubscript{1,\beta} (10, 18) and the binding and catalytic sites for guanine nucleotides (25, 26). The N and C termini contain binding sites for calmodulin (CaM), 14-3-3, and phosphatidylinositol lipids, which regulate the subcellular distribution and function of RGK proteins (7–9, 27–31). The role of the interactions with these modulators in RGK inhibition of HVA Ca\textsuperscript{2+} channels is controversial and is not fully characterized (4, 11–13, 15, 32).

In this study we investigated the molecular determinants in Gem that are important for its inhibition of P/Q-type Ca\textsuperscript{2+} channels expressed in Xenopus oocytes. We identified two distinct regions in Gem, a 12-amino acid stretch in the C terminus and a 3-amino acid motif in the core, that are critical for Gem inhibitory action and may form two separate inhibitory sites.
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**Experimental Procedures**

Constructs and Cloning—For electrophysiology experiments in *Xenopus* oocytes, cDNAs encoding various constructs were subcloned into a modified oocyte expression vector pGEMHE. The constructs included rabbit brain Cav2.1 (GenBank\(^{\text{TM}}\) accession number X57477), rat skeletal muscle \(\alpha_{\text{d}}, \delta\), rat brain \(\beta_3\) (GenBank\(^{\text{TM}}\) accession number M88751), a mutant \(\beta_3\) named \(\beta_3_{\text{Mut2}}\) bearing the M196A/L200A mutation, and WT or mutated human skeletal muscle Gem (GenBank\(^{\text{TM}}\) accession number BC022010).

For protein synthesis in *Escherichia coli*, DE3 bacteria were used for cDNA transformation and protein expression. Gem-(Ser-68–Arg-264) cDNA encoding residues Ser-68 to Arg-264 and Gem-(Ser-68–Lys-276) cDNA encoding residues Ser-68 to Lys-Lys-276 were subcloned into the pAcycDuet vector (Novagen). Peptides GCPI and GCPI2 were purchased from GL Biochem, and peptide GCPI_5 was purchased from GenScript. For pulldown assays, GCPI, GCPI_5, and Ca2.1-(Val-1899–Glu-1989) cDNA encoding residues from Val-1899 to Glu-1989 were subcloned into modified pET26b (Novagen) with an MBP tag at the N terminus and His tag at the C terminus. *Rattus norvegicus* calmodulin (GenBank\(^{\text{TM}}\) accession number NM_017326) was subcloned into pETDuet-1 (Novagen).

For co-immunoprecipitation experiments in HEK 293T cells (gift of Dr. Hiroaki Matsunami at Duke University), an HA (hemagglutinin) tag was added to the N terminus of full-length Gem cDNA (mutant) with a flexible linker of three glycines in between, and the whole construct was cloned into the pCDNA3.1(–) vector (Invitrogen). An Myc tag was added to the N terminus of full-length rat brain \(\beta_3\) with three glycines in between, and the whole construct was cloned into the pCDNA3.1(–) vector (Invitrogen). Rabbit brain Cav2.1 was used for cDNA transformation and protein expression. Gem-(Ser-68–Arg-264) cDNA encoding residues Ser-68 to Arg-264 was subcloned into a modified oocyte expression vector pGEMHE. For protein synthesis in *Escherichia coli*, DE3 bacteria were transformed (Novagen) with a solution containing 96 mM NaCl, 2.5 mM KCl, 1 mM MgCl2, 1 mM MgATP, and a resistance of 0.2–0.5 megaohms when filled with a solution containing 45 mM BaCl2, 80 mM KCl, and 10 mM HEPES (pH 7.3 with KOH). The bath (i.e. cytoplasmic) solution contained 125 mM KCl, 4 mM NaCl, 10 mM HEPES, and 10 mM EGTA (pH 7.3 with KOH). 0.3 mM phosphatidylinositol diphosphate and 3 mM Mg-ATP were added freshly to the bath solution to attenuate rundown. Macroscopic currents were evoked from a holding potential at −80 mV by 10-ms depolarizations ranging from −30 to +90 mV in 10-mV increments at a 1-s interval.

To obtain the β-less channels described in Fig. 3F, a fast perfusion speed (1.5 ml/min) was used. Wash-off of \(\beta_3\) Mut2 was determined by monitoring the positive shift of the activation curve. To obtain the activation curve, macroscopic currents were evoked by 20-ms depolarizations ranging from −40 to +100 mV in 10-mV increments at a 6-s interval. Tail currents were recorded by repolarization to −40 mV regardless of the preceding test pulse, normalized by that after the depolarization to +100 mV, and plotted against the test potentials.

Currents were sampled at 10 kHz and filtered at 2.5 kHz. All data were analyzed with Clampfit and were represented as the mean ± S.D. (number of observations). Statistical significance was determined using two-tailed Student's t tests.

**Protein Synthesis in E. coli**—Transformed DE3 bacteria were cultured at 37 °C until A\(_{600}\) reached 0.6 and then induced at room temperature by 0.5–1 mM isopropyl 1-thio-β-D-galactopyranoside for overnight. Cells were collected at 4000 rpm for 15 min and resuspended in a lysis solution containing 50 mM Tris-HCl, 250 mM NaCl, 2.5% glycerol, and 7 mM β-mercaptoethanol (pH 7.8). Resuspended bacteria were sonicated with a Branson digital sonifier followed by centrifugation at 14,000 rpm for 40 min. For Gem-(Ser-68–Lys-276) and Gem-(Ser-68–Arg-264) protein purification, the supernatant was collected and incubated with nickel-nitrilotriacetic acid His-Bind beads (Novagen) in the presence of 20 mM imidazole at 4 °C for 1 h. Proteins were sonicated and incubated with nickel-nitrilotriacetic acid His-Bind beads (Novagen) in the presence of 20 mM imidazole at 4 °C for 1 h. Proteins were then eluted from the beads with 200 mM imidazole in the lysis solution. For CaM purification, most bacterial proteins were denatured by incubation at 70 °C for 3 min and then washed with phenyl-Superose resin for 1 h. After washing the resin with a buffer containing 2 mM CaCl\(_2\) and 1 mM MgCl\(_2\), calmodulin protein was eluted with a buffer containing 2 mM EGTA and 1 mM MgCl\(_2\). Proteins were concentrated and further purified by a Superdex 75 gel-filtration chromatography (GE Healthcare).

**Pulldown Assay—**MBP fusion peptides were expressed in DE3 and purified by incubation with amylose resin (New England Biolabs). After elution from the resin with 20 mM maltose, MBP fusion peptides were immobilized on nickel-nitrilotriacetic acid His-Bind beads in PBS buffer with or without Ca\(^{2+}\) (4 mM). The CaM protein bound to peptides was eluted from the beads and denatured at 100 °C for 15 min. The elution was detected with Coomassie Blue staining on SDS-PAGE gel.

**Cell Culture and Transfection**—HEK 293T cells were maintained at 37 °C in a DMEM medium containing 10% FBS, 100
units/ml penicillin, and 100 µg/ml streptomycin. Lipofectamine 2000 (Invitrogen) was used for all transfections.

**Oocyte Preparation for Western Blot**—Oocytes were collected after TEVC recordings, washed in cold PBS, and lysed with 15–20 µl/oocyte of lysis buffer (PBS, 10% glycerol, 1% Triton X-100, 1–2% protease inhibitor) using trituration through a syringe with a 25-gauge needle (10 times). Lysates were incubated at 4 °C for 1 h while rotating, with brief vortexing every 15–20 min. Aliquots of the lysates were then mixed with 5× SDS and boiled at 55 °C for 15–20 min. 15–20 µl lysates were loaded onto an acrylamide gel for Western blot.

**SDS-PAGE and Western Blot**—SDS-PAGE was performed in a Tris-glycine-SDS buffer with 8 or 12% acrylamide gel. Precision Plus Protein All Blue Standards (Bio-Rad) were used as molecular weight markers. For the Western blot, after electrophoresis, the protein gel was transferred to the PVDF membrane and processed with the Odyssey Western-blot kit (Li-Cor). The monoclonal mouse anti-HA antibody HA.11 (Covance), the monoclonal mouse anti-Myc antibody (Sigma), or the monoclonal mouse anti-FLAG antibody M2 (Sigma) was used as the primary antibody (usually overnight incubation at 4 °C). After triple washes with PBS, 1% Tween, membranes were incubated with a secondary antibody (Alexa Fluor 680-conjugated goat anti-mouse IgG; Invitrogen). Images were scanned and analyzed with the Odyssey Infrared Imaging System (Li-COR). The intensity of the protein bands was analyzed with the software ImageJ (NIH).

**Coinmunoprecipitation**—About 30–48 h later, after transfection, cells were collected in the PBS buffer (Invitrogen (pH 7.2)) at 4 °C. Cell lysates were obtained by adding 1% triton X-100.

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**FIGURE 1.** Mapping regions of Gem critical for the inhibition of P/Q-type Ca²⁺ channels. A, shown is a schematic diagram of WT Gem and various truncation mutants. B–D, left panels, shown are whole oocyte currents recorded at +10 mV by TEVC from oocytes expressing Cav2.1, α₂δ, and β₃ without Gem (control), with WT Gem (Gem_WT), or with the indicated truncated Gem. In these and the following panels showing TEVC currents the number of recordings is indicated above the bar, and all the results shown in each panel were obtained from the same batch of oocytes. B and D, middle panels, a Western blot shows protein expression of WT Gem and the indicated mutant Gem in the lysates of the oocytes recorded in the corresponding left panels. β₃ expression is shown as the loading control. B and D, right panels, bar graphs show the normalized intensity of the corresponding Gem bands in the middle panels.

**FIGURE 2.** A 12-amino acid region in the C-terminus is crucial for Gem inhibition of P/Q-type Ca²⁺ channels on plasma membrane. A and B, left panels, shown is time course of inhibition of currents (recorded at +20 mV) by 5 µM purified Gem-(Ser-68–Lys-276) protein (A) or Gem-(Ser-68–Arg-264) protein (B) in inside-out membrane patches from oocytes expressing Cav2.1, α₂δ and β₃, GK. n = 5. Right panels, current traces selected from a representative patch for each condition are shown. Currents were evoked by a depolarization to +20 mV from a holding potential of −80 mV and were obtained immediately before (red), during (blue), or after (green) perfusion of the indicated purified mutant Gem proteins. The dashed line indicates zero current level.
(V/V) Triton X-100 (Sigma) and 1/50 (V/V) Protease Inhibitor Mixture (Sigma) and rotating at 4 °C for 1 h. Supernatant was obtained by centrifuging at 14,000 rpm for 20 min and was mixed with monoclonal anti-HA antibody (HA.11) coated beads (Covance). After incubation at 4 °C overnight, the beads were spun down and washed 3 times with a solu-

**FIGURE 3.** A 12-amino acid Gem peptide is sufficient to inhibit P/Q-type Ca\(^{2+}\) channels on plasma membrane. A, shown is an amino acid sequence of three Gem-derived peptides. GCP1 and GCP2 correspond to Lys-265–Lys-276 and Asn-277–Leu-296 of Gem, respectively; GCP1\_S is a sequence-scrambled version of GCP1. B–D and F, left panels, shown is time course of inhibition of currents (recorded at +20 mV) by 100 \(\mu\)M GCP1 (B), 200 \(\mu\)M GCP2 (C), 5 \(\mu\)M GCP1 (D), or 100 \(\mu\)M GCP1\_S (F) in inside-out membrane patches from oocytes expressing Cav2.1, \(\alpha\)\_delta, and \(\beta\_3\). \(\beta\_3\) Mut2. Before time 0, the patch had been perfused for 5 min such that the channels had lost \(\beta\_3\) Mut2 and become \(\beta\)-less. \(\bar{n} = 4\). B–F, right panels, shown are current traces selected from a representative patch for each condition. Currents were evoked by a depolarization to +20 mV from a holding potential of –80 mV and were obtained immediately before (red), during (blue), or after (green) perfusion of the indicated Gem peptides. The dashed line indicates zero current level. G, left panel, whole oocyte currents were recorded at +10 mV by TEVC from oocytes expressing Cav2.1, \(\alpha\)\_delta, and \(\beta\_3\) Mut2. \(\beta\_3\) Mut2. Before time 0, the patch had been perfused for 5 min such that the channels had lost \(\beta\_3\) Mut2 and become \(\beta\)-less. \(\bar{n} = 4\). B–F, right panels, shown are current traces selected from a representative patch for each condition. Currents were evoked by a depolarization to +20 mV from a holding potential of –80 mV and were obtained immediately before (red), during (blue), or after (green) perfusion of the indicated Gem peptides. The dashed line indicates zero current level. G, left panel, whole oocyte currents were recorded at +10 mV by TEVC from oocytes expressing Cav2.1, \(\alpha\)\_delta, and \(\beta\_3\) Mut2. Before time 0, the patch had been perfused for 5 min such that the channels had lost \(\beta\_3\) Mut2 and become \(\beta\)-less. \(\bar{n} = 4\). B–F, right panels, shown are current traces selected from a representative patch for each condition. Currents were evoked by a depolarization to +20 mV from a holding potential of –80 mV and were obtained immediately before (red), during (blue), or after (green) perfusion of the indicated Gem peptides. The dashed line indicates zero current level. G, left panel, whole oocyte currents were recorded at +10 mV by TEVC from oocytes expressing Cav2.1, \(\alpha\)\_delta, and \(\beta\_3\) Mut2. Before time 0, the patch had been perfused for 5 min such that the channels had lost \(\beta\_3\) Mut2 and become \(\beta\)-less. \(\bar{n} = 4\).
tion containing 1 × PBS with a total NaCl concentration of 250 mM and 1/50 (V/V) protease inhibitor mixture (Sigma). The bound proteins were then eluted by adding 0.4 mg/ml HA peptide (Genscript), boiled with an SDS loading buffer for 10 min, and analyzed by SDS-PAGE and Western blot.

RESULTS

12-Amino Acid C-terminal Region in Gem Is Critical for Gem Inhibition—We first carried out systematic deletions to roughly delineate the structural elements of Gem critical for its inhibition of P/Q-type Ca\(^{2+}\) channels expressed in *Xenopus* oocytes (Fig. 1A). WT or mutant Gem was coexpressed with Ca\(_{\text{a}2.1}\), \(\alpha_\delta\), and \(\beta_\beta\). Ba\(^{2+}\) was used as the charge carrier, and whole-oocyte currents were recorded by TEVC. WT Gem completely abolished P/Q channel currents (Fig. 1, B–D). The core region of Gem (Gem-(68–247)) was totally ineffective (Fig. 1B, left panel) even though its protein level was much higher than that of WT Gem (Fig. 1B, middle and right panels), indicating that the N and/or C termini were required. Deleting the entire N terminus, however, did not affect Gem inhibition (Gem-(68–296) in Fig. 1C), indicating that the N terminus is not necessary, consistent with previous work showing that the N terminus is not required for Rem2 inhibitory effect (15). In contrast, Gem-(1–264), in which the last 32 amino acids in the C terminus was deleted, was completely inactive (Fig. 1D, left panel) despite its much higher protein level (Fig. 1D, middle and right panels), indicating that this 32-amino acid region is critical for Gem inhibitory action. This result agrees with previous work showing that the corresponding last 32–34 C-terminal residues of Rem and Rad are crucial for their inhibitory effects (20, 21, 23). Another deletion construct, Gem-(68–276), which lacks the last 20 amino acids in the C terminus as well as the entire N terminus, was still fully active (Fig. 1C). The complete lack of effect of Gem-(1–264) and the potent effect of Gem-(68–276) demonstrate that the 12-amino acid region from Lys-265 to Lys-276 in the C terminus is indispensable for Gem inhibition.

To further test this notion, we applied purified Gem-(Ser-68–Arg-264) and Gem-(Ser-68–Lys-276) proteins to inside-out membrane patches excised from oocytes expressing Ca\(_{\text{a}2.1}\), \(\alpha_\delta\), and \(\beta_\beta\), an approach employed in our previous study (18). Whereas Gem-(Ser-68–Lys-276) acutely and markedly inhibited macroscopic current (Fig. 2). This result reinforces the critical importance of the Lys-265–Lys-276 region. The lack of effect of Gem-(Ser-68–Arg-264) is unlikely caused by protein misfolding, as a shorter protein, Gem-(Met-73–Arg-264), was able to be crystallized and exhibited a structure similar to that of canonical Ras (26).

Lys-265–Lys-276 C-terminal Region in Gem Functions as Candidate Inhibitory Site—How is the Lys-265–Lys-276 region involved in Gem inhibition? One possibility is that it forms an inhibitory site that interacts directly with P/Q channels. To test this hypothesis, a peptide corresponding to this region named GCP1 (Gem C-terminal Peptide 1, Fig. 3A) was synthesized and applied to inside-out membrane patches excised from oocytes expressing Ca\(_{\text{a}2.1}\), \(\alpha_\delta\), and \(\beta_\beta\). GCP1 at 100 \(\mu\)M greatly reduced the currents (Fig. 3B). In contrast, a peptide corresponding to the last 20 amino acids (Asn-277–Leu-296) of Gem (named GCP2, Fig. 3A) had no effect even at a doubled concentration (Fig. 3C). These results suggest that the inhibitory effect of GCP1 is specific and that the Lys-265–Lys-276 region indeed forms an inhibitory site. The inhibitory effect of Gem, however, became much weaker when its concentration was decreased to 5 \(\mu\)M (Fig. 3D), a concentration at which purified Gem-(Ser-68–Lys-276) was fully effective (Fig. 2A). An explanation of this discrepancy is that Gem-(Ser-68–Lys-276) can associate with surface P/Q channels through Gem-\(\alpha_\gamma\) and Gem-\(\alpha_\alpha\) interactions, which would greatly increase the effective concentration of the Lys-265–Lys-276 inhibitory site near the channels.

In our previous study we showed that although purified Gem-(Ser-68–Lys-276) protein inhibited surface P/Q channels containing Ca\(_{\beta}\) it did not affect channels lacking Ca\(_{\beta}\) (18). The latter, termed \(\beta\)-less channels, were created by washing off a mutant Ca\(_{\beta}\) that was able to traffic Ca\(_{\text{a}2.1}\) to the plasma membrane but dissociated quickly from the surface channel upon vigorous perfusion (18). Using this approach, we found that GCP1, like Gem-(Ser-68–Lys-276), did not inhibit surface \(\beta\)-less P/Q channels (Fig. 3E). This result further demonstrates the specificity of GCP1 and suggests that GCP1 binds to a Ca\(_{\beta}\)-induced inhibitory site on Ca\(_{\text{a}2.1}\).

Remarkably, when GCP1 was scrambled into a new peptide named GCP1_S with the same amino acid content but a different sequence, it retained its inhibitory effect (Fig. 3F). This result suggests that the amino acid composition of this region rather than its sequence per se is important for its inhibitory effect.

To examine whether the inhibitory action of GCP1_S was a fortuitous effect of the peptide in isolation, we linked GCP1_S to Gem-(1–264), which as shown in Fig. 1D was inactive. The resulting construct, named Gem-(1–264)+GCP1_S, was able to inhibit P/Q channels, albeit it was slightly less potent than WT Gem was (Fig. 3G, left panel). In contrast, Gem-(1–264)+GCP2, in which the inactive GCP2 peptide was linked to Gem-(1–264), failed to cause inhibition at a similar protein level (Fig. 3G). These results further support GCP1 as a genuine inhibitory site in the full-length Gem.

CaM Binding to Gem Is Not Involved in Gem Inhibition—The identified Lys-265–Lys-276 inhibitory site has been reported to bind CaM (7–9, 14, 27). We confirmed this finding by using purified MBP-tagged GCP1 (MBP_GCP1) to pull down purified CaM in the presence of Ca\(^{2+}\) (Fig. 4A). A fragment of the C terminus of Ca\(_{\text{a}2.1}\) containing a canonical CaM binding IQ motif, denoted as Ca\(_{\text{a}2.1}\)-(Val-1899–Glu-1989), served as a positive control, whereas MBP-tagged GCP2 (MBP_GCP2) served as a negative control (Fig. 4A). CaM binding to Gem is severely attenuated by mutations of Trp-270 (14). Thus, to examine whether CaM binding to the Lys-265–Lys-276 site plays a role in Gem inhibition, we mutated four large hydrophobic residues (Phe-269, Trp-270, Ile-273, and Val-274) in the Lys-265–Lys-276 region to alanines in the full-length Gem. The resultant Gem mutant (Gem_M1) showed complete inhibition of P/Q channels (Fig. 4B), indicating that
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CaM binding to the Lys-265–Lys-276 site is not required for Gem inhibition.

**Inhibitory Site Exists in Core Region of Gem**—To investigate the role of the Lys-265–Lys-276 site in the inhibition of P/Q channels by full-length Gem, we created two Gem mutants named Gem_10G and Gem_11A, in which 9 of the 12 residues in this region were simultaneously mutated to glycine or alanine, resulting in 10 glycines or 11 alanines in the region (Fig. 5A). Surprisingly, however, both Gem mutants were still capable of inhibiting P/Q channels (Fig. 5, B and C), albeit the magnitude of inhibition by Gem_10G varied in different experiments, ranging from 60 to 100%. The underlying cause for the variation was unclear, but a potential factor was the variation in the effective concentrations of the Gem_10G protein in different batches of oocytes. Regardless of the varying inhibition, the results in Fig. 5, B and C, suggest the existence of an additional inhibitory site in Gem. To map this site, further deletions and point mutations were made in Gem_10G or Gem_11A. Deleting the N terminus of Gem_10G or Gem_11A did not abolish the inhibition (Fig. 5D) nor did simultaneously mutating 4 or 5 amino acids in the C terminus of Gem_10G to alanine either upstream or downstream of Lys-265–Lys-276 (Fig. 6). These results suggest that the additional inhibitory site resides in the core region of Gem.

To pinpoint this site, we selectively mutated residues in the core region that are conserved among RGK proteins but are distinct from the corresponding Ras residues (labeled red and I, 2, 3, and 4 in Fig. 7A). To minimize undesirable structural changes, these residues were mutated to their counterparts in Ras in the Gem_10G background (Fig. 7B). Three of the resulting Gem mutants (Gem_10G_CM1, Gem_10G_CM2, and Gem_10G_CM3) were still able to cause inhibition, albeit less potently than WT Gem but comparable to Gem_10G itself (Fig. 7C), but one mutant, Gem_10G_CM4 (carrying the L241K/R242H/R243K mutation), completely lost its activity (Fig. 7D), indicating that residues Leu-241, Arg-242, and Arg-243 play an important role in Gem inhibition. However, when these residues were mutated in WT Gem (producing a mutant named Gem_CM4), the inhibitory effect was fully preserved (Fig. 7, D and E, left panel). This result suggests that the inhibitory site in the core region and the Lys-265–Lys-276 site in the C terminus can act indepen-
ently and that each site functioning alone is sufficient to produce full-fledged inhibition.

There are at least four possible explanations for the complete lack of inhibition by Gem_10G_CM4. (i) It cannot fold properly. (ii) It expresses poorly in oocytes. (iii) It cannot interact with Cav2.1 or Cav/H9252. (iv) It no longer possesses a functional inhibitory site. To test the first three possibilities, we examined Gem_10G_CM4 expression in oocytes and its interaction with Cav2.1 or Cav/H9252 by co-immunoprecipitation in HEK 293T cells.

DISCUSSION

In our previous study we proposed a “Caβ-priming” model for Gem inhibition of surface P/Q-type Ca2+ channels (18). This model postulates that Gem associates directly and independently of Cav/H9252 with Cav2.1 through an anchoring site on both Gem and Cav2.1 and that Cav/H9252 induces the formation of an inhibitory site.
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FIGURE 8. Gem\(_{10G}\) CM4 can still associate with Ca\(_{2.1}\) and β\(_2\). A and B, a Western blot shows co-immunoprecipitation of HA-Gem\(_{10G}\) CM4 and FLAG-Ca\(_{2.1}\) (A) or HA-Gem\(_{10G}\) CM4 and Myc-β\(_2\) (B). Immunoprecipitation (IP) of Gem was carried out using an anti-HA antibody from the lysates of HEK 293T cells expressing FLAG-Ca\(_{2.1}\) alone (A, left lane) or Myc-β\(_2\) alone (B, left lane), co-expressing HA-Gem\(_{10G}\) CM4 and FLAG-Ca\(_{2.1}\) (A, right lane), or co-expressing HA-Gem\(_{10G}\) CM4 and Myc-β\(_2\) (B, right lane). Ca\(_{2.1}\) was detected with an anti-FLAG antibody and β\(_2\) with an anti-Myc antibody. Each experiment was repeated twice.

inhibitory site in Ca\(_{2.1}\) where a corresponding inhibitory site in Gem binds to cause inhibition. In this study, through systematic deletions and targeted point mutations, we identify two putative inhibitory sites in Gem (key constructs, their functional effects, and the functional states of the two putative inhibitory sites are summarized in supplemental Fig. 1). One site is formed by a 12-amino acid region (Lys-265–Lys-276) in the C terminus, as evidenced by its ability to directly, acutely, specifically, and in a Ca\(_{2}\)β-dependent manner inhibit surface P/Q-type channels on its own as a peptide (Fig. 3). That this 12-amino acid region can inhibit P/Q channels on its own is in line with the report that a Gem fragment containing the last ~80 residues of the C terminus was sufficient to inhibit P/Q channels (19). Eight of the 12 residues in this 12-amino acid region are fully conserved or highly similar among RGK proteins (Fig. 7A), and this region overlaps with a 13-amino acid region in Rem (corresponding to Phe-271–Arg-283 of human Rem) that was found to be critical for Rem inhibition of L-type channels (23). Thus, it is highly likely that this region also forms an inhibitory site in other RGK proteins.

The Lys-265–Lys-276 region falls within the polybasic motif that has been postulated to be involved in membrane targeting, which has been shown to be critical for Ca\(^{2+}\) channel inhibition by Rem and Rem2 (15, 20, 23). However, the observation that Gem\(_{10G}\) and Gem\(_{11A}\), in which all the positively charged residues were mutated to either glycine or alanine, were still capable of inhibiting P/Q-type channels (Fig. 5) indicates that the core inhibitory site not only in Gem but also in other RGK proteins is indicated by previous findings that Rem and Rem2 truncation mutants missing most of the C terminus (including the equivalent Lys-265–Lys-276 inhibitory site) can inhibit Ca\(^{2+}\) channels when they are fused to an exogenous membrane-targeting sequence (15, 20, 23). Our results suggest, but do not prove, that in Gem residues Leu-241/Arg-242/Arg-243 may contribute to form this inhibitory site (Fig. 7). These residues are pivoted at the C-terminal end of an α-helix, with their side chains exposed and ample room to possibly engage in interactions with another protein, as revealed by the crystal structure of a GDP-bound Gem protein fragment (Met-73–Arg-264) containing most of the core (26) (Fig. 9). These residues are completely conserved among RGK proteins but are substituted by other residues in Ras that do not directly regulate HVA Ca\(^{2+}\) channels. Together, these observations suggest that residues Leu-241/Arg-242/Arg-243 form a candidate inhibitory site not only in Gem but also in other RGK proteins. However, more direct evidence is needed to firmly establish this conclusion.

Our results further show that the core inhibitory site requires the distal C terminus to function properly. Deleting the last 32 C-terminal residues (including Lys-265–Lys-276) rendered this site totally ineffective (Fig. 1D), and mutating various clusters of residues among the last 20 amino acids attenuated its potency (Fig. 6). Furthermore, although the core inhibitory site was capable of producing full inhibition in Gem\(_{10G}\), this site became completely ineffective in...
Gem_10GΔC, which lacks the last 20 amino acids but was expressed strongly in oocytes (Fig. 10). These results suggest that the last 20 amino acids are necessary for the proper function of the core inhibitory site. How this distal C-terminal region modulates the function of the core inhibitory site remains to be elucidated. The distance between this region and the core inhibitory site seems to be an important factor, as linking the last 20 amino acids to the inactive Gem-(1–264) did not restore inhibition (Fig. 3G, left panel), although this mutant construct (Gem-(1–264)+GCP2) expressed robustly in oocytes (Fig. 3G, middle and right panels).

The notion that RGK proteins contain more than one inhibitory site is in harmony with a report that Rem inhibits L-type Ca2+ channels through three distinct mechanisms, including reducing the number of surface channels, decreasing channel open probability, and immobilizing channel voltage sensors (13). It remains to be investigated how different inhibitory sites are functionally coordinated and regulated.

Various regions of RGK proteins have been found to play a role in the inhibition of HVA Ca2+ channels (15, 19, 21, 23, 24). The N and C termini of RGK proteins contain binding sites for CaM and 14-3-3, and interactions with CaM and 14-3-3 have been shown to regulate the subcellular distribution of RGK proteins (7–9, 28–31). In Gem, these sites include Trp-269 (for CaM) and Ser-22 and Ser-288 (for 14-3-3) (corresponding to Trp-270, Ser-23, and Trp-289 in human Gem) (9, 14, 30, 33). A Gem mutant, W269G (Trp-270 in our studies), which is defective in binding CaM (33), has been reported to weaken Gem regulation of Ca2+ channels (9, 14). Overexpression of Gem in tsA201 cells attenuates Rem inhibition of Ca1.2 currents (34). However, we found that Gem_10G, whose mutations include W270G and are much more severe than W269G, was fully capable of inhibiting P/Q channels (Fig. 5B), indicating that CaM binding to Gem is not required for Gem inhibitory effect. A possible explanation for the discrepancy lies in the observation that the magnitude of RGK regulation of Ca2+ channels depends on the concentration of the RGK protein (35). Our observation that Gem-(Ser-68–Lys-276), which lacks both the N- and C-terminal binding sites for 14-3-3, was fully active in inhibiting P/Q channels (Figs. 1 and 2) indicates that interaction with 14-3-3 is not required for Gem inhibition, in agreement with earlier studies (9, 14).

The Lys-265–Lys-276 region and the Leu-241/Arg-242/Arg-243 tri-amino acid motif do not appear to be involved in the anchoring interaction between Gem and Ca2_1.1, as Gem_10G_CM4, which contains mutations in both sites, was still able to associate with Ca2_1.1 in the absence of CaM (Fig. 8A). Thus, the anchoring site in Gem remains to be identified. Also remaining to be found are the anchoring site and the Caβ-induced inhibitory site in Ca2_1.2.

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REFERENCES
1. Felix, R. (2005) Molecular regulation of voltage-gated Ca2+ channels. J. Recept. Signal Transduct. Res. 25, 57–71
2. Catterall, W. A. (2000) Structure and regulation of voltage-gated Ca2+ channels. Annu. Rev. Cell Dev. Biol. 16, 521–555
3. Kelly, K. (2005) The RGK family. A regulatory tail of small GTP-binding proteins. Trends Cell Biol. 15, 640–643
4. Correll, R. N., Pang, C., Niedowicz, D. M., Finlin, B. S., and Andres, D. A. (2008) The RGK family of GTP-binding proteins. Regulators of voltage-dependent calcium channels and cytoskeleton remodeling. Cell. Signal. 20, 292–300
5. Buraei, Z., and Yang, J. (2010) The β subunit of voltage-gated Ca2+ channels. Physiol. Rev. 90, 1461–1506
6. Flynn, R., and Zamponi, G. W. (2010) Regulation of calcium channels by RGK proteins. Channels 4, 434–439
7. Béguin, P., Mahalakshmi, R. N., Nagashima, K., Cher, D. H., Ikeda, H., Yamada, Y., Seino, Y., and Hunziker, W. (2006) Nuclear sequestration of β-subunits by Rad and Rem is controlled by 14-3-3 and calmodulin and reveals a novel mechanism for Ca2+ channel regulation. J. Mol. Biol. 355, 34–46
8. Béguin, P., Mahalakshmi, R. N., Nagashima, K., Cher, D. H., Kuwamura, N., Yamada, Y., Seino, Y., and Hunziker, W. (2005) Roles of 14-3-3 and calmodulin binding in subcellular localization and function of the small G-protein Rem2. Biochem. J. 390, 67–75
9. Béguin, P., Mahalakshmi, R. N., Nagashima, K., Cher, D. H., Takahashi, A., Yamada, Y., Seino, Y., and Hunziker, W. (2005) 14-3-3 and calmodulin control subcellular distribution of Kir/Gem and its regulation of cell shape
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and calcium channel activity. *J. Cell Sci.* **118**, 1923–1934

10. Béguin, P., Ng, Y. J., Krause, C., Mahalakshmi, R. N., Ng, M. Y., and Hunziker, W. (2007) RGK small GTP-binding proteins interact with the nucleotide kinase domain of Ca\(^{2+}\) channel \(\beta\)-subunits via an uncommon effector binding domain. *J. Biol. Chem.* **282**, 11509–11520

11. Sasaki, T., Shibasaki, T., Béguin, P., Nagashima, K., Miyazaki, M., and Seino, S. (2005) Direct inhibition of the interaction between \(\alpha\)-interaction domain and \(\beta\)-interaction domain of voltage-dependent Ca\(^{2+}\) channels by *Gem*. *J. Biol. Chem.* **280**, 9308–9312

12. Yada, H., Murata, M., Shimoda, K., Yuasa, S., Kawaguchi, H., Ieda, M., Adachi, T., Murata, M., Ogawa, S., and Fukuda, K. (2007) Dominant negative suppression of Rad leads to QT prolongation and causes ventricular arrhythmias via modulation of L-type Ca\(^{2+}\) channels in the heart. *Circ. Res.* **101**, 69–77

13. Yang, T., Xu, X., Kernan, T., Wu, V., and Colecraft, H. M. (2010) Rem, a member of the RGK GTPases, inhibits recombinant CaV1.2 channels using multiple mechanisms that require distinct conformations of the GTPase. *J. Physiol.* **588**, 1665–1681

14. Béguin, P., Nagashima, K., Goni, T., Shibasaki, T., Takahashi, K., Kashima, Y., Ozaki, N., Geering, K., Iwanaga, T., and Seino, S. (2001) Regulation of Ca\(^{2+}\) channel expression at the cell surface by the small G-protein kir/Gem. *Nature* **411**, 701–706

15. Chen, H., Puhl, H. L., 3rd, Niu, S. L., Mitchell, D. C., and Ikeda, S. R. (2005) Expression of Rem2, an RGK family small GTPase, reduces N-type calcium current without affecting channel surface density. *J. Neurosci.* **25**, 9762–9772

16. Finlin, B. S., Correll, R. N., Pang, C., Crump, S. M., Satin, J., and Andres, D. A. (2006) Analysis of the complex between Ca\(^{2+}\) channel \(\beta\)-subunit and the Rem GTPase. *J. Biol. Chem.* **281**, 23557–23566

17. Finlin, B. S., Mosley, A. L., Crump, S. M., Correll, R. N., Ozcan, S., Satin, J., and Andres, D. A. (2005) Regulation of L-type Ca\(^{2+}\) channel activity and insulin secretion by the Rem2 GTPase. *J. Biol. Chem.* **280**, 41864–41871

18. Fan, M., Bureai, Z., Luo, H. R., Levenson-Palmer, R., and Yang, J. (2010) Direct inhibition of P/Q-type voltage-gated Ca\(^{2+}\) channels by Gem does not require a direct Gem/Cav\(\beta\) interaction. *Proc. Natl. Acad. Sci. U.S.A.* **107**, 14887–14892

19. Leyris, J. P., Gondeau, C., Charnet, A., Delattre, C., Rousset, M., Cens, T., and Charnet, P. (2009) RGK GTPase-dependent CaV2.1 Ca\(^{2+}\) channel inhibition is independent of CaV\(\beta\)-subunit-induced current potentiation. *FASEB J.* **23**, 2627–2638

20. Yang, T., Suhail, Y., Dalton, S., Kernan, T., and Colecraft, H. M. (2007) Genetically encoded molecules for inducibly inactivating CaV channels. *Nat. Chem. Biol.* **3**, 795–804

21. Finlin, B. S., Crump, S. M., Satin, J., and Andres, D. A. (2003) Regulation of voltage-gated calcium channel activity by the Rem and Rad GTPases. *Proc. Natl. Acad. Sci. U.S.A.* **100**, 14469–14474

22. Correll, R. N., Botzet, G. J., Satin, J., Andres, D. A., and Finlin, B. S. (2008) Analysis of the Rem2-voltage-dependent calcium channel \(\beta\) subunit interaction and Rem2 interaction with phosphorylated phosphatidylinositol lipids. *Cell. Signal.* **20**, 400–408

23. Correll, R. N., Pang, C., Finlin, B. S., Dailey, A. M., Satin, J., and Andres, D. A. (2007) Plasma membrane targeting is essential for Rem-mediated Ca\(^{2+}\) channel inhibition. *J. Biol. Chem.* **282**, 28431–28440

24. Flynn, R., Chen, L., Hameed, S., Spafford, J. D., and Zamponi, G. W. (2008) Molecular determinants of Rem2 regulation of N-type calcium channels. *Biochem. Biophys. Res. Commun.* **368**, 827–831

25. Yanuar, A., Sakurai, S., Kitano, K., and Hakoshima, T. (2006) Crystal structure of human Rad GTPase of the RGK family. *Genes Cells* **11**, 961–968

26. Slingard, A., Ménétriet, J., Perderiset, M., Cicolari, J., Regazzoni, K., Hamoudi, F., Cabanié, L., El Marjou, A., Wells, A., Houdusse, A., and de Gunzburg, J. (2007) Biochemical and structural characterization of the gem GTPase. *J. Biol. Chem.* **282**, 1905–1915

27. Meyers, J. S., Bilan, P. J., Zhu, J., and Kahn, C. R. (1997) Rad and Rad-related GTPases interact with calmodulin and calmodulin-dependent protein kinase II. *J. Biol. Chem.* **272**, 11832–11839

28. Ward, Y., Spinelli, B., Quon, M. J., Chen, H., Ikeda, S. R., and Kelly, K. (2004) Phosphorylation of critical serine residues in Gem separates cytoskeletal reorganization from down-regulation of calcium channel activity. *Mol. Cell. Biol.* **24**, 651–661

29. Finlin, B. S., and Andres, D. A. (1999) Phosphorylation-dependent association of the Ras-related GTP-binding protein Rem with 14–3–3 proteins. *Arch. Biochem. Biophys.* **368**, 401–412

30. Mahalakshmi, R. N., Nagashima, K., Ng, M. Y., Inagaki, N., Hunziker, W., and Béguin, P. (2007) Nuclear transport of Kir/Gem requires specific signals and importin \(\alpha\)5 and is regulated by calmodulin and predicted serine phosphorylations. *Traffic* **8**, 1150–1163

31. Mahalakshmi, R. N., Ng, M. Y., Guo, K., Qi, Z., Hunziker, W., and Béguin, P. (2007) Nuclear localization of endogenous RGK proteins and modulation of cell shape remodeling by regulated nuclear transport. *Traffic* **8**, 1164–1178

32. Xu, X., Marx, S. O., and Colecraft, H. M. (2010) Molecular mechanisms, and selective pharmacological rescue, of Rem-inhibited CaV1.2 channels in heart. *Circ. Res.* **107**, 630–630

33. Fischer, R., Wei, Y., Anagli, J., and Berchtold, M. W. (1996) Calmodulin binds to and inhibits GTP binding of the ras-like GTPase Kri/Gem. *J. Biol. Chem.* **271**, 25067–25070

34. Pang, C., Crump, S. M., Jin, L., Correll, R. N., Finlin, B. S., Satin, J., and Andres, D. A. (2010) Rem GTPase interacts with the proximal CaV1.2 C terminus and modulates calcium-dependent channel inactivation. *Channels* **4**, 192–202

35. Seu, L., and Pitt, G. S. (2006) Dose-dependent and isoform-specific modulation of Ca\(^{2+}\) channels by RGK GTPases. *J. Gen. Physiol.* **128**, 605–613