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Functional Role of a C-Terminal Gβγ-Binding Domain of $Ca_v2.2$ Channels

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ABSTRACT

Presynaptic Ca^{2+} channels are inhibited by neurotransmitters acting through G protein-coupled receptors via a membrane-delimited pathway. Inhibition is reversed by strong depolarization, resulting in prepulse facilitation. Activated G protein βγ subunits (Gβγ) are required for maximal prepulse facilitation. Gβγ binds to multiple sites on $Ca_v2.1$, $Ca_v2.2$, and $Ca_v2.3$ α1 subunits. Here we examine the functional relevance of a C-terminal binding site for Gβγ on $Ca_v2.2b$ channels, which mediate N-type Ca^{2+} currents. In vitro binding studies showed that Gβγ subunits bind to the intracellular loop connecting domains I and II and the C-terminal domain of $Ca_v2.2b$ but not the intracellular loops connecting domains II and III or III and IV. Deletion analysis revealed that the binding site is located near the C terminus, within amino acid residues 2257 to 2336. Directed yeast two-hybrid analysis confirmed this specific binding interaction in vivo in yeast cells. $Ca_v2.2b$ channels with this site deleted had normal function properties, and they were inhibited essentially normally by strong activation of G proteins with guanosine 5′-3-*O*-(thio)triphosphate (GTPγS) and were facilitated nearly normally by depolarizing prepulses. Similarly deletion of this site had small, statistically insignificant effects on inhibition of Ca^{2+} current and on prepulse facilitation in the presence of somatostatin to stimulate receptor-mediated activation of G proteins. In contrast, deletion of the C-terminal Gβγ site substantially reduced the low level of intrinsic prepulse facilitation present at the basal level of G protein activation in tsA-201 cells. Thus, this C-terminal Gβγ binding site contributes to the affinity or efficacy of Gβγ regulation at basal levels of G protein activation. The simplest interpretation of our results is that the C-terminal binding site increases the affinity of Gβγ for the channel but is not required for Gβγ action. C-terminal binding of Gβγ may influence the physiological responsiveness of Ca^{2+} channels to low-level G protein activation.

Presynaptic Ca^{2+} channels of the Ca_v2 family conduct N-, P/Q-, and R-type Ca^{2+} currents (Ertel et al., 2000) and are inhibited by neurotransmitters acting through G protein-coupled receptors (Hille, 1994). This negative feedback mechanism has an important effect on regulation of neurotransmitter release and synaptic transmission (Lipscombe et al., 1989; Brody and Yue, 2000). G protein-mediated inhibition is caused by a positive shift in the voltage dependence of activation (Bean, 1989) and is reversed by strong positive prepulses or by trains of action potential-like stimuli, producing prepulse facilitation of Ca^{2+} channel activity (Marchetti et al., 1986; Tsunoo et al., 1986; Brody and Yue, 2000). These effects are mediated by binding of G protein βγ subunits (Gβγ) to Ca_v2 channels (Herlitze et al., 1996; Ikeda, 1996). Gβγ binds to at least three sites on Ca_v2 channels. The primary site, located in the intracellular loop connecting domains I and II ($L_{I\text{-}II}$), was identified by mutagenesis studies and G protein binding studies (De Waard et al., 1997; Herlitze et al., 1997; Page et al., 1997; Zamponi et al., 1997). A second site of interaction is located in the C-terminal domain of $Ca_v2.3$ subunits (Zhang et al., 1996; Qin et al., 1997; Furukawa et al., 1998; Simen et al., 2001). The third site of interaction is located at the N terminus and in domain I of $Ca_v2.2$ and $Ca_v2.3$ channels (Page et al., 1998; Simen and Miller, 1998, 2000; Canti et al., 1999). All these binding sites appear to be involved in G protein binding and subsequent modulation of channel gating, but their relative functional roles have not been clearly resolved.

N-type Ca^{2+} currents in nerve terminals play a central role in regulating the release of neurotransmitters (Hirning at al., 1988: Turner et al., 1993; Dunlap et al., 1995). N-type Ca^{2+}

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ABBREVIATIONS: CMV, cytomegalovirus; nt, nucleotide(s); PCR, polymerase chain reaction; aa, amino acid; GST, glutathione *S*-transferase; Ni-NTA, nickel-nitrilotriacetic acid; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate; NEM, *N*-ethylmaleimide; SST, somatostatin; GBD, GAL4 DNA-binding domain; GAD, GAL4 activation domain; GRK, G protein receptor kinase; PAK, p21-activated kinase; PAGE, polyacrylamide gel electrophoresis.

currents are conducted by $Ca_v2.2$ channels (Dubel et al., 1992; Williams et al., 1992; Ertel et al., 2000), which are highly concentrated in nerve terminals (Westenbroek et al., 1992, 1995). Like the other members of the Ca_v2 family, $Ca_v2.2$ channels are inhibited by activation of G protein-coupled receptors (Hille 1994; Dunlap et al., 1995) through binding of $G\beta\gamma$ subunits (Herlitze et al., 1996; Ikeda, 1996). Localization of G protein interaction sites and determination of their functional effects on $Ca_v2.2$ channels is important for understanding the molecular mechanisms of modulation of the activity of the Ca^{2+} channel and subsequent regulation of synaptic transmission. The initial evidence for a functional role of the C-terminal domain in G protein modulation came from studies of a family of $Ca_v2.2/Ca_v1.2$ chimeras (Zhang et al., 1996), which led to the conclusion that domain I, $L_{I\text{-}II}$, and the C-terminal domain are required for establishment of G protein modulation in an unresponsive $Ca_v1.2$ channel. Consistent with these results, Qin et al. (1997) defined a C-terminal G protein binding site that is required for modulation in $Ca_v2.3$, which has weak G protein modulation compared with $Ca_v2.1$ and $Ca_v2.2$ channels (Toth et al., 1996); however, Furukawa et al. (1998) found that neither a deletion of the C-terminal region nor a replacement of the C-terminal domain of $Ca_v2.2$ by that of $Ca_v1.2$ affected $G\beta\gamma$-dependent facilitation of the channel. In contrast, they found that a $Ca_v2.2$ chimera containing a substitution of $L_{I\text{-}II}$ from $Ca_v1.2$ displayed a complete loss of $G\beta\gamma$-dependent prepulse facilitation. From these reports, the functional roles of the G protein binding sites in the C-terminal domains of $Ca_v2.2$ and related Ca^{2+} channels remain unresolved, and the location of the $G\beta\gamma$ binding site in the C-terminal domain of $Ca_v2.2$ is unclear. In this study, we have identified a $G\beta\gamma$ binding site near the C terminus of $Ca_v2.2b$ channels through in vitro binding of fusion proteins and in vivo binding in yeast cells, and we have tested the functional significance of the C-terminal $G\beta\gamma$-binding domain by whole-cell voltage clamp recording. Our results show that $G\beta\gamma$ subunits bind to a segment of 80 amino acid residues near the C terminus of $Ca_v2.2b$, which contributes to prepulse facilitation of Ca^{2+} channel activity primarily at the basal level of G protein activation.

Materials and Methods

Construction of Ca^{2+} Channel Plasmids. A recombinant cDNA encoding $Ca_v2.2b$ from rat brain (originally called rbB-II; Dubel et al., 1992) was subcloned into pMRC-CMV (Invitrogen; Zhong et al., 2001). The β1b subunit was in the vector pMT2XS, $\alpha2\delta$ in pZEM228, and CD8 in EBO-pcD (Zhong et al., 2001). To construct the C-terminal deletion mutant of $Ca_v2.2b$ ($Ca_v2.2b\Delta CT5$), C at nt 6768 was mutated to G by a two-step PCR mutagenesis such that a TAG stop codon was introduced at residue 2256. A PCR fragment (nt 6526–7011) containing the mutation was subcloned into the $Ca_v2.2b$ expression plasmid by replacing its wild-type counterpart. To identify the G protein binding sites on the Ca^{2+} channel, NT (nt 1–285, aa 1–95), $L_{I\text{-}II}$ (nt 1069–1449, aa 357–483), $L_{II\text{-}III}$ (nt 2131–3429, aa 711-1143), $L_{III\text{-}IV}$ (nt 4252–4422, aa 1418–1474), CT1 (nt 5128–6130, aa 1710–2043), CT2 (nt 6130–7011, aa 2044–2336), CT3 (nt 6628–7011, aa 2211–2336), CT4 (nt 6130–6747, aa 2044–2249), and CT5 (nt 6768–7022, aa 2257–2336) were subcloned into the pAS2–1 vector (BD Biosciences Clontech, Palo Alto, CA) for expression of GAL4 DNA-binding domain (GBD) hybrids in yeast. Plasmids expressing the GAL4 activation domain (GAD)-Gβ hybrids and the yeast strain for the yeast two-hybrid assay have been described by Garcia et al. (1998). Plasmids expressing hexahistidine-tagged channel fragments were described earlier (Sheng et al., 1994). $L_{I\text{-}II}$ was also cloned into the pGEX4T-1 (Pfizer, Inc., New York, NY) as a EcoRI-SalI fragment and CT5 into pGEX3X (Pfizer, Inc.) as a BamHI-PstI fragment to express GST fusion proteins.

Protein Purification. $Ca_v2.2b$ fragments were subcloned in both pTrcHisC and the GST-fusion protein expression vectors pGEX3X or pGEX4T-1 by PCR amplification and subcloning. The constructs including the PCR-amplified Ca^{2+} channel inserts were confirmed by DNA sequencing. These plasmids were transformed into B21DE3 (Novagen, Madison, WI) for protein expression. To purify the hexahistidine-tagged fusion protein, 500 ml of LB medium containing 75 μg/ml ampicillin was seeded with a 10-ml overnight culture containing the expression plasmid. Isopropyl β-D-thiogalactoside (0.1 mM) was added to the culture when the cell density generated an optical density at 600 nm of 0.7. After a 3-h induction, the culture was harvested, and the cell pellet was resuspended in the phosphate buffer (QIAGEN, Valencia, CA) containing protease inhibitors (200 μm phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml aprotinin). Following sonication, 1% Triton X-100 was added to the lysate. The lysate was incubated on ice for 30 min and subjected to centrifugation for 50 min at 15,000 rpm at 4°C. The supernatant was saved and incubated for 2 h with Ni-NTA beads (QIAGEN) that had been equilibrated with phosphate buffer. After washing by phosphate buffer and the same buffer containing 20 mM imidazole, the hexahistidine-tagged protein was eluted by a series of concentrations of imidazole from 50 to 300 mM. The fraction containing concentrated pure protein was used for in vitro binding assays. The GST fusion proteins were expressed similarly in bacteria and harvested. Cell pellets were resuspended in phosphate-buffered saline containing 5 mM EDTA and protease inhibitors mentioned above. Cell lysates were obtained by sonication and centrifugation as described above. The lysates were incubated for 1 h with glutathione-Sepharose (Pfizer, Inc.) equilibrated with PBS. Following incubation, the beads were washed with PBS containing 0.1% Triton X-100, 5% glycerol, 0.5 mM EDTA, and 0.2 mM phenylmethylsulfonyl fluoride, and the GST-fusion protein was eluted with 20 mM reduced glutathione in 50 mM Tris-HCl, pH 8.0. The untagged wild-type $G\beta_1\gamma_2$ protein, hexahistidine-tagged wild-type $G\beta_1\gamma_2$, and the five hexahistidine-tagged $G\beta_1$ mutants in complex with the γ_2 subunit were purified as described (Kozasa and Gilman, 1995).

In Vitro Binding Assay. Affinity-purified wild-type $G\beta_1\gamma_2$ complex (50 nM) was incubated with 100 nM hexahistidine-tagged Ca^{2+} channel fragments in a volume of 400 μl of buffer A (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween 20, 20 mM imidazole) for 2 h at 4°C. Ni-NTA beads (20 μl) that had been equilibrated with buffer A were then added to the preincubated protein complexes followed by tumbling for another 2 h at 4°C. The beads were then centrifuged briefly at top speed in a microcentrifuge, and washed with buffer A four times. The hexahistidine-tagged Ca^{2+} channel fragments and their associated proteins were eluted from the Ni-NTA beads with 600 mM imidazole in Tris-buffered saline (10 mM Tris-HCl, pH 8.0, 150 mM NaCl) at room temperature for 30 min on a vortex mixer. The eluates were then subjected to Western blot analysis with anti-$G\beta_1$ antibodies.

In experiments that tested the interactions of $L_{I\text{-}II}$ and CT5 with $G\beta_1$, binding assays were performed in a similar fashion. In brief, approximately 100 nM GST-fusion proteins were incubated with increasing amounts of wild-type or mutant hexahistidine-tagged $G\beta_1$ in complex with γ_2. After 2 h, Ni-NTA beads were added to the protein mix and incubated for another 2 h. Beads were pelleted and washed with buffer A. Bound proteins were eluted with 600 mM imidazole in Tris-buffered saline. Eluates were subjected to Western blotting with anti-GST antibodies (Zymed Laboratories, South San Francisco, CA).

Yeast Two-Hybrid Assay. The assay was performed as described (Garcia et al., 1998). In brief, plasmids expressing GBD and GAD hybrids were cotransformed into the yeast reporter strain,

PJ69-4A. Transformants carrying these two plasmids were first isolated and subsequently transferred to the yeast medium to select those that express the reporter genes as a result of two-hybrid interactions.

Transfection and Electrophysiology. tsA-201 cells were maintained in Dulbecco's modified Eagle's medium/Ham's F-12 medium (1:1) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) at 37°C under 10% CO_2. Cells plated in 35-mm tissue culture dishes were grown to 60 to 80% confluence and transfected by the Ca^{2+} phosphate method with a total of 4 μg of DNA including a 1:1 M ratio of cDNAs encoding Ca^{2+} channel subunits and 0.3 μg of a CD8 expression plasmid for identification of transfected cells. The cells were subcultured at 24 h after the transfection. At least 48 h after transfection, positive transfected cells were visually identified by labeling with anti-CD8 antibody. Barium currents were recorded by whole-cell patch clamp technique using a List EPC-7 amplifier and filtered at 5 kHz with an eight-pole Bessel filter. Leak and capacitance currents were measured using hyperpolarizing pulses and subtracted using the p/-4 method. The extracellular recording solution contained 120 mM Tris, 4 mM $MgCl_2$, and 10 mM $BaCl_2$ with pH adjusted to 7.3 by methanesulfonic acid. The internal pipette solution consisted of 120 mM aspartic acid, 5 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM Hepes, 10 mM EGTA, and 2 mM Mg-ATP with pH adjusted to 7.3 by CsOH. In some experiments, guanosine 5′-3-*O*-(thio)triphosphate (GTPγS) was added to the internal solution at a concentration of 0.2 mM. Somatostatin (SST) was dissolved in water to give a stock solution of 1 mM and added to the extracellular solution at a final concentration of 1 μM. *N*-Ethylmaleimide (NEM) was prepared in distilled water at 50 mM and added to the extracellular solution to give a final concentration of 50 μM. All agents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise mentioned. All averaged values represent mean ± S.E.M.

Results

Interaction of the Intracellular Loops and C Terminus of $Ca_v2.2$ with Gβγ. To understand the mechanism by which Gβγ subunit complexes modulate the N-type Ca^{2+} channels, we sought to identify the regions on Ca^{2+} channel that interact with the Gβγ subunits. We used an in vitro biochemical binding assay and the in vivo yeast two-hybrid assay in intact yeast cells (Fields and Song, 1989). In the biochemical assay, intracellular loops were expressed in bacteria as hexahistidine-tagged fusion proteins and were purified by nickel affinity columns. The interactions between the fusion proteins and affinity-purified $G\beta_1\gamma_2$ protein were assessed by examining whether $G\beta_1\gamma_2$ could be coprecipitated by the Ni-NTA agarose beads that bind to the hexahistidine moiety. In these experiments, the intracellular loop connecting domains II and III (L_{II-III}) of the skeletal muscle Ca^{2+} channel $Ca_v1.1$ was used as a negative control. Consistent with previous results, our experiments showed that L_{I-II} of $Ca_v2.2b$ interacted strongly with the $G\beta_1\gamma_2$ subunits, whereas the L_{II-III} and L_{III-IV} did not interact (Fig. 1, A and B). We next focused on the C-terminal tail of $Ca_v2.2b$, which contains the last 666 amino acid residues. To facilitate the purification of hexahistidine-tagged fusion proteins of this region, the C-terminal domain was divided in half and expressed and purified as two separate proteins for assay. We observed that $G\beta_1\gamma_2$ interacted effectively with the C-terminal half (CT2, aa 2044–2336), but not the N-terminal half of the C-terminal domain (CT1, aa 1710–2043; Fig. 1B), suggesting that this may be a region involved in mediating G protein modulation.

Binding of L_{I-II} and CT2 to Gβγ was also confirmed by the yeast two-hybrid assay, which detects protein-protein interactions in intact cells. In our assays, the Ca^{2+} channel fragments were expressed as GBD hybrids and the $G\beta_1$ subunit was expressed as a GAD hybrid. Interactions between the GBD and GAD hybrids in the yeast reporter strain can reconstitute GAL4 transcriptional activator function to activate the expression of two reporter genes, *ADE2* and *HIS3*, that are involved in adenine and histidine synthesis, respectively. Thus, yeast cells expressing interacting hybrid proteins are able to grow on medium lacking these two amino

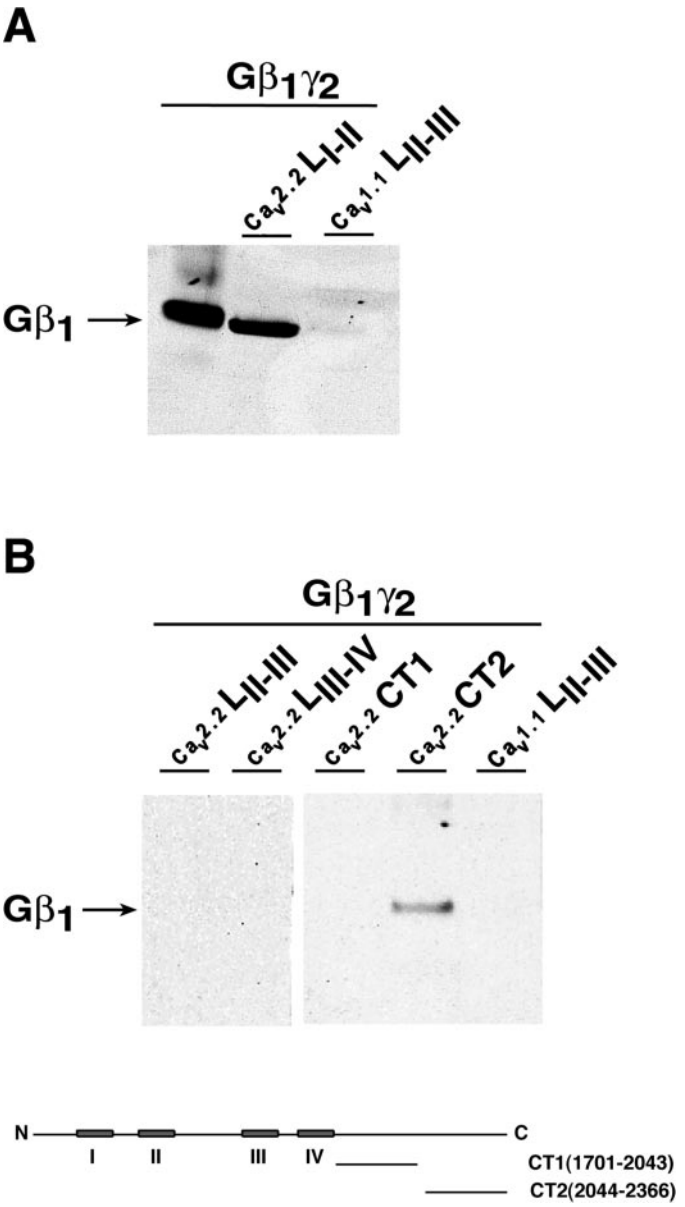

Fig. 1. Specific interactions of L_{I-II} and the C terminus of $Ca_v2.2b$ with Gβγ. A, $G\beta_1\gamma_2$ protein was analyzed by SDS-PAGE and immunoblotting alone (lane 1) or affinity purified $G\beta_1\gamma_2$ protein (50 nM) was incubated with hexahistidine-tagged $Ca_v2.2b$ L_{I-II} (100 nM, lane 2) or hexahistidine-tagged $Ca_v2.2.b$ L_{II-III} (100 nM, lane 3) and precipitated by Ni-NTA agarose beads. The precipitated protein complexes were subjected to SDS-PAGE and immunoblotting with anti-$G\beta_1$ antibodies to detect a complex with the Ca^{2+} channel fragments. B, hexahistidine-tagged $Ca_v2.2b$ $L_{II-III,}$ (lane 1), $L_{III-IV,}$ (lane 2), CT1 ($Ca_v2.2b$[1710–2043], lane 3), CT2 ($Ca_v2.2b$[2044–2336], lane 4) and $Ca_v2.2$ L_{II-III} (lane 5) were incubated with $G\beta_1\gamma_2$ and complexes were isolated and immunblotted as in panel A. Bottom, linear map of the $Ca_v2.2b$ α1 subunit.

acids. When we tested the intracellular loops and the C-terminal tail, we observed that only yeast cells coexpressing $G\beta_1$ and $L_{I\text{-}II}$ or CT2 grew on the selection medium, indicating specific interactions (Fig. 2). In these experiments, no mammalian γ subunit was introduced into the yeast cells. Therefore, the $G\beta_1$ subunit may complex with the endogenous yeast γ subunit, or the overexpressed GAD-$G\beta_1$ fusion protein may be stable enough to interact separately with Ca^{2+} channel fragments.

To identify the minimal domain in the C terminus of the channel that is required for the interaction with $G\beta\gamma$, three deletion constructs were made in the CT2 region (aa 2044–2366) (Fig. 3A) and introduced into the yeast reporter strain with the GAD-$G\beta_1$-expressing plasmid. The two-hybrid analyses showed that the last 80 residues (CT5, aa 2257–2336) of $Ca_v2.2b$ are necessary and sufficient for interaction with the $G\beta_1$ subunit (Fig. 3A). This region was also shown to bind to the G protein in the in vitro biochemical assay in which the last 80 residues were expressed as a GST-fusion protein and the $G\beta_1$ subunit was tagged with hexahistidine. GST-CT5, but not GST was coprecipitated with hexahistidine-$G\beta\gamma$ by the Ni-NTA beads (Fig. 3B). These results confirmed that the C-terminal $G\beta$ binding site is located within the last 80 amino acid residues at the C terminus of the polypeptide. The location of this site is different from the previously reported $G\beta$ binding site in $Ca_v2.3$, which is located in the middle of the C-terminal region (Qin et al., 1997); however, these sites contain a conserved sequence motif (see *Discussion*).

Specificity of Interaction of the C-Terminal $G\beta\gamma$-Binding Site with Different $G\beta$ Subunits. It has been reported that effectors modulated by the $\beta\gamma$ subunits of the G proteins have differential affinities toward different subtypes of G proteins. For example, a G protein receptor kinase (GRK), GRK2, binds to $G\beta_1$ and $G\beta_2$, but not $G\beta_3$ (Daaka et al., 1997). We examined the interaction of the last 80 residues of $Ca_v2.2b$ with the known five subtypes of $G\beta$ subunit. In contrast to $L_{I\text{-}II}$, which showed differential affinities toward these $G\beta$ subunits (Garcia et al., 1998), yeast cells cotransformed with GBD-CT5 and the five GAD-$G\beta$-expressing plasmids showed similar growth rates on the selection medium (Fig. 4), indicating similar interaction strength between the five paired hybrids. Since the five $G\beta$ subunit hybrids were expressed at a similar level (Garcia et al., 1998), it is probable that CT5 interacts with the five $G\beta$ subtypes with similar affinities. These data indicate that the C-terminal $G\beta$ binding site is unable to distinguish among the different subtypes of G protein β subunits. It may function together with $L_{I\text{-}II}$ to mediate specific modulation by these G proteins.

Kinetics and Voltage Dependence of Activation of $Ca_v2.2b$ and $Ca_v2.2b\Delta CT5$ Channels. Ca^{2+} channels consisting of the $Ca_v2.2b$ or $Ca_v2.2b\Delta CT5$ $\alpha1$ subunits, $\alpha2\delta$ subunits, and $\beta1b$ subunits were expressed in the tsA-201 subclone of human embryonic kidney 293 cells as described under *Materials and Methods*. These two Ca^{2+} channels exhibit similar fast activation kinetics (Fig. 5A). Although the midpoint of the conductance-voltage curve of $Ca_v2.2b$ is more

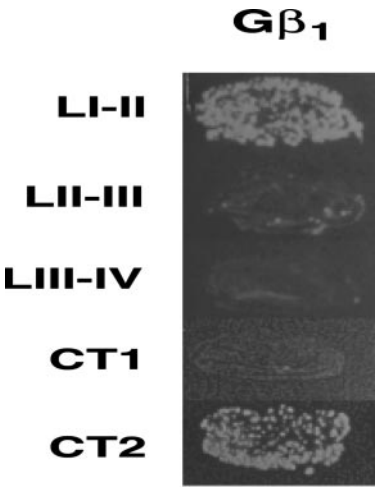

Fig. 2. Interactions of $Ca_v2.2b$ intracellular domains with the $G\beta_1$ subunit in the yeast two-hybrid assay. A plasmid expressing the GBD-$Ca_v2.2b$ hybrid and one expressing GAD-$G\beta_1$ hybrid or the vector alone was cotransformed into the yeast reporter strain PJ69-4A. Transformants from the medium SS-Trp-Leu were transferred onto the selection medium SS-Trp-Leu-His-Ade to detect the growth of these tranformants, indicative of two-hybrid interactions.

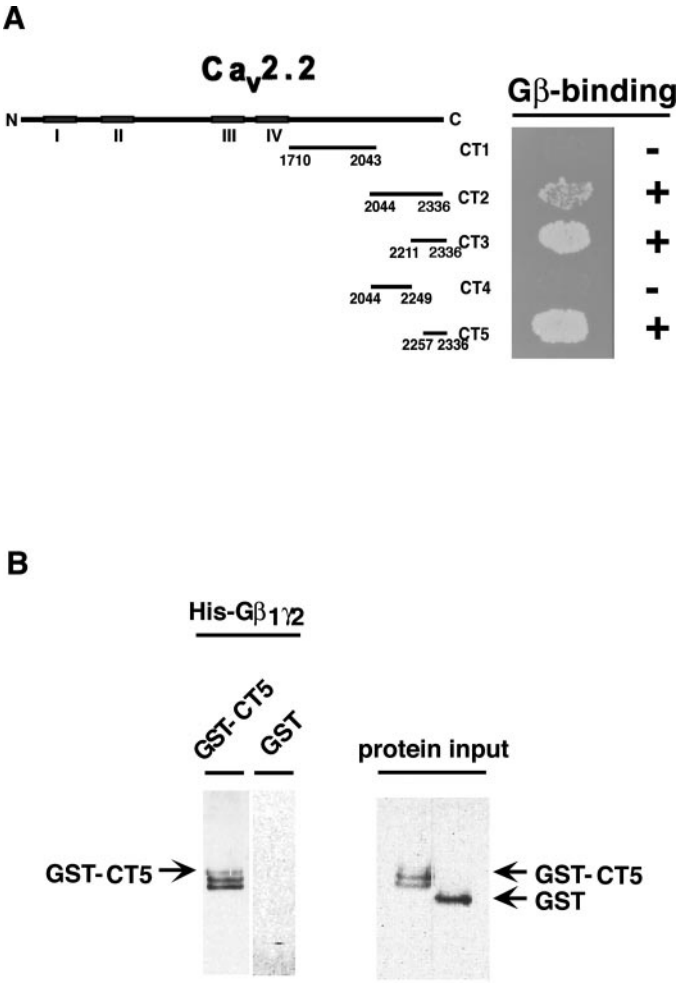

Fig. 3. Identification of the C-terminal binding site for $G\beta_1$. A, plasmids expressing GBD-CT1, GBD-CT2, GBD-CT3 ($Ca_v2.2b$[2211–2336]), GBD-CT4 ($Ca_v2.2b$[2044–2249]), and GBD-CT5 ($Ca_v2.2$[2257–2336]) were transformed individually into PJ69-4A with a plasmid expressing GAD-$G\beta_1$ or the vector alone. Two-hybrid interactions were detected on the selection medium as shown in Fig. 2. B, GST-CT5 or GST was incubated with hexahistidine-tagged $G\beta_1\gamma_2$, followed by addition of Ni-NTA agarose beads. Precipitated protein complexes were subjected to SDS-PAGE and immunoblotting with anti-GST antibodies to detect complexes containing GST or GST fusion proteins.

positive (V_a = 18.6 ± 2.2 mV, n = 24; Fig. 5B) and less steep in comparison to $Ca_v2.2b\Delta CT5$ (V_a = 15.4 ± 1.6 mV, n = 28; Fig. 5B), the difference is not statistically significant ($p > 0.05$). These results show that deletion of CT5 has no effect on basic Ca^{2+} channel function.

Intrinsic and Somatostatin-induced G Protein Activation and Prepulse Facilitation of $Ca_v2.2b$ and $Ca_v2.2b\Delta CT5$ Channels. To examine G protein modulation of these Ca^{2+} channels, we induced prepulse facilitation without and with SST in the bath solution to activate endogenous SST receptors and G proteins (see Fig. 6A, inset). Facilitation during the prepulse was assessed by comparing the tail current following Test Pulse 2 with the tail current following Test Pulse 1. In the absence of SST, $Ca_v2.2b$ channels activate rapidly at relatively negative membane potentials (Fig. 6, A and B). A depolarizing prepulse has no obvious effect on the rate of activation and causes only a small shift in the voltage dependence activation (Fig. 6, A and B). Depolarizing prepulses cause a small degree of facilitation, which is maximum at +30 mV (9.7 ± 2.3%; Fig. 6B) and is significant compared with the baseline variation in barium current (Fig. 10). As this facilitation is observed without exogenous activation of G proteins, we have designated it intrinsic facilitation. Intrinsic facilitation of $Ca_v2.2$ channels has been observed previously in neuroblastoma/glioma cells and transfected tsA-201 cells (Kasai, 1991; Zhong et al., 2001). The intrinsic facilitation indicates that, in the absence of exogenous activators, the cells have constitutively active G proteins that cause tonic inhibition of Ca^{2+} currents and allow prepulse facilitation. Activation of G proteins with SST (1 μM), which reacts with receptors constitutively expressed in the tsA 201 cells, slows activation of $Ca_v2.2b$ and shifts its voltage dependence of activation to more positive membrane potentials (Fig. 6, C and D). Depolarizing prepulses cause an obvious facilitation (22.7 ± 1.8% at +30 mV; Fig. 6, C and D), resulting from voltage-dependent reversal of G protein inhibition.

In the absence of SST, $Ca_v2.2b\Delta CT5$ channels activate rapidly at relatively negative membrane potentials, similar to $Ca_v2.2b$ (Fig. 7, A and B). A depolarizing prepulse has no obvious effect on the rate or voltage dependence of activation (Fig. 7, A and B). Using a single test pulse to +30 mV, depolarizing prepulses cause a 2.6 ± 2.5% facilitation of $Ca_v2.2b\Delta CT5$, signifcantly less than wild-type intrinsic facilitation ($p < 0.02$) and not significantly different from zero (Fig. 10). These results revealed that the C-terminal Gβγ binding site contributes to intrinsic facilitation of $Ca_v2.2b$. Activation of G proteins with SST (1 μM) slows activation of $Ca_v2.2b\Delta CT5$, shifts its voltage dependence of activation to more positive membrane potentials, and increases prepulse facilitation (Fig. 7, C and D) as in wild type. At +30 mV test pulse, the level of facilitation (19.3 ± 1.6%; Fig. 7) is slightly, but not significantly, less than that of wild-type $Ca_v2.2b$ (22.7 ± 1.8%; p = 0.11; Fig. 10).

GTPγS-Induced G Protein Activation and Prepulse Facilitation of $Ca_v2.2b$ and $Ca_v2.2b\Delta CT5$ Channels. Activation of G proteins by SST may only activate part of the

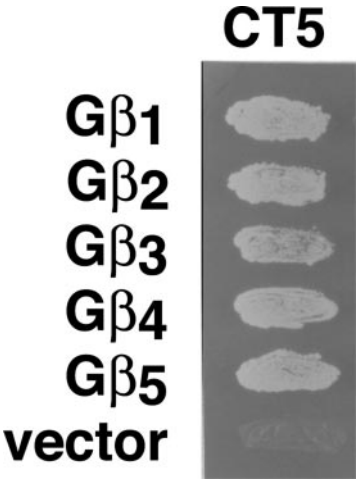

Fig. 4. The C terminus of $Ca_v2.2b$ interacts with all subtypes of Gβ subunit. The plasmid expressing GBD-α_{1B} CT5 ($Ca_v2.2b$[2257–2336]) was transformed into the yeast strain PJ69-4A individually with the plasmids expressing GAD-$G\beta_1$, GAD-$G\beta_2$, GAD-$G\beta_3$, GAD-$G\beta_4$, and GAD-$G\beta_5$. Cell growth in the yeast two-hybrid assay was detected on the selection medium as shown in Fig. 2.

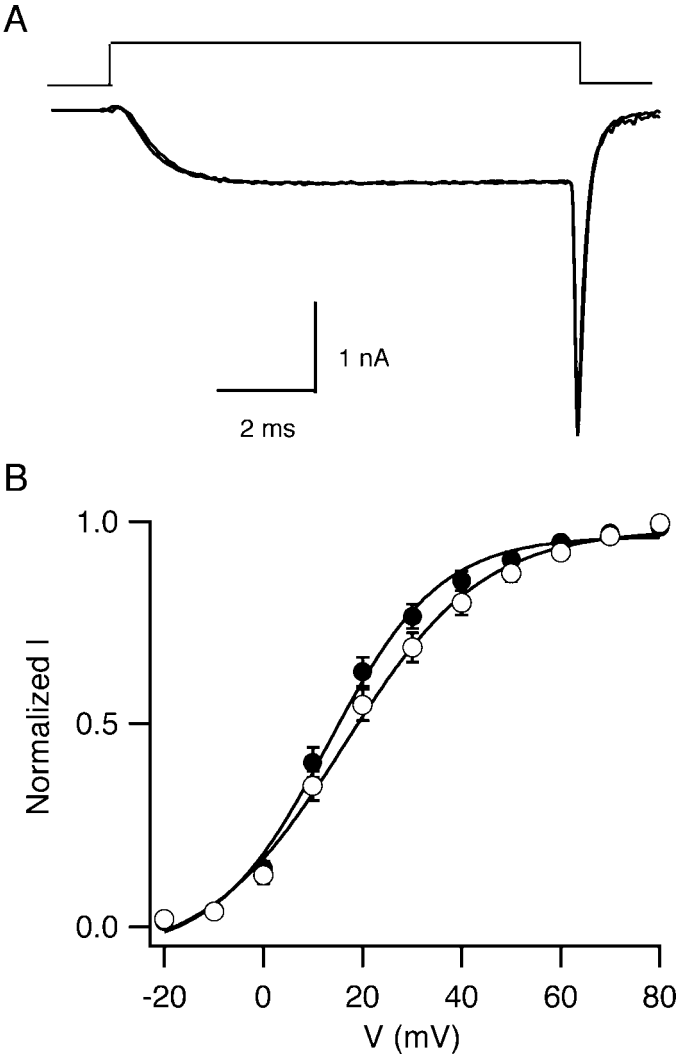

Fig. 5. Kinetics and voltage dependence of activation of $Ca_v2.2b$ and mutant $Ca_v2.2\Delta CT5$ channels. A, ionic currents of $Ca_v2.2b$ and mutant $Ca_v2.2b\Delta CT5$ channels. Example current traces were recorded during a 10-ms test pulse to +30 mV from the holding potential of −80 mV and overlapped. Inset, voltage clamp protocol. B, voltage dependence of activation of $Ca_v2.2b$ (open circles) and $Ca_v2.2b\Delta CT5$ (filled circles). Tail currents were recorded after test pulses to the indicated potentials from a holding potential of −80 mV as shown in panel A and normalized to the largest tail current in each series of test pulses. Mean ± S.E.M. were plotted against test pulse potentials.

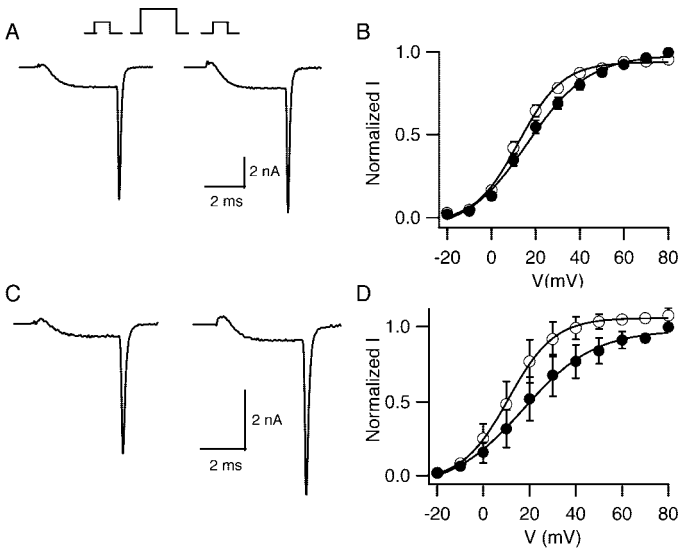

Fig. 6. Intrinsic and SST-induced facilitation of $Ca_v2.2b$ channels. A, prepulse facilitation of activation without SST. A 4-ms test pulse (test 1) to +30 mV was applied from the holding potential of −80 mV. After 3 s, a 10-ms prepulse to 100 mV was applied, the cell was repolarized to −80 mV for 10 ms, and a second 4-ms test pulse (test 2) identical to the first test pulse was applied. Inset, voltage clamp protocol. B, prepulse facilitation of the voltage dependence of activation without SST. Tail currents were recorded after test 1 (filled circles) and test 2 (open circles) at the indicated membrane potentials as described in panel A. Mean ± S.E.M. of tail currents are plotted versus test potential. C, prepulse facilitation of the rate of activation with 1 μM SST, measured as in panel A. D, prepulse facilitation of the voltage dependence of activation with 1 μM SST, measured as in panel B. Scale bars: 2 nA, 4 ms.

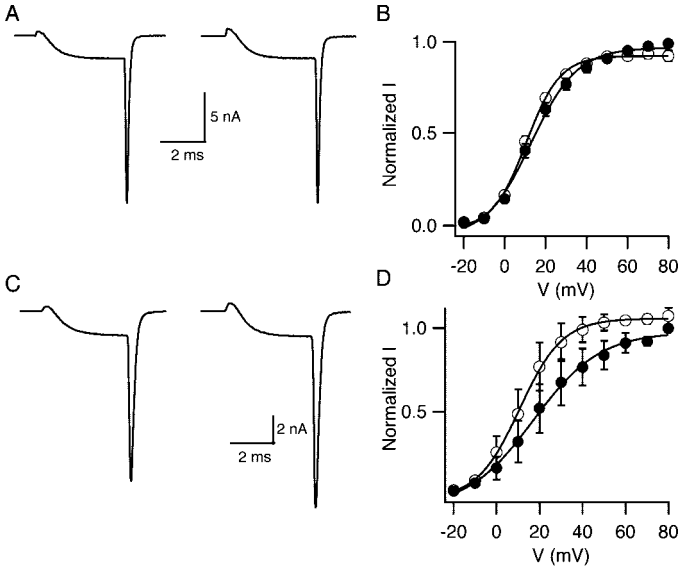

Fig. 7. Intrinsic and SST-induced facilitation of $Ca_v2.2b\Delta CT5$ channels. A, prepulse facilitation of the rate of activation without SST. A 4-ms test pulse (test 1) to +30 mV was applied from the holding potential of −80 mV. After 3 s, a 10-ms conditional prepulse to 100 mV was applied, the cell was repolarized to −80 mV for 10 ms, and a second 4-ms test pulse (test 2) identical to the first test pulse was applied. B, prepulse facilitation of the voltage dependence of activation without SST. Tail currents were recorded following test 1 (filled circles) and test 2 (open circles) at the indicated membrane potentials as described in Fig. 6B. Mean ± S.E.M. of tail currents are plotted versus test potential. C, prepulse facilitation of the rate of activation with 1 μM SST, measured as in panel A. D, prepulse facilitation of the voltage dependence of activation with 1 μM SST, measured as in panel B. Scale bars: 2 nA, 4 ms.

intrinsic G protein pool that is available for modulation of Ca^{2+} channels. To examine modulation by the full complement of G proteins, we used GTPγS in the recording pipette to fully activate endogenous G proteins (Fig. 8). In $Ca_v2.2b$ channels, activation of G proteins with GTPγS slows activation and shifts its voltage dependence to more positive membrane potentials (Fig. 8, A and B). Depolarizing prepulses cause an obvious facilitation, with a maximum at +30 mV (36.0 ± 2.9%; Fig. 8, A and B). Similar to the results with $Ca_v2.2b$, $Ca_v2.2b\Delta CT5$ channels are slowly activated and have strong prepulse facilitation with G protein activation by GTPγS (Fig. 8, C and D). Prepulse facilitation +30 mV is 32.2 ± 1.2%, slightly but not significantly less than wild-type ($p > 0.05$; Fig. 10). Thus, as for activation by SST, there is a small but not statistically significant reduction in modulation by GTPγS with deletion of CT5.

To test whether the prepulse facilitation of $Ca_v2.2b$ is truly dependent on G protein action, we used a G protein inhibitor. The sulfhydryl reagent NEM is a potent inhibitor of G protein action on $Ca_v2.2$ channels (Shapiro et al., 1994). Addition of 50 μM NEM to the extracellular solution blocked both SST-induced and intrinsic facilitation of $Ca_v2.2b$ in our transfected cells (Fig. 9). Previous reports suggested that NEM rapidly eliminates agonist-induced inhibition whereas sparing the tonic inhibition of N-type Ca^{2+} currents in neuroblastoma/glioma cells (Kasai, 1991). Our results show that NEM blocks both somatostatin-induced and intrinsic facilitation of these channels in tsA-201 cells and that both these forms of modulation are dependent on G protein activation.

Primary Role of the C-Terminal Gβγ Binding Site in Intrinsic Facilitation. Comparison of the effects of deletion of the C-terminal Gβγ binding site in $Ca_v2.2$ on intrinsic,

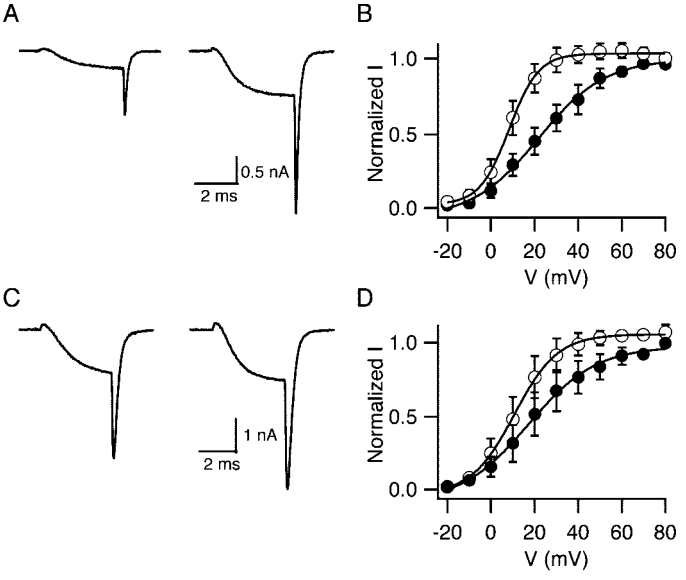

Fig. 8. GTPγS induced facilitation of $Ca_v2.2b$ and $Ca_v2.2b\Delta CT5$ channels. A, prepulse facilitation of the rate of activation with 0.2 mM GTPγS, recorded from $Ca_v2.2b$ as described in Fig. 6A. B, prepulse facilitation of the voltage dependence of activation with GTPγS of $Ca_v2.2b$. Tail currents were recorded following test 1 (filled circle) and test 2 (open circle) at the indicated membrane potentials as described in panel A. Mean ± S.E.M. of tail currents are plotted versus test potential. C, prepulse facilitation of the rate of activation with 0.2 mM GTPγS in the intracellular solution, recorded from $Ca_v2.2b\Delta CT5$ as described in Fig. 6A. D, prepulse facilitation of the voltage dependence of activation with 0.2 mM GTPγS, measured as in Fig. 6B. Scale bars: 0.1 nA, 1 nA, 4 ms.

SST-induced, and GTPγS-induced facilitation revealed that the primary effects of the C-terminal Gβγ binding site is on intrinsic facilitation (Fig. 10). Intrinsic facilitation was reduced from 9.7% to essentially zero. In contrast, there was a trend toward reduction of SST-induced and GTPγS-induced facilitation but it was not statistically significant at the $p < 0.05$ confidence level. Possible mechanisms underlying these effects are considered under *Discussion*.

Discussion

Gβγ-Binding Sites on Ca^{2+} Channels. Ca^{2+} channels are modulated by G proteins through direct binding of Gβγ subunits to the α1 subunits. In this article, we studied two regions of the N-type Ca^{2+} channel $Ca_v2.2b$ subunit that can interact with the βγ subunits of the G protein. Our results confirmed Gβγ binding to L_{I-II}, consistent with previous reports (De Waard et al., 1997; Herlitze et al., 1997; Zamponi et al., 1997; Furukawa et al., 1998). In addition, we have identified a novel site of Gβγ interaction located near the C terminus. Four separate lines of evidence support our identification of this Gβγ binding site. First, we observed specific binding interactions between fusion proteins containing segments of the C-terminal domain and Gβγ. Second, we measured this specific binding interaction in vivo in yeast cells with the yeast two-hybrid assay. Third, we detected a specific functional effect, loss of basal G protein modulation in transfected cells, upon deletion of this C-terminal Gβγ binding site. Fourth, we found that this Gβγ binding site has substantial amino acid sequence similarity to G protein regulatory sites in $Ca_v2.3$ channels and in the family of PAK kinases (see below). Together, these four lines of evidence constitute strong support for the conclusion that this segment of the distal C terminus of $Ca_v2.2$ channels is a genuine binding site for Gβγ.

We tested the specificity of G protein binding in vivo in yeast cells using the yeast two-hybrid assay. Previous work showed that L_{I-II} binds Gβγ subunits with the same specificity as modulation of channel function (Garcia et al., 1998). $G\beta_1$ and $G\beta_2$ interacted strongly with L_{I-II}, $G\beta_5$ interacted weakly, and $G\beta_3$ and $G\beta_4$ failed to interact in a yeast two-hybrid assay. In contrast to L_{I-II} (Garcia et al., 1998), our present results show that the C-terminal binding site of $Ca_v2.2b$ has similar interaction with all five Gβ subunits. These data suggest that the C terminus does not contribute to the differential effects of the five subtypes of Gβ subunits on voltage-dependent facilitation, in contrast to the site in L_{I-II} (Garcia et al., 1998). The C-terminal site in $Ca_v2.2$ channels may interact with a conserved site on the Gβ subunit.

Comparison of the Gβγ-Binding Site in the C-Terminal Domains of $Ca_v2.2b$ and $Ca_v2.3$. Comparing the Gβγ binding site we discovered near the C terminus of the $Ca_v2.2b$ with the previously identified Gβγ binding site in C-terminal domain of the $Ca_v2.3$ channel (Qin et al., 1997) reveals 40% amino acid sequence identity (Scheme 1). Both of these sequences contain an SSL motif, which is also found in the Gβ binding of PAK kinase family members (Leeuw et al., 1998), strongly suggesting the site in $Ca_v2.2$ identified in this study is an authentic one. Interestingly, the C-terminal Gβγ binding site in the $Ca_v2.3$ channel is located near the middle of the C-terminal domain (Qin et al., 1997), rather than near the end of the C terminus as in $Ca_v2.2b$. R-Type currents mediated by $Ca_v2.3$ are not as strongly modulated by G proteins as N- and P/Q-type currents mediated by $Ca_v2.1$ and $Ca_v2.2$ (Toth et al., 1996; Simen and Miller, 1998).

The low level of modulation of $Ca_v2.3$ is entirely dependent on the C-terminal Gβγ binding site (Qin et al., 1997), in

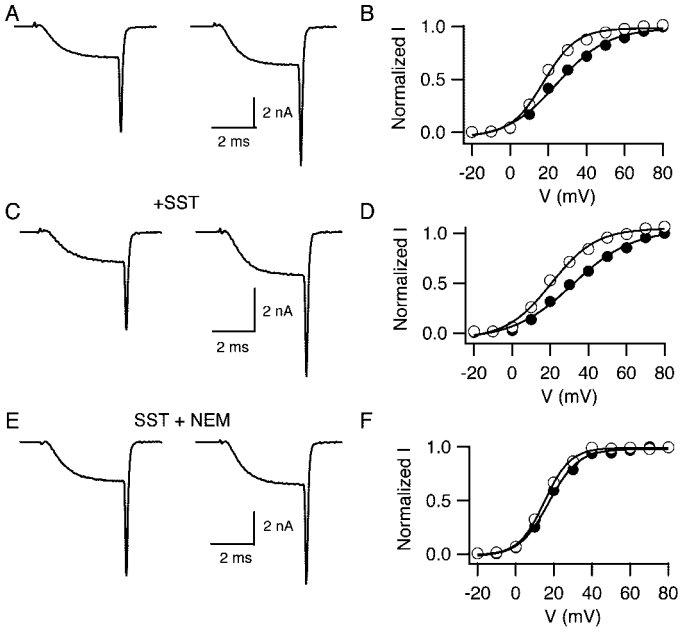

Fig. 9. Effect of NEM on prepulse facilitation of $Ca_v2.2b$ channels. A, prepulse facilitation of the rate of activation, recorded as in Fig. 6A. B, prepulse facilitation of the voltage dependence of activation. Tail currents were recorded from the same cell as in panel A after test 1 (filled circles) and test 2 (open circles) at the indicated membrane potentials as described in Fig. 6B. C, prepulse facilitation of the rate of activation with SST recorded from the same cell as in panel A. D, prepulse facilitation of the voltage dependence of activation with SST. Tail currents were recorded from the same cell as in panel A after test 1 (filled circles) and test 2 (open circles) at the indicated membrane potentials. E, prepulse facilitation of the rate of activation with SST and NEM. F, prepulse facilitation of the voltage dependence of activation of $Ca_v2.2b$ with SST and NEM. Tail currents were recorded from the same cell as in panel A after test 1 (filled circles) and test 2 (open circles) at the indicated membrane potentials. Scale bars: 2 nA, 2 ms.

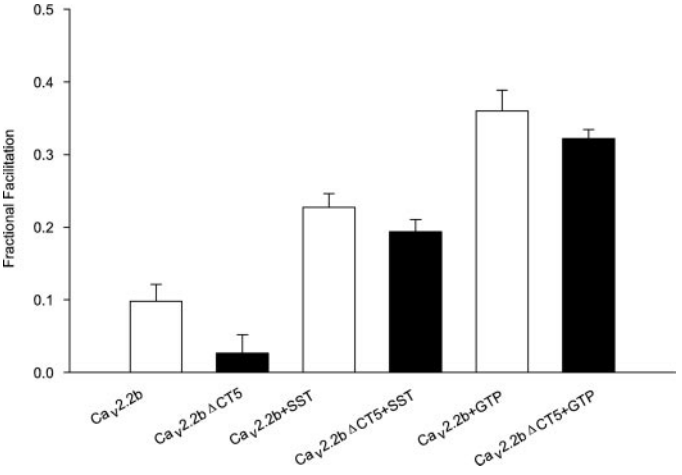

Fig. 10. Intrinsic, GTPγS-, and SST-induced maximal prepulse facilitation of $Ca_v2.2b$ and $Ca_v2.2b\Delta$ CT5 channels. Tail currents were recorded after test 1 and test 2 at the membrane potential (+30 mV) which gives maximal facilitation as described in Fig. 6A. Fractional facilitation from comparing the tail currents of test 1 and test 2 is plotted as mean ± S.E.M. Clear bar represents wild-type $Ca_v2.2b$ channels. Black bar represents mutant $Ca_v2.2b\Delta$ CT5 channels. **, $p < 0.05$.

contrast to $Ca_v2.2$ where the $G\beta\gamma$ site in L_{I-II} is more important when G proteins are strongly activated and the C-terminal $G\beta\gamma$ site is significant primarily for basal modulation due to the low level of intrinsic G protein activity. It is possible that the comparatively weak modulation of the $Ca_v2.3$ channel reflects the weak regulatory capacity of the C-terminal $G\beta\gamma$ sites compared with the site in L_{I-II}, which is not functional in $Ca_v2.3$.

Function of the C-Terminal Gβγ-Binding Site. To understand the functional importance of the C-terminal $G\beta\gamma$ binding site in G protein modulation, we analyzed the deletion mutant $Ca_v2.2b\Delta CT5$. The current amplitude, voltage-dependent activation, and inactivation of the mutant channel were similar to wild type, suggesting that the deletion does not produce global structural changes; however, prepulse facilitation of this mutant channel is significantly smaller than that of the wild-type channel under basal conditions where intrinsic G protein activity modulates the channel (Fig. 10). These data can be interpreted in terms of a reversible equilibrium between "willing" and "reluctant" channel states with differing kinetics and voltage dependence of activation (Bean, 1989). Many factors alter G protein modulation by affecting the equilibrium between these two states, including G proteins, membrane depolarization, $Ca_v\beta$ subunits, and protein kinase C (Herlitze et al., 2001). G protein activation shifts the equilibrium to the reluctant state, whereas depolarizing prepulses shift the channel back to the willing state and relieve the G protein-dependent inhibition resulting in prepulse facilitation. Based on this model, our experimental data suggest that the C-terminal $G\beta\gamma$ binding site regulates voltage-dependent activation primarily by enhancing the G protein-dependent shift of the channel state when G protein activation is at the basal level, perhaps by enhancing the affinity for $G\beta\gamma$ binding. Prepulse facilitation is a sensitive method to test the changes of channel activity due to G protein modulation, even at the basal level. Although the intrinsic facilitation of $Ca_v2.2b$ is less than 10%, the prepulse facilitation protocol clearly detected the difference between the wild-type and $Ca_v2.2b\Delta CT$ mutant. Moreover, our results showed that both SST-induced and intrinsic facilitation are blocked by NEM, providing strong support for the conclusion that intrinsic facilitation is dependent on endogenous G protein activation. The simplest explanation of the data is that when the C-terminal site is present, a fraction of $Ca_v2.2$ channels are reluctant to open due to the inhibition by $G\beta\gamma$ bound at least in part to the C-terminal site. When this region is deleted, $G\beta\gamma$ binds with lower affinity; the $Ca_v2.2$ channel is less inhibited by endogenous activated $G\beta\gamma$ and is less available for prepulse facilitation because fewer of the channels are in the reluctant state.

Although experiments probing G protein regulation are usually designed to activate a single dominant G protein-coupled signaling pathway, the situation is often more complex in vivo since numerous signaling pathways are likely to be activated at a low level simultaneously. For example, central neurons receive simultaneous input from low level activation of G protein signaling pathways at synapses from many presynaptic neurons using different neurotransmitters. Enhanced regulation of $Ca_v2.2$ channels at low levels of G protein activation is likely to be physiologically significant in this in vivo situation. The function of the C-terminal $G\beta\gamma$-binding domain in basal modulation of $Ca_v2.2$ channels in our experiments is consistent with a physiologically significant role at low levels of G protein activation in central neurons and other cell types in vivo.

Molecular Mechanisms of G Protein Binding and Function. Our data support the proposal that the functional target of voltage-dependent $G\beta\gamma$ interaction includes the loop L_{I-II} (De Waard et al., 1997; Herlitze et al., 1997; Page et al., 1997; Zamponi et al., 1997). However, our results do not support the proposal that C-terminal binding site is the primary one that mediates the action of $G\beta\gamma$ for $Ca_v2.2$, as suggested for $Ca_v2.3$ by Qin et al. (1997), since we find that deletion of this site in $Ca_v2.2$ reduces intrinsic facilitation but does not have a statistically significant effect on modulation by SST and GTPγS. Whether the C-terminal region comprises an independent $G\beta\gamma$ binding site or is part of a single multifaceted site is not clear. Our working hypothesis is that the C terminus of $Ca_v2.2b$ may function as a secondary anchoring site for the $G\beta\gamma$, which enhances its affinity for $Ca_v2.2$ channels and allows regulation by $G\beta\gamma$ interaction with L_{I-II} at lower concentrations of activated $G\beta\gamma$. Consistent with this idea, Zhao et al. (2003) have recently reported that amino acid residues in two of the β-propellor domains of $G\beta\gamma$ are involved in regulation of inwardly rectifying potassium channels. By analogy, L_{I-II} and the C-terminal domain of $Ca_v2.2$ channels may bind to two different β-propellors of $G\beta\gamma$ subunits to give optimal regulation at low levels of G protein activation. Alternatively, more than one $G\beta\gamma$ complex may bind to the channel with additive or synergistic effects. The C-terminal region may have a higher affinity for $G\beta\gamma$ and contribute primarily to intrinsic facilitation at low level activation of G proteins. When G proteins are activated more strongly by G protein-coupled receptors or by GTPγS, L_{I-II}, and the N-terminal $G\beta\gamma$ binding site may dominate channel regulation and exceed the effects of $G\beta\gamma$ binding to the C-terminal region. These multiple functional sites may allow response to a wider range of activation of G protein signaling pathways. Further studies on the binding affinity of the N terminus, L_{I-II} and the C-terminal site to $G\beta\gamma$ in intact mammalian cells using fluorescence resonance energy transfer or bioluminescence resonance energy transfer methods with $Ca_v2.2$ channels labeled in these three $G\beta\gamma$-binding domains may shed more light on the mechanism of G protein modulation. It is likely that structural studies will be required to finally resolve the number of distinct G protein binding sites and the segments of the $Ca_v2.2$ channels that form them.

Gβ protein binding site in Ca^{2+} channels

```
Cav2.2b[CT5]    EAVATNSGRSSRTSYVSSLTSQSHPL
Cav2.3          ERSSENTYKSRRRSYHSSLRLSAHRL
                *    *   * * ** ***    * *
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Gβ binding site in PAK family members

```
Ste20           ANSSLAPLVKLARL
mouse PAK3      PLSSLTPLIIAAKE
rat PAK         PLSSLTPLIAAAKE
```

Scheme 1.

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