Direct inhibition of P/Q-type voltage-gated Ca^{2+} channels by Gem does not require a direct Gem/$Ca_v\beta$ interaction

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The Rem, Rem2, Rad, and Gem/Kir (RGK) family of small GTP-binding proteins potently inhibits high voltage-activated (HVA) Ca^{2+} channels, providing a powerful means of modulating neural, endocrine, and muscle functions. The molecular mechanisms of this inhibition are controversial and remain largely unclear. RGK proteins associate directly with Ca^{2+} channel β subunits ($Ca_v\beta$), and this interaction is widely thought to be essential for their inhibitory action. In this study, we investigate the molecular underpinnings of Gem inhibition of P/Q-type Ca^{2+} channels. We find that a purified Gem protein markedly and acutely suppresses P/Q channel activity in inside-out membrane patches, that this action requires $Ca_v\beta$ but not the Gem/$Ca_v\beta$ interaction, and that Gem coimmunoprecipitates with the P/Q channel α_1 subunit ($Ca_v\alpha_1$) in a $Ca_v\beta$-independent manner. By constructing chimeras between P/Q channels and Gem-insensitive low voltage-activated T-type channels, we identify a region encompassing transmembrane segments S1, S2, and S3 in the second homologous repeat of $Ca_v\alpha_1$ critical for Gem inhibition. Exchanging this region between P/Q and T channel $Ca_v\alpha_1$ abolishes Gem inhibition of P/Q channels and confers $Ca_v\beta$-dependent Gem inhibition to a chimeric T channel that also carries the P/Q I-II loop (a cytoplasmic region of $Ca_v\alpha_1$ that binds $Ca_v\beta$). Our results challenge the prevailing view regarding the role of $Ca_v\beta$ in RGK inhibition of high voltage-activated Ca^{2+} channels and prompt a paradigm in which Gem directly binds and inhibits $Ca_v\beta$-primed $Ca_v\alpha_1$ on the plasma membrane.

β subunit | electrophysiology | modulation | Rem, Rem2, Rad, and Gem/Kir proteins | T-type Ca^{2+} channels

High voltage-activated (HVA) Ca^{2+} channels, which include L-, N-, P/Q-, and R-type channels, are essential for diverse biological processes, ranging from gene transcription and neurotransmission to hormone secretion and heart beat. They contain a pore-forming α_1 subunit ($Ca_v\alpha_1$), a membrane anchored $\alpha_2\delta$ subunit, and a cytosolic β subunit ($Ca_v\beta$; review in ref. 1). $Ca_v\alpha_1$ has four homologous repeats, each consisting of six transmembrane segments (S1–S6) and a pore-forming loop. $Ca_v\alpha_1$ is the principal subunit of HVA Ca^{2+} channels and is the main determinant of the unique pharmacological and biophysical properties of each channel type. $Ca_v\beta$ is an auxiliary subunit that is indispensible for transporting $Ca_v\alpha_1$ to the plasma membrane and fine-tuning channel gating (reviews in refs. 2 and 3). Both effects depend critically on the binding of $Ca_v\beta$ to the α interacting domain (AID) in the cytoplasmic loop (referred to as the I–II loop) connecting the first two homologous repeats of $Ca_v\alpha_1$ (2–11). Gating regulation by $Ca_v\beta$ also needs a continuous α-helix between the AID and the S6 segment of the first repeat (IS6) of $Ca_v\alpha_1$ (3, 8, 9, 12).

The activity of HVA Ca^{2+} channels is regulated by numerous signaling pathways and interacting proteins with profound functional consequences (review in ref. 1). Recently, members of the Rem, Rem2, Rad, and Gem/Kir (RGK) family of Ras-related monomeric small GTP-binding proteins, which are known to regulate cytoskeleton remodeling through the Rho/Rho kinase signaling cascade (review in ref. 13), have emerged as the most potent protein inhibitors of HVA Ca^{2+} channels (14–31). RGK proteins are present in many tissues and cells where HVA Ca^{2+} channels are expressed, including the brain and cardiac, skeletal, and smooth muscles (review in ref. 13). Accordingly, they are emerging as strong regulators of hormone secretion and cardiac and brain physiology, both in vivo and in vitro. For example, in the heart, dominant negative suppression of endogenous Rad increases L-type Ca^{2+}-channel currents and action-potential duration in cardiac cells and produces longer QT intervals and arrhythmias (24). Rem2 prevents glucose-stimulated insulin secretion in pancreatic β cells (19) and regulates the development of both excitatory and inhibitory synapses, presumably through a feedback loop that controls Ca^{2+} influx (32). Finally, alteration in Gem regulation of $Ca_v1.2$ L-type Ca^{2+} channels may contribute to certain neural phenotypes displayed in Timothy Syndrome, a genetic disorder characterized by cardiac and neurological defects and autism (33).

All RGK proteins associate directly with $Ca_v\beta$ in vitro and in cells (14–17, 19–21, 23, 25, 27–29, 34), and $Ca_v\beta$ is required for RGK-induced inhibition of HVA Ca^{2+} channels (14, 15, 22). Two modes of action have been reported: (*i*) RGK proteins reduce the number of HVA Ca^{2+} channels on the cell surface, either by interfering with channel trafficking to the plasma membrane or increasing endocytosis of surface channels (14, 16, 17, 20, 24, 31, 34, 35), and (*ii*) RGK proteins inhibit channels already on the plasma membrane (18, 19, 21, 25, 31). The molecular mechanisms of either mode of action are unknown, but because of the central role of $Ca_v\beta$ in HVA Ca^{2+}-channel trafficking and gating, it is widely assumed that both forms of inhibition rely on the RGK/$Ca_v\beta$ interaction (13–29, 31). This key hypothesis, however, has not been tested.

In this study, we investigated the molecular mechanism of Gem inhibition of P/Q-type Ca^{2+} channels expressed in *Xenopus* oocytes. We unambiguously show that Gem directly inhibits P/Q-type Ca^{2+} channels on the plasma membrane and that this inhibition requires $Ca_v\beta$. Surprisingly, we discover that Gem inhibition does not require a direct Gem/$Ca_v\beta$ interaction or a structural element critical for $Ca_v\beta$ regulation of channel gating. Instead, $Ca_v\beta$ seems required only to prime $Ca_v\alpha_1$ for Gem inhibition. We find that Gem and P/Q channel $Ca_v\alpha_1$ coimmunoprecipitate, and we identify a region in

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$Ca_v\alpha_1$ crucial for Gem inhibition. Our results show an essential and hitherto unrecognized contribution from $Ca_v\alpha_1$ to RGK inhibition and lead us to propose a model for RGK inhibition of HVA Ca^{2+} channels.

Results

Gem Directly Inhibits P/Q-Type Ca^{2+} Channels on the Plasma Membrane. To date, there is no report of direct inhibition of surface HVA Ca^{2+} channels by Gem. Thus, we first investigated whether Gem inhibited P/Q-type Ca^{2+} channels on the plasma membrane. To accomplish this, we designed a Gem construct (S68-K276 of human Gem) that contained most of the Gem protein and could be readily purified. Gem(S68-K276), when purified and applied to the intracellular face of giant inside-out membrane patches from *Xenopus* oocytes expressing P/Q channels, markedly and quickly (in ~1 min) reduced the macroscopic currents (Fig. 1). This inhibition was only partially reversible (Fig. 1 *A* and *B*), partly because of the difficulty of washing out the applied protein in this recording configuration, and it was largely voltage-independent, as indicated by the current–voltage relation (Fig. 1*C Right*). This result shows that Gem(S68-K276) acutely inhibits P/Q channels on the plasma membrane. As expected, Gem(S68-K276) inhibited whole-oocyte P/Q-channel currents when tonically expressed (Fig. S1).

$Ca_v\beta$ Is Required for Gem Inhibition of Surface P/Q-Type Ca^{2+} Channels. We next investigated whether $Ca_v\beta$ was required for the acute and direct inhibition of surface P/Q channels by Gem. To obtain large populations of surface P/Q channels that did not contain a $Ca_v\beta$ (β-less channels), we created, following our previous strategy (12), a mutant β_3 named β_3_Mut2 (bearing the M196A/L200A mutation). β_3_Mut2 retained the ability to chaperone $Ca_v2.1$ to the plasma membrane but with vigorous perfusion, could quickly dissociate from the surface channels in inside-out patch recordings, leaving functional β-less channels behind. This dissociation was ascertained by monitoring the positive shift of the activation curve and its subsequent restoration (i.e., negative shift) after the application of purified β_3_core (amino acids G16–G366 of β_3) (Fig. 2*A*). Purified Gem(S68-K276) did not inhibit the β-less channels but quickly and strongly suppressed the same population of channels after they bound β_3_core (Fig. 2 *B* and *C*). The inhibition developed well beyond the current level before the application of β_3_core, indicating that Gem(S68-K276) did not simply reverse the gating modulation by β_3_core. These results indicate that $Ca_v\beta$ is necessary for Gem-induced acute inhibition.

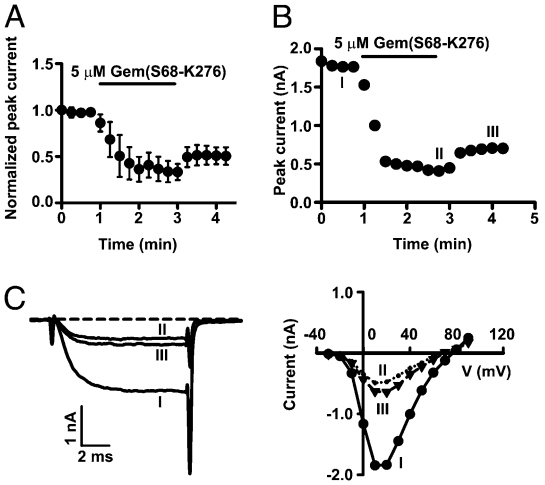

Fig. 1. Gem acutely inhibits surface P/Q-type Ca^{2+} channels. (*A*) Time course of current inhibition (recorded at +20 mV) by 5 μM purified Gem(S68-K276) in inside-out membrane patches from oocytes expressing $Ca_v2.1$, $\alpha_2\delta$, and β_3. In this figure and subsequent similar figures, currents were normalized and then averaged (n = 5). (*B*) Exemplar time course of inhibition in a single patch, with the same conditions as in *A*. (*C*) Current traces recorded at +20 mV (*Left*) and current–voltage relationships (*Right*) taken at the times indicated by I, II, and III in *B*.

Role of $Ca_v\beta$ in Gem Inhibition. Why is $Ca_v\beta$ necessary for Gem inhibition of P/Q channels? $Ca_v\beta$ regulates Ca^{2+}-channel gating through high-affinity binding of its guanylate kinase (GK) domain to the AID in the I–II loop of $Ca_v\alpha_1$ and through low-affinity interactions involving other regions of $Ca_v\beta$ and $Ca_v\alpha_1$ (2–11). Thus, it is possible that $Ca_v\beta$'s mandatory role arises from $Ca_v\beta$-induced gating changes and the underlying low-affinity $Ca_v\beta/Ca_v\alpha_1$ interactions. To test this possibility, we examined the effect of Gem on channels formed by $\alpha_2\delta$, β_3, and a mutant $Ca_v2.1$ named $Ca_v2.1\_7G$, in which a seven-glycine linker was inserted between the AID and IS6. The hepta-glycine linker disrupts the rigid α-helix between IS6 and the AID and renders $Ca_v\beta$ completely impotent in regulating channel gating without compromising its chaperone effect (8–10, 12). However, $Ca_v2.1\_7G$ channels were inhibited by Gem(S68-K276) in both whole oocytes (Fig. 3*A*) and inside-out patches (Fig. 3*B*). Thus, $Ca_v\beta$-induced gating changes are not a prerequisite for Gem inhibition. Furthermore, in agreement with a recent study in whole oocytes (29),

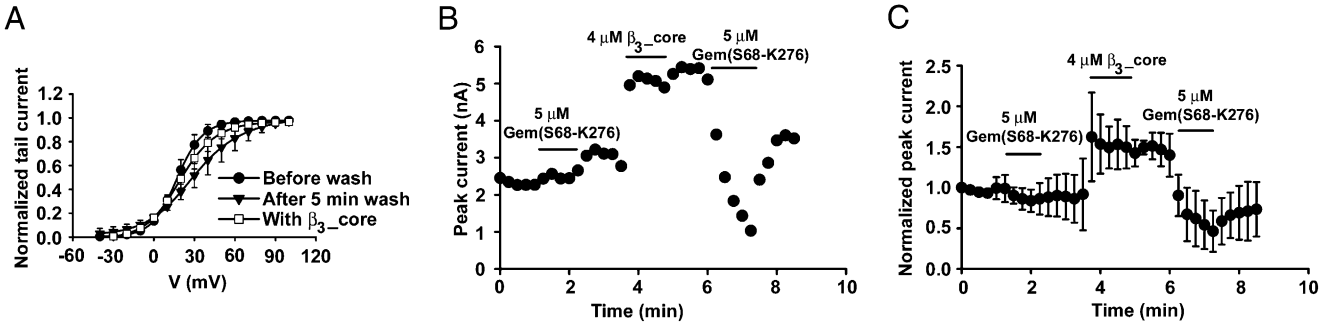

Fig. 2. $Ca_v\beta$ is required for Gem inhibition of surface P/Q-type Ca^{2+} channels. (*A*) Voltage-dependence of activation under the indicated conditions for channels composed of $Ca_v2.1$, $\alpha_2\delta$, and β_3_Mut2. Activation curves were obtained in inside-out patches before the wash out of β_3_Mut2, after 5 min of wash, and 1 min after subsequent application of β_3_core (n = 5). (*B*) Time course of Gem action on β-less and β-containing channels. Currents (recorded at +20 mV) were obtained from an inside-out patch from an oocyte expressing $Ca_v2.1$, $\alpha_2\delta$, and β_3_Mut2. Before time 0, the patch had been washed for 5 min such that the channels had lost β_3_Mut2 and become β-less; 5 μM Gem(S68-K276) had no effect on the β-less channels. Subsequent application of 4 μM purified β_3_core increased the current, which was suppressed, with partial reversibility, by the second application of 5 μM Gem(S68-K276). (*C*) Same plot as in *B* for data pooled from five patches. For each patch, the current was normalized by that at time 0.

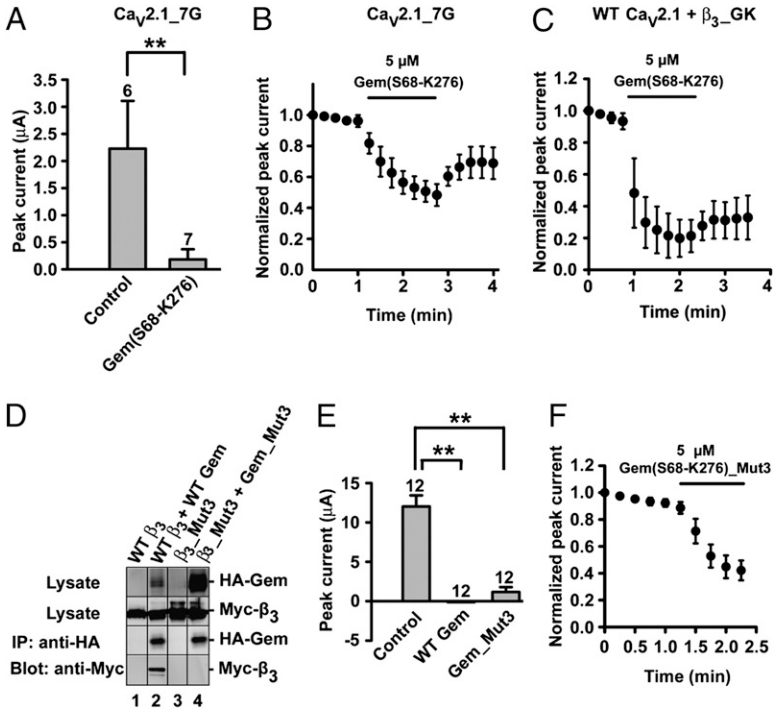

Fig. 3. The role of Ca$_V$β in Gem inhibition of P/Q channels. (*A*) Inhibition of P/Q channels composed of Ca$_V$2.1_7G, α$_2$δ,and β$_3$ by constitutively expressed Gem(S68-K276) in whole oocytes. (*B*) Time course of inhibition of Ca$_V$2.1_7G channels by 5 μM purified Gem(S68-K276) in inside-out patches (n = 5). (*C*) Time course of inhibition of P/Q channels containing β$_3$_GK by 5 μM purified Gem(S68-K276) in inside-out patches (n = 5). (*D*) Western blot showing the abolishment of Gem/β$_3$ interaction by targeted mutations. Immunoprecipitation (IP) of Gem was carried out using an anti-HA antibody from the lysates of HEK 293T cells expressing the constructs indicated on the top of each lane. HA-Gem and Myc-β$_3$ coimmunoprecipitated (lane 2), but HA-Gem_Mut3 and Myc-β$_3$_Mut3 did not (lane 4). Similar results were obtained in two other experiments. (*E*) Inhibition of P/Q channels containing β$_3$_Mut3 by constitutively expressed WT Gem or Gem_Mut3 in whole oocytes. In this figure and other similar figures, asterisks indicate $P < 0.01$, the number of recordings is indicated above the bar, all recordings were obtained from the same batch of oocytes, and similar results were obtained from at least two different batches of oocytes. (*F*) Time course of inhibition of β$_3$_Mut3-containing P/Q channels by 5 μM purified Gem(S68-K276)_Mut3 in inside-out patches (n = 5).

we found that the GK domain of β$_3$ (β$_3$_GK) alone was sufficient to support Gem inhibition of P/Q channels in inside-out patches (Fig. 3*C*), indicating that low-affinity interactions engaging Ca$_V$2.1 and other regions of β$_3$ are not required.

All members of RGK proteins interact directly with Ca$_V$β (14–17, 19–21, 23, 25, 27–29, 34). Thus, a prevalent hypothesis is that RGK proteins need to bind Ca$_V$β to exert their inhibitory effect (13–29, 31). To test this hypothesis, we abolished Gem/β$_3$ interaction by simultaneously mutating to alanine three residues on Gem and three residues on β$_3$ that have been shown to be critical for this interaction (34). Coimmunoprecipitation confirmed that the mutant Gem (R196A/V223A/H225A named Gem_Mut3) and the mutant β$_3$ (D194A/D270A/D272A named β$_3$_Mut3) did not interact with each other (Fig. 3*D* and Fig. S2). This result is in agreement with a previous biochemical study showing that mutating any one of these residues abolished or severely weakened Gem/β$_3$ interaction (34). Strikingly, Gem_Mut3 and Gem(S68-K276)_Mut3 were fully capable of inhibiting channels formed by Ca$_V$2.1, α$_2$δ and β$_3$_Mut3 when either constitutively expressed (Fig. 3*E*) or acutely applied (Fig. 3*F*). These results indicate that the Gem/β$_3$ interaction is not required for Gem-induced acute inhibition.

Gem and Ca$_V$α$_1$ Coimmunoprecipitate. Because Gem_Mut3 did not bind β$_3$_Mut3, the observation that Gem_Mut3 inhibited surface P/Q channels containing β$_3$_Mut3 (Fig. 3 *E* and *F*) suggested that Gem_Mut3 might directly interact with Ca$_V$2.1. In an initial test for this possibility, we found that Ca$_V$2.1 and β$_3$ coimmunoprecipitated with Gem (lane 3 of Fig. 4*A* and Fig. S3). This result is in accord with previous results showing the existence of RGK/Ca$_V$β/Ca$_V$α$_1$ tripartite complexes (21, 25, 27, 34). However, coimmunoprecipitation of Ca$_V$2.1, β$_3$, and Gem could simply be a result of the Gem/β$_3$ and β$_3$/Ca$_V$2.1 interactions. Remarkably, however, further experiments show that Ca$_V$2.1 and β$_3$_Mut3 coimmunoprecipitated with Gem_Mut3 (Fig. 4*A*, lane 6), although Gem_Mut3 and β$_3$_Mut3 did not bind each other, as shown in Fig. 3*D*. Moreover, Ca$_V$2.1 coimmunoprecipitated with either Wild Type (WT) Gem or Gem_Mut3 in the absence of an exogenously expressed Ca$_V$β (Fig. 4*A*, lanes 2 and 5). These results suggest that either Gem directly associates with Ca$_V$2.1 or Gem and Ca$_V$2.1 both associate with an unknown protein in the same complex. In either case, this association is Ca$_V$β-independent.

Model for Gem Inhibition of Surface P/Q-Type Ca^{2+} Channels. The preceding results lead us to propose a Ca$_V$β-priming model for Gem inhibition of P/Q-type Ca^{2+} channels on the plasma membrane (Fig. 4*B*). The most distinct feature of this model is that the interaction between Gem and Ca$_V$β is not necessary for Gem's inhibitory effect, but a direct association between Gem and Ca$_V$2.1 is essential. In this model, Gem interacts directly with Ca$_V$2.1 through an anchoring site, with or without Ca$_V$β present. In the presence of Ca$_V$β and Gem, Ca$_V$2.1 forms a multimeric complex with both proteins on the plasma membrane (Fig. 4*B*, row I). Binding of Ca$_V$β to Ca$_V$2.1 produces a conformational change, resulting in the formation of an inhibitory binding site in Ca$_V$2.1 where Gem contacts to produce inhibition (Fig. 4*B*, row I). When Ca$_V$β dissociates or is washed off from the surface Ca$_V$2.1, the inhibitory binding site disappears, and Gem becomes unable to inhibit Ca$_V$2.1, although it can still be attached to Ca$_V$2.1 through the anchoring site (Fig. 4*B*, row II). With mutant forms of Ca$_V$β

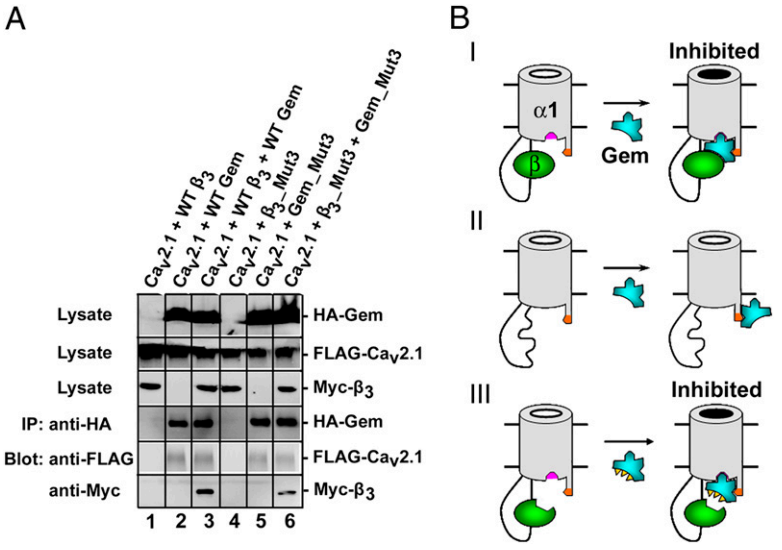

Fig. 4. Model of Gem inhibition of surface P/Q channels. (*A*) Gem coimmunoprecipitates with $Ca_v2.1$ in a $Ca_v\beta$-independent manner. IP of Gem was carried out using an anti-HA antibody from the lysates of HEK 293T cells expressing the constructs indicated on the top of each lane. $Ca_v2.1$ and β_3 were detected by an anti-FLAG and anti-Myc antibody, respectively. Western blot shows coimmunoprecipitation of Gem and $Ca_v2.1$ (lane 2), Gem_Mut3 and $Ca_v2.1$ (lane 5), and Gem_Mut3, $Ca_v2.1$, and β_3_Mut3 (lane 6). In groups without Gem, no coimmunoprecipitation of $Ca_v2.1$ was observed (lanes 1 and 4). As a positive control, $Ca_v2.1$ and WT β_3 are coimmunoprecipitated by WT Gem (lane 3). Similar results were observed in three other experiments. (*B*) Model of Gem inhibition of surface P/Q channels. Gem associates directly with $Ca_v2.1$ through an anchoring site on $Ca_v2.1$ (indicated by the orange patch), with (I and III) or without (II) $Ca_v\beta$. Binding of WT $Ca_v\beta$ (I) or β_3_Mut3 (III) to $Ca_v2.1$ induces an inhibitory binding site in $Ca_v2.1$ (indicated by the pink patch) where WT Gem (I) or Gem_Mut3 (II) binds to cause inhibition.

and Gem that cannot bind each other (Fig. 4*B*, row III), inhibition can still proceed, because the ability of $Ca_v\beta$ and Gem to bind $Ca_v2.1$ is uncompromised. According to this model, the essential role of $Ca_v\beta$ is to convert $Ca_v2.1$ into a state permissive for Gem inhibition.

Identification of a $Ca_v\alpha_1$ Region Critical for Gem Inhibition. To further test the $Ca_v\beta$-priming model, we attempted to identify the molecular determinants in $Ca_v\alpha_1$ that are critical for Gem inhibition. To this end, we took advantage of the observation that RGK proteins do not regulate the activity of low voltage-activated T-type Ca^{2+} channels, which do not associate with $Ca_v\beta$ or require $Ca_v\beta$ for their activity (15, 18), and we constructed chimeras between $Ca_v2.1$ and $Ca_v3.1$, a T-type channel α_1 subunit.

Expression of $Ca_v3.1$ in *Xenopus* oocytes produced typical, small, and fast inactivating T-type currents, which were unaffected by the coexpression of β_3 (Fig. 5 *A* and *B*). Consistent with previous reports (15, 18), Gem did not affect T-type currents, either with or without the coexpression of β_3 (Fig. 5*B*). Replacing the I–II loop of $Ca_v3.1$ (from I394 to I739) with that of $Ca_v2.1$ (from L359 to M483) produced a mutant named $T_{(PQ\ I\text{-}II\ loop)}$ that had larger currents (Fig. 5 *C* and *E*). Coexpression of β_3 with $T_{(PQ\ I\text{-}II\ loop)}$ did not significantly increase the peak current amplitude but markedly slowed the speed of inactivation (Fig. 5 *C* and *D*), indicating that β_3 was able to bind $T_{(PQ\ I\text{-}II\ loop)}$. This result was expected, because the I–II loop of $Ca_v2.1$ contains the $Ca_v\beta$-binding AID. However, coexpression of Gem with $T_{(PQ\ I\text{-}II\ loop)}$ had no effect on the amplitude of the current, either in the presence or absence of β_3 (Fig. 5*E*). These results indicate that binding of β_3 to the α_1 subunit alone is not sufficient to confer Gem inhibition to T-type channels.

Next, we created a chimeric $Ca_v3.1$ in which the I–II loop and the immediately adjacent downstream S1, S2, and S3 transmembrane segments of the second homologous repeat (from I394 to G828) were replaced by those of $Ca_v2.1$ (from L359 to F577); this mutant is named $T_{(PQ\ I\text{-}II\ loop—IIS3)}$ (Fig. 5*F*). In the absence of an exogenous $Ca_v\beta$, the current produced by $T_{(PQ\ I\text{-}II\ loop—IIS3)}$ was small and unaffected by Gem (Fig. 5 *F* and *H*). Coexpression of β_3 with $T_{(PQ\ I\text{-}II\ loop—IIS3)}$ greatly increased the peak current (Fig. 5 *F* and *H*) and slowed the inactivation speed (Fig. 5 *F* and *G*). Remarkably, in the presence of β_3, coexpression of Gem drastically suppressed the peak current (Fig. 5*H*). The magnitude of this inhibition is nearly as large as Gem inhibition of WT P/Q channels (Fig. 5*I*). Thus, by grafting the I–II loop, IIS1, IIS2, and IIS3 of $Ca_v2.1$ to $Ca_v3.1$, we were able to confer full-fledged Gem inhibition to an otherwise completely resistant T-type channel. These results indicate that IIS1, IIS2, and IIS3 are critical for Gem inhibition. They also reiterate the critical role of $Ca_v\beta$ in Gem inhibition.

We then examined whether the Gem/$Ca_v\beta$ interaction is necessary for Gem inhibition of $T_{(PQ\ I\text{-}II\ loop—IIS3)}$ by testing the effect of Gem_Mut3. As with P/Q channels (Fig. 3 *E* and *F*), Gem_Mut3 strongly suppressed the channels formed by $T_{(PQ\ I\text{-}II\ loop—IIS3)}$ and β_3_Mut3 (Fig. 5*H*), indicating that a direct physical interaction between Gem and $Ca_v\beta$ is not required for Gem inhibition.

In a complimentary experiment, we replaced a region harboring IIS1, IIS2, and IIS3 of $Ca_v2.1$ (from R482 to F577) with its counterpart from $Ca_v3.1$ (from K738 to G828); this mutant is referred to as $PQ_{(T\ IIS1—IIS3)}$ (Fig. 5*I*). Expression of this mutant $Ca_v2.1$ alone in oocytes produced little or no current, much like WT P/Q channels (Fig. S4). Coexpression of β_3 greatly increased the current (Fig. S4), indicating that $PQ_{(T\ IIS1—IIS3)}$ binds β_3. Strikingly, Gem had little or no effect on this current while strongly suppressing WT P/Q channel currents in the same batch of oocytes (Fig. 5*I*). These results identify a region in $Ca_v\alpha_1$ (IIS1–IIS3) that is essential for Gem inhibition.

Discussion

In this study, we show the following key features of Gem inhibition of P/Q-type Ca^{2+} channels: (*i*) Gem directly inhibits channels on the plasma membrane (Fig. 1), (*ii*) this inhibition requires $Ca_v\beta$ (Fig. 2), (*iii*) this inhibition does not require a direct physical interaction between Gem and $Ca_v\beta$ or $Ca_v\beta$-induced gating changes (Fig. 3), (*iv*) Gem coimmunoprecipitates with $Ca_v2.1$ (Fig. 4*A*), and (*v*) the region encompassing IIS1–IIS3 in $Ca_v2.1$ is essential for Gem inhibition (Fig. 5 *H* and *I*). Several of these key features (including *ii*, *iii*, and *v*) are reinforced by the results obtained from various $Ca_v2.1$/$Ca_v3.1$ chimeras (Fig. 5). These results, in aggregate, strongly support the $Ca_v\beta$-priming model proposed in Fig. 4*B*.

In the first study of RGK inhibition of HVA Ca^{2+} channels, Gem was reported to suppress the activity of L-, N-, and P/Q-type channels expressed in *Xenopus* oocytes and baby hamster kidney cells by decreasing their expression at the cell surface (14). By directly applying purified Gem proteins to the cytoplasmic face of inside-out membrane patches containing expressed P/Q channels, we show that the surface channels are rapidly and directly inhibited by Gem. Inhibition of surface HVA Ca^{2+} channels has also been observed for two other RGK proteins. Thus, Rem2 inhibits endogenous surface N-type channels in sympathetic and dorsal-root ganglion neurons (18), Rem inhibits surface L-type

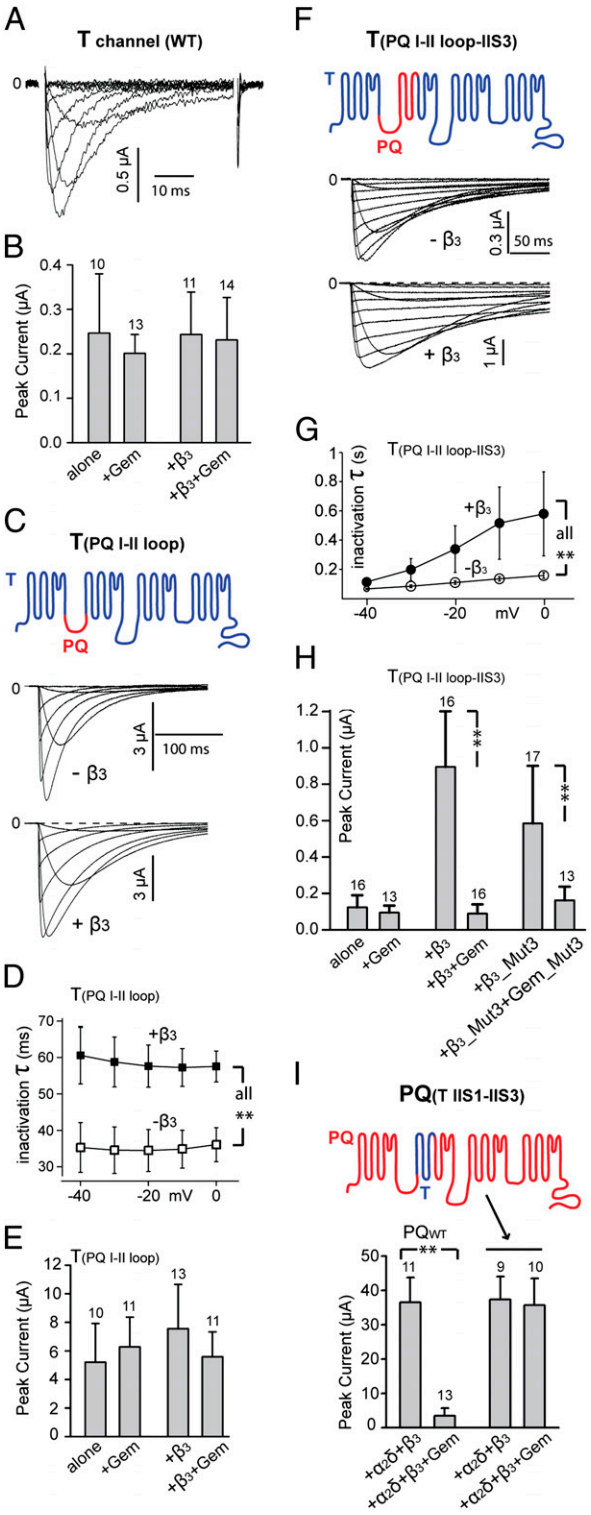

Fig. 5. The region encompassing IIS1–IIS3 of $Ca_v2.1$ is essential for Gem inhibition. (*A*) Representative family of currents from WT T-type channels. (*B*) Comparison of peak currents in oocytes expressing $Ca_v3.1$ and the indicated proteins. (*C*) Representative family of currents from channels formed by $T_{(PQ\ I\text{-}II\ loop)}$ (schematized in *Top*), with or without coexpression of β_3. (*D*) Comparison of the time constant of inactivation of channels formed by $T_{(PQ\ I\text{-}II\ loop)}$ alone and by $T_{(PQ\ I\text{-}II\ loop)}$ + β_3 (n = 5–6). (*E*) Comparison of peak currents in oocytes expressing $T_{(PQ\ I\text{-}II\ loop)}$ and the indicated proteins. (*F*) Exemplar family of currents from channels formed by $T_{(PQ\ I\text{-}II\ loop—IIS3)}$ (schematized in *Top*), with or without coexpression of β_3. (*G*) Comparison of the time constant of inactivation of channels formed by $T_{(PQ\ I\text{-}II\ loop—IIS3)}$ alone and $T_{(PQ\ I\text{-}II\ loop—IIS3)}$ + β_3 (n = 5–6). (*H*) Comparison of peak currents in oocytes expressing $T_{(PQ\ I\text{-}II\ loop—IIS3)}$ and the indicated proteins, showing inhibition of channels containing $T_{(PQ\ I\text{-}II\ loop—IIS3)}$ and β_3 by WT Gem and inhibition of channels containing $T_{(PQ\ I\text{-}II\ loop—IIS3)}$ and β_3_Mut3 by Gem_Mut3. (*I*) Comparison of the effect of Gem on currents produced by WT P/Q channels formed by $Ca_v2.1$, $\alpha_2\delta$, and β_3 and mutant channels formed by $PQ_{(T\ IIS1—IIS3)}$ (schematized in *Upper*), $\alpha_2\delta$, and β_3, showing the complete lack of inhibition of the mutant channels. Similar results were observed in at least three batches of oocytes.

channels expressed in pancreatic β-cells (19, 21) and HEK 293 cells (31), and rapid translocation of a recombinant Rem derivative acutely inhibits L- and N-type channels expressed in tsA201 cells (25).

Previous studies show that RGK proteins do not affect Ca^{2+} channels in heterologous systems expressing only $Ca_v\alpha_1$ (14, 15, 22), suggesting that $Ca_v\beta$ is necessary for RGK inhibition of Ca^{2+} channels. Consistent with this notion, it has been shown and is reproduced in this study that T-type Ca^{2+} channels, which do not contain an associated $Ca_v\beta$, are not modulated by RGK proteins (Fig. 5*A*) (15, 18). However, until now, it was unclear whether $Ca_v\beta$ is required for RGK inhibition of surface Ca^{2+} channels. By successfully producing large populations of β-less Ca^{2+} channels on the plasma membrane, we show unequivocally that $Ca_v\beta$ is essential for this direct inhibitory effect (Fig. 2).

A striking finding of this work is that the direct association between Gem and $Ca_v\beta$ is not necessary for Gem inhibition (Fig. 3). This finding contradicts the general belief that RGK proteins exert their inhibitory action on HVA Ca^{2+} channels through $Ca_v\beta$ (13–29, 31). However, a direct physical interaction between RGK proteins and $Ca_v\beta$ has been well-documented (14–17, 19–21, 23, 25, 27–29, 34). Therefore, what is the functional importance of this interaction? One possibility is that in native cells, with physiological levels of RGK proteins, the RGK/$Ca_v\beta$ interaction serves to facilitate RGK inhibition by bringing RGK proteins close to surface Ca^{2+} channels, hence increasing the effective local concentration of RGK proteins near the channels. Another completely disparate function of this interaction could be to translocate full-length $Ca_v\beta$ into the nucleus (20), where $Ca_v\beta$ could engage in transcriptional regulation (36).

The $Ca_v\beta$-priming model proposed here for Gem may also be applicable to Rem and Rem2, because they both have been shown to inhibit surface Ca^{2+} channels (18, 19, 21, 25, 31). This model remains speculative, and many questions remain to be answered, including the locations of the proposed anchoring site and inhibitory binding site for Gem in $Ca_v\alpha_1$. These binding sites are likely located in the cytoplasmic regions of $Ca_v\alpha_1$, given that Gem is a cytosolic protein. Indeed, a new study reports that a proximal C-terminal region of $Ca_v1.2$ can directly bind Rem, Rem2, and Rad in vitro (30). Because this binding occurs in the absence of a $Ca_v\beta$, it could serve as the anchoring interaction proposed in our model. This hypothesis needs further testing. A mutant $Ca_v1.2$ containing the S1928A mutation was reported to be resistant to inhibition by Rem2 (37), which would suggest that this C-terminal region and protein kinase A (PKA) phosphorylation of $Ca_v\alpha_1$ regulate Rem2 inhibition of L-type channels. However, deleting the distal C terminus of $Ca_v1.2$ (from K1906 to L2171) has no effect on Rem inhibition of $Ca_v1.2$, although this is a region that also binds Rem in vitro (30). Thus, the role of $Ca_v\alpha_1$ C terminus in RGK inhibition remains largely unclear. The molecular mechanism of the involvement of the IIS1–IIS3 region, which is highly conserved among the $Ca_v\alpha_1$ of HVA Ca^{2+} channels (Fig. S5), in Gem inhibition is also unclear. One possible role of this region is mediating the conformational changes of $Ca_v\alpha_1$ that likely occur after $Ca_v\beta$ and/or Gem binding.

Although many of the molecular details remain to be elucidated, our findings and the $Ca_v\beta$-priming model shift the focus from the RGK/$Ca_v\beta$ interaction to RGK/$Ca_v\alpha_1$ interactions. This shift may prompt new investigations into and expand our understanding of the molecular mechanisms of RGK regulation of HVA Ca^{2+} channels.

Materials and Methods

WT and mutant P/Q- and T-type Ca^{2+} channels were expressed in *Xenopus* oocytes with or without WT or mutant Gem. Ba^{2+} currents were recorded with inside-out patch clamp or two-electrode voltage clamp. Purified Gem protein fragments were applied to membrane patches in inside-out patch-clamp recordings. Coimmunoprecipitation was carried out in HEK 293T cells.

Details for the methods described above and construct cloning, cell culture and transfection, SDS/PAGE and Western blot, oocyte preparation and injection, protein expression and purification, coimmunoprecipitation, and electrophysiology are provided in *SI Materials and Methods*.

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