Direct Inhibition of the Interaction between α-Interaction Domain and β-Interaction Domain of Voltage-dependent Ca^{2+} Channels by Gem

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The Ras-related small G-protein Gem regulates voltage-dependent Ca^{2+} channels (VDCCs) through interaction with the β-subunit of the VDCC. This action of Gem is mediated by regulated α-subunit expression at the plasma membrane. In the present study, we examined the mechanism of the inhibition of VDCC activity by Gem. The β-interaction domain (BID) of the β-subunit, which specifically interacts with the α-interaction domain (AID) of the α-subunit, is shown to be essential for the interaction between Gem and β-subunits. In addition, the AID peptide inhibited interaction between Gem and β-subunits in a dose-dependent manner. GemSS88N mutant, which has low binding affinity for guanine nucleotide, did not interact with β-subunits, allowing α2-subunit expression at the plasma membrane. This inhibitory effect of wild-type Gem on VDCC activity was reduced in cells expressing GemSS88N. The overexpression of wild-type Gem in pancreatic β-cell line MIN6 cells suppressed Ca^{2+}-triggered secretion, whereas overexpression of GemSS88N induced Ca^{2+}-triggered secretion to control level. These results suggest that GTPase activity of Gem is required for the binding of Gem to BID that regulates VDCC activity through interaction with AID.

Voltage-dependent Ca^{2+} channels (VDCCs)† permit the entry of Ca^{2+} into excitable cells, coupling membrane potential changes to biological activities including muscle contraction, hormone and neurotransmitter release, neuronal migration, and gene expression (1–4). Based on the pharmacological and electrophysiological properties, VDCCs have been grouped into five subclasses, T, L, N, P/Q, and R (5–7). The VDCCs are multisubunit proteins composed of α1, β, α2γδ, and γ-subunits.

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§ The abbreviations used are: VDCC, voltage-dependent calcium channel; AID, α-interaction domain; BID, β-interaction domain; GST, glutathione S-transferase; tsA201 cells, large T-antigen-transformed human embryonic kidney cells; bGH, human growth hormone; GEF, guanine nucleotide exchange factor; RGK protein, Rem, Rad, and Gem/kir protein; EGFP, enhanced green fluorescent protein; MOPS, 3-(N-morpholino)propanesulfonic acid; SH, Src homology.

The α-subunit includes the pore of the channel, the voltage sensor, and the binding sites of drugs and toxins that modulate channel activity. Although the biophysical diversity of native VDCCs is conferred by α1-subunits, auxiliary subunits β, β2/δ, and γ modulate channel properties including current amplitude, voltage dependence, and kinetics of activation and inactivation (8–11). Ten α1-subunit isoforms have been identified (4). Four β-subunit isoforms, β1, β2, β3, and β4 have been identified (12). Knock-out mice lacking the β1, β2, or β4-subunits demonstrate that the β-subunit is essential for formation and functional expression of VDCCs (13–15).

Coexpression of a β-subunit with an α1-subunit results in an increase of peak current amplitude and an increase in the number of binding sites for drugs and toxins, acceleration of activation and inactivation kinetics, and an increase the number of α1-subunits in plasma membrane (16–19). The effects of β-subunits are suggested to be mediated by interaction with the intracellular I-II loop of α1-subunits (20–22). The I-II loop of the α1-subunit includes the endoplasmic reticulum retention signal and inhibits trafficking of α1-subunits to the plasma membrane. The interaction of the loop with the β-subunit masks the retention signal in the loop, leading to channel expression at the plasma membrane (23). β-Subunits function as chaperone-like molecules in the trafficking of α1-subunits to plasma membrane (17). α1- and β-subunits interact with each other at the α-interaction domain (AID) and the β-interaction domain (BID), respectively (see Fig. 1A) (20, 24). AID comprises a minimal 18 amino acids in the I-II loop of the α-subunit. BID comprises the amino-terminal 30 amino acids of the second conserved domain of the β-subunit.

Gem was identified originally in mitogen-induced peripheral blood T cells (25) and oncogenic tyrosine kinase-transformed cells (26). Gem is a member of the RGK (Rem, Rad, and Gem/kir) family consisting of a long amino-terminal region, a Ras-related core domain, and a carboxy-terminal region lacking a CAAX motif (27, 28). Subcellular localization of Gem requires interaction of its carboxyl-terminal region with Ca^{2+}/calmodulin. We previously reported that Gem inhibits the trafficking of the α1-subunits of N, P/Q, and L-type VDCCs to the plasma membrane by interacting with the β-subunits (29), inhibiting channel activities. Inhibition of cell surface expression also is involved in the interaction of the Gem carboxyl terminus with Ca^{2+}/calmodulin. Recently, it has been shown that Rad and Rem, members of the RGK family, also inhibit VDCC activity by binding to the β-subunit (30).

In the present study, we examined the mechanism of regu-
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EXPERIMENTAL PROCEDURES

Constructions—Rabbit α1,2-subunit, rat wild-type β-subunit, and β-subunit lacking BID (amino acid residues 163–203) (β$_{BID}$) were subcloned in pCMV expression vector as FLAG-tagged protein (Sigma). AID was subcloned in pCMV expression vector as EGFP-tagged protein (Clontech Laboratories, Inc.). Rat wild-type β$_{subunit}$ was subcloned in pCMV expression vector. Mouse Gem was subcloned in pCMV expression vector as Myc-tagged protein. Site-directed mutagenesis of Gem was performed by the PCR-based method.

Recombinant Proteins—Mouse Gem was expressed as glutathione S-transferase (GST) fusion protein and purified by affinity chromatography on glutathione-agarose (Amersham Biosciences Corp.). GST-FLAG AID peptides (amino acid residues 458–475) in the I-II loop of rabbit α$_1$-subunit were synthesized and purified using reverse-phase chromatography.

Cell Culture and Transfection—COS-1 cells, MIN6 cells, and tsA201 cells were maintained in Dulbecco’s modified Eagle’s medium (Sigma) containing 10% fetal bovine serum (Sigma) and 5% fetal bovine serum (Sigma) at 37 °C in 10% CO$_2$. Transfections were performed using the Lipofectamine Reagent (Invitrogen) and 5% fetal bovine serum (Sigma) at 37 °C in 10% CO$_2$. Serum (Invitrogen) and 1 mM MEM sodium pyruvate were used as a charged carrier.

Subcloned in pCMV expression vector as FLAG-tagged protein (Sigma). The bound proteins were separated by SDS-PAGE, followed by immunoblotting with anti-Gem antibody. The blots were visualized using goat anti-mouse (AID), goat anti-rabbit (Gem), and goat anti-mouse (EGFP) secondary antibodies.

Recombinant Proteins—Mouse Gem was expressed as glutathione S-transferase (GST) fusion protein and purified by affinity chromatography on glutathione-agarose (Amersham Biosciences Corp.). GST-FLAG AID peptides (amino acid residues 458–475) in the I-II loop of rabbit α$_1$-subunit were synthesized and purified using reverse-phase chromatography.

Immunoprecipitation and Immunoblotting—Transfected COS-1 cells were homogenized in hypotonic buffer (10 mM HEPES-NaOH, pH 7.8, 0.5 mM ethylenediamine tetraacetic acid (EDTA), 1.5 mM MgCl$_2$, 10 mM KCl, 10 mM Tris, 10 μM phenylmethylsulfonyl fluoride). The homogenate was centrifuged at 800 × g for 5 min. The supernatant was centrifuged at 15,000 × g for 20 min. The resulting pellet was extracted in extract buffer (20 mM Tris-HCl, pH 7.8, 1 mM EDTA, 0.5 mM dithiothreitol, 1.5 mM MgCl$_2$, 100 mM NaCl, 0.5% Tween 20, 10 μM phenylmethylsulfonyl fluoride). The extracts were subjected to immunoprecipitation with anti-FLAG M2 antibody (Sigma). The bound proteins were separated by SDS-PAGE, followed by immunoblotting with anti-Gem antibody. The blots were visualized using goat anti-mouse (AID), goat anti-rabbit (Gem), and goat anti-mouse (EGFP) secondary antibodies.

Electrophysiology—The whole-cell VDCC currents in PC12 cells were recorded as described (29). Briefly, Ba$^{2+}$ was used as a charged carrier for measurement of VDCC currents. The extracellular solution contained 110 mM NaCl, 11.2 mM KCl, 1.8 mM CaCl$_2$, 1.0 mM MgCl$_2$, 5.0 mM HEPES, pH 7.2. The pipette solution contained 110 mM NaCl, 11.2 mM KCl, 1.8 mM CaCl$_2$, 1.0 mM MgCl$_2$, 5.0 mM HEPES, pH 7.2. Cells were maintained at a holding potential of −60 mV. For recording VDCC currents, square pulses of 400-ms duration at potentials between −40 and +60 mV in steps of 10 mV were applied every 4 s. Currents were normalized by dividing the membrane capacitance for each cell (31). Recordings were performed using the Axopatch200B amplifier (Axon Instruments, Foster City, CA).

Measurement of Growth Hormone (GH) Secretion—MIN6 cells were cotransfected with pXGH5-human hGH (Nicholas Institute, San Juan Capistrano, CA) and pSV2 wild-type Gem or pSV2-GemSS8N. As a control, the cells were cotransfected with pXGH5 and pSV2-α-galactosidase. One day after transfection, MIN6 cells were harvested and split to a 12-well plate at a density of 5.0 × 10$^5$ cells/well. Two days after transfection, MIN6 cells were washed three times with KR8 G1.0 (140 mM NaCl, 4.7 mM KCl, 1.2 mM KH$_2$PO$_4$, 1.2 mM MgSO$_4$, 5.0 mM NaHCO$_3$, 2.5 mM CaCl$_2$, 10 mM HEPES, pH 7.4, 0.1% bovine serum albumin, and 1.0% fetal bovine serum) and then preincubated at 37 °C for 30 min. After washing with KR8 G1.0, the cells were incubated at 37 °C for 10 min with a high K$^+$ solution (KR8 G1.0 containing 40 mM KCl and 85 mM NaCl) or a low K$^+$ solution (KR8 G1.0 containing 4.7 mM KCl and 140 mM NaCl). Amounts of bGH released into medium were measured using enzyme-linked immunosorbent assay kit (Roche Applied Science). Secretion was expressed as percent of the amount of bGH released into medium relative to the total cellular GH amounts (32).

RESULTS

Binding of Gem to β$_{subunit}$—It has been shown that the binding of β-subunits participates in modulation of VDCC activity through interaction with AID in α-subunits (24). We investigated the involvement of BID in the interaction between Gem and the β$_{subunit}$. FLAG-tagged wild-type β$_{subunit}$ or β$_{subunit}$ lacking BID were expressed in COS-1 cells. The cell lysate was subjected to immunoprecipitation with anti-FLAG M2 antibody and visualized by immunoblotting with anti-living colors A.v. peptide antibody. In vivo binding of β$_{subunit}$ and Gem by AID. FLAG-tagged β$_{subunit}$ expressed in COS-1 cells was incubated with GST-tagged Gem immobilized on glutathione-resin at a dosage of 0–1 μg of AID peptide. Samples were subjected to pull-down assay and visualized by immunoblotting with anti-FLAG M2 antibody.

Immunofluorescence Staining and Confocal Microscopy—The tsA201 cells were transfected with FLAG-tagged α$_1$,2-subunit, β$_{subunit}$, and wild-type Gem or GemSS8N. The transfected cells were fixed with 4% paraformaldehyde, followed by permeabilization with 0.2% Triton X-100, and incubated with 10% normal goat serum in phosphate-buffered saline. The cells were incubated at room temperature for 60 min with anti-FLAG M2 antibody. As the secondary antibody, Cy3-conjugated goat anti-mouse IgG antibody (Chemicon International, Temecula, CA) was used. The cells were mounted on cover slips using PermaFlour (Immunon Pittsburgh, PA) and observed using LSM510/Ver3.0 laser scanning microscope (Carl Zeiss Co., Ltd.).

Because both AID and Gem bind to BID in the β$_{subunit}$, we investigated AID inhibition of the binding of Gem and BID. As shown in Fig. 1D, the binding of GST-tagged Gem and FLAG-tagged β$_{subunit}$ was inhibited in an AID peptide dose (0–1 μg) dependent manner, suggesting that the inhibitory effect of Gem on the trafficking of α$_{subunit}$ to the plasma membrane is because of its blocking of the interaction between BID and AID.

Requirement of Guanine Nucleotide Binding of Gem for Interaction between Gem and β$_{subunit}$—Activation of small G-proteins leads to stimulation of the downstream pathway
through effector proteins. Because the RasS17N mutant, which has low binding affinity for the guanine nucleotide, cannot bind to Ras effector proteins and functions in a dominant negative manner, the mutant has been used to investigate the effect of GTP-bound form (active form) of Ras on its physiological functions (33). By analogy to RasS17N mutant, we constructed Gem mutant in which Ser at 88 was substituted by Asn (GemS88N), as described previously (34, 35). The mutant has been shown to have reduced guanine nucleotide-binding affinity (35). We investigated to find out whether the guanine nucleotide-binding of Gem is required for binding Gem and the β-subunit using GemS88N. GST-tagged GemS88N and FLAG-tagged β3-subunit expressed in COS-1 cells was subjected to an in vitro binding assay. The binding of GST-tagged GemS88N to the FLAG-tagged β3-subunit was significantly decreased compared with that of wild-type Gem to FLAG-tagged β3-subunit (Fig. 2A). In COS-1 cells coexpressing Myc-tagged GemS88N and FLAG-tagged β3-subunit, Myc-tagged GemS88N did not bind to FLAG-tagged β3-subunit (Fig. 2B). These results suggest that guanine nucleotide-binding of Gem is required for the interaction between Gem and the β3-subunit.

**Subcellular Localization of α1.2-Subunit in tsA201 Cells Expressing Wild-type Gem and GemS88N**—We investigated to find out whether the guanine nucleotide-binding of Gem participates in inhibition of α1-subunits at cell surface expression. Subcellular localization of the FLAG-tagged α1.2-subunit in tsA201 cells expressing GemS88N, FLAG-tagged α1.2-subunit, and β3-subunit were analyzed by confocal microscopy. In cells expressing FLAG-tagged α1.2-subunit alone, the α1.2-subunit was not localized to the plasma membrane (Fig. 3A). In cells expressing the FLAG-tagged α1.2-subunit and β3-subunit, the α1.2-subunit was localized to plasma membrane (Fig. 3B). When wild-type Gem was expressed together with the FLAG-tagged α1.2-subunit and β3-subunit, the α1.2-subunit was not localized to plasma membrane (Fig. 3C), as described previously (29). However, when GemS88N was expressed together with the FLAG-tagged α1.2-subunit and β3-subunit, the α1.2-subunit was mainly localized to plasma membrane (Fig. 3D). No effects of GemS88N on the subcellular localization of the α1.2-subunit were found in any of the transfected tsA201 cells.

**Effect of GemS88N on VDCC Activity and Hormone Secretion**—To elucidate the effect of guanine nucleotide-binding of Gem on inhibition of VDCC activity, we examined the effects of Gem in PC12 cells, in which Ca2+-induced exocytosis is triggered through the activation of L-type VDCC (36). In PC12 cells expressing wild-type Gem or GemS88N, the J-V relationship of VDCC was measured (Fig. 4). The overexpression of wild-type Gem in PC12 cells resulted in a significant inhibition of endogenous Ca2+ channel currents, whereas overexpression of the GemS88N had only a small inhibitory effect (Fig. 4).

Overexpression of wild-type Gem in PC12 cells and MIN6 cells prevents Ca2+-triggered exocytosis through inhibition of VDCC activity (29). We examined the effect of GemS88N on Ca2+-triggered exocytosis in MIN6 cells. For this purpose, we utilized MIN6 cells transfected with hGH (32) and monitored secretion by measuring the hGH release from hGH-transfected MIN6 cells under high K+ stimulation. The overexpression of wild-type Gem in MIN6 cells inhibited hGH secretion from 10.6 ± 0.6% of total (control, MIN6 cells overexpressing β-galactosidase) to 7.9 ± 0.8% of total. In contrast, in MIN6 cells overexpressing GemS88N, the hGH secretion (11.5 ± 0.9% of total) was comparable with the control level (Fig. 5). These results suggest that inhibitory effects of Gem on both VDCC activity and hormone secretion are involved in its guanine nucleotide-binding.

**DISCUSSION**

The β-subunits of VDCCs are critical in determining the properties of the channels including modification of kinetics, amplitude, and trafficking of α-subunits to the plasma membrane (16–19). We previously reported that interaction of β-subunits with Gem prevents the trafficking of α-subunits to the plasma membrane in the regulation of VDCC activity (29). It also has been found that infection of guinea pig heart with adenovirus carrying Gem markedly decreased L-type VDCC density at the sarcolemma of isolated ventricular myocytes (37). However, the mechanism of the inhibition of VDCC activity by Gem was not known. BID in β-subunits has been shown to interact with AID in the I-II loop of α-subunits in the modulation of VDCC activity (24). We found that BID also is required for interaction with Gem and that the AID peptide inhibits the binding of Gem to the β-subunits. These findings...
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suggest a competitive interaction between Gem and the α1-subunits at the BID site. In addition to BID, the β3-subunit contains Src homology 3 (SH3) and guanylate kinase domains (38). The tandem arrangement of these domains is characterized in membrane-associated guanylate kinase, which serves as the scaffold proteins that organize the signaling pathway (39). These motifs are required in the modulation of VDCC activity (40). The finding that the mutant β3-subunit (β3ΔBID) lacking BID but containing both SH3 and guanylate kinase domains cannot bind to Gem indicates that neither the SH3 nor the guanylate kinase domain is required for the inhibitory effect of Gem on VDCC activity.

The GTP-bound form (active form) of small G-proteins binds with high affinity to a set of effector proteins that initiate distinct intracellular signaling cascades. The active form of Gem specifically interacts with the β-subunits in vitro (29). We found that mutant GemS88N, which has reduced guanine nucleotide-binding affinity, did not bind to the β3-subunit and had no inhibitory effect on the trafficking of α1-subunits to the plasma membrane. These findings suggest that the GTP form of Gem is required for its inhibitory effect on interaction of the α1- and β-subunits. Indeed, overexpression of wild-type Gem in PC12 cells inhibited endogenous VDCC currents, whereas overexpression of mutant GemS88N reduced them only slightly.

We also examined the effect of mutant GemS88N in MIN6 cells, in which insulin granule exocytosis is triggered by Ca2+ influx through activation of L-type VDCCs (41), and found that Ca2+-triggered hormone secretion was inhibited in MIN6 cells expressing wild-type Gem, whereas secretion in MIN6 cells expressing GemS88N was not inhibited. This suggests that cycling between the GTP- and GDP-form is important in the effect of Gem on Ca2+-triggered hormone secretion most likely through regulation of the activity of the VDCCs.

NM23, which was identified as GTPase-activating protein and guanine nucleotide exchange factor (GEP) for Rad, acts as Gem-GEF in vitro (42). It has been shown that NM23 inhibits Rad-induced growth and tumorigenicity of breast cancer in breast tissues (43). However, it is not known whether NM23 activates Gem in intact cells. The GTPase-activating protein and GEF for Gem, which have yet to be identified, may play important roles in Gem-mediated cellular functions. Gem interacts with the small G-protein Rho effector, Rho kinase, and negatively regulates cytoskeletal organization mediated by Rho/Rho kinase signaling (44). Rho/Rho kinase signaling has been shown to modulate the translocation of plasma membrane proteins including Na+,K+-ATPase and water channel aquaporin-2 (45, 46). Although the effect of Rho kinase on the interaction between Gem and the β-subunits is not clear, translocation to the plasma membrane of α1-subunits regulated by Gem/β-subunits may well be involved in Rho/Rho kinase signaling.

Recently, it has been shown that Rad and Rem, members of the RGA family, also regulate VDCC activity through interaction with the β-subunits of the VDCCs (30). As RGA proteins Rad, Rem, and Gem share conserved structural features (25–28), a similar mechanism may well be involved in regulation of VDCC activities by these small G-proteins.

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