

**The  $\beta 1$  subunit of L-type voltage-gated  $\text{Ca}^{2+}$  channels  
independently binds to and inhibits the gating of large-  
conductance  $\text{Ca}^{2+}$ -activated  $\text{K}^{+}$  channels**

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Non-standard abbreviations

AID	Alpha subunit interaction domain
BK <sub>Ca</sub> channels	Large-conductance Ca <sup>2+</sup> -activated K <sup>+</sup> channels
Ca <sub>v</sub> β1	β1 subunit of voltage-activated Ca <sup>2+</sup> channels
CG	ciliary ganglion
GFP	green fluorescence protein
GK	guanylate kinase-like domain
GST	glutathione transferase
HA	hemagglutinin epitope
MAGUK	membrane-associated guanylate kinase (MAGUK) protein
RFP	red fluorescent protein
RCK	Regulator of conductance of K <sup>+</sup> channels domain
SH3	Src Homology 3 domain
Slo1	Pore-forming subunit of BK <sub>Ca</sub> channels

## Abstract

Large conductance  $\text{Ca}^{2+}$ -activated  $\text{K}^{+}$  channels ( $\text{BK}_{\text{Ca}}$  channels) encoded by the *Slo1* gene are ubiquitously expressed, and play a role in regulation of many cell types. In excitable cells,  $\text{BK}_{\text{Ca}}$  channels and voltage-activated  $\text{Ca}^{2+}$  channels often form functional complexes that allow the cytoplasmic domains of  $\text{BK}_{\text{Ca}}$  channels to lie within spatially discrete calcium microdomains. Here we report a novel protein interaction between the  $\beta 1$ -subunit of L-type voltage-activated calcium channels ( $\text{Ca}_v\beta 1$ ) and critical regulatory domains of Slo1 that can occur in the absence of other proteins. This interaction was identified by a yeast two-hybrid screen, and confirmed by confocal microscopy in native neurons, co-immunoprecipitation, and by direct binding assays. The  $\text{Ca}_v\beta 1$  subunit binds within the calcium bowl domain of Slo1 that mediates a portion of high-affinity  $\text{Ca}^{2+}$ -binding to  $\text{BK}_{\text{Ca}}$  channels, and also to a non-canonical Src Homology 3 (SH3) domain-binding motif within Slo1. Binding of  $\text{Ca}_v\beta 1$  markedly slows Slo1 activation kinetics and causes a significant decrease in  $\text{Ca}^{2+}$  sensitivity in inside-out and in dialyzed cells, even in the absence of pore-forming subunits of  $\text{Ca}_v$  channels. The guanylate kinase (GK) domain of  $\text{Ca}_v\beta 1$  mediates Slo1 regulation through its binding to calcium bowl domains, and this domain of  $\text{Ca}_v\beta 1$  is necessary and sufficient for the observed effects on  $\text{BK}_{\text{Ca}}$  activation. Binding of  $\text{Ca}_v\beta 1$  to SH3-binding motifs may stabilize the interaction with Slo1, or contribute to formation of other complexes, but does not appear to affect  $\text{Ca}^{2+}$ -dependent gating of Slo1. Binding of  $\text{Ca}_v\beta 1$  does not affect cell surface expression of Slo1 in HEK293T cells.

## Introduction

Large-conductance  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  ( $\text{BK}_{\text{Ca}}$ ) channels are expressed in excitable and non-excitabile tissues, including neurons, myocytes, secretory cells and epithelial cells. These channels are cooperatively activated by membrane depolarization and binding of cytosolic  $\text{Ca}^{2+}$ , which in the presence of other channels contributes to regulation of the waveform and frequency of action potential discharge, the initiation and spread of dendritic  $\text{Ca}^{2+}$  spikes, the control of membrane potential oscillations, the release of hormones and neurotransmitters, and modulation of muscle contraction (Salkoff et al., 2006).

The pore-forming subunits of  $\text{BK}_{\text{Ca}}$  channels are encoded by the *Slo1* gene (also known as *KCNMA1*), and contain a core region with seven membrane-spanning domains. In addition, Slo1 proteins have an unusually large cytoplasmic tail that is subjected to extensive alternative splicing, yielding channel variants with markedly different voltage- and  $\text{Ca}^{2+}$ -sensitivities (Shipston, 2001). The allosteric relationship between membrane potential and cytosolic  $\text{Ca}^{2+}$  in the control of  $\text{BK}_{\text{Ca}}$  gating has been extensively studied (Magleby, 2003). Voltage regulation of channel gating is associated with domains in the core regions of  $\text{BK}_{\text{Ca}}$  channels (Diaz et al., 1998), whereas the regulator of conductance of  $\text{K}^+$  (RCK) (Xia et al., 2002; Jiang et al., 2001) and calcium-bowl domains (Schreiber et al., 1997; Bao et al., 2002; Zeng et al., 2005) in the cytoplasmic tail account for most of the  $\text{Ca}^{2+}$  regulation, although additional low-affinity  $\text{Ca}^{2+}$  binding sites may also modulate  $\text{Ca}^{2+}$  sensitivity (Zhang et al., 2001; Xia et al., 2002; Bao et al., 2004).

Although some  $\text{BK}_{\text{Ca}}$  channels can respond over a broad range of free  $\text{Ca}^{2+}$  concentrations (Thurm et al., 2005), most variants typically require micromolar

concentrations for robust activation. For this reason, Slo1 channels must be close to a significant source of  $\text{Ca}^{2+}$  in order for the necessary  $\text{Ca}^{2+}$  concentrations to occur in space and time. In most excitable cells, the usual sources are voltage-gated  $\text{Ca}^{2+}$  channels ( $\text{Ca}_v$ ). Indirect evidence for the presence of  $\text{BK}_{\text{Ca}}$  channels within  $\text{Ca}_v$ -dependent “calcium microdomains” appeared several years ago (Gola and Crest, 1993; Wisgirda and Dryer 1994; Marrion and Tavalin, 1998), and these types of observations have been quantitatively refined in recent years (Prakriya and Lingle, 2000). A more recent study directly demonstrated the existence of macromolecular complexes between Slo1 and  $\text{Ca}_v1.2$  (L-type),  $\text{Ca}_v2.1$  (P/Q-type), and  $\text{Ca}_v2.2$  (N-type) subunits in rat brain and in heterologous expression systems (Berkefeld et al., 2006).

In parasympathetic neurons of the chick ciliary ganglion,  $\text{BK}_{\text{Ca}}$  channels are functionally coupled to dihydropyridine-sensitive L-type  $\text{Ca}_v$  channels (Wisgirda & Dryer, 1994). L-type  $\text{Ca}^{2+}$  channels are comprised of a single pore-forming subunit ( $\text{Ca}_v1.1$  or  $\text{Ca}_v1.2$ ), often in a complex with  $\beta$ -subunits ( $\text{Ca}_v\beta$ ) which regulate the gating properties and trafficking of the resulting channels (DeWaard and Campbell, 1995; Brice et al., 1997; Dolphin, 2003). The net effect of  $\text{Ca}_v\beta$  subunits is to increase L-type current and the resulting voltage-evoked  $\text{Ca}^{2+}$  influx in cells where they are expressed. The  $\text{Ca}_v\beta$  subunits are members of the membrane-associated guanylate kinase (MAGUK) family of proteins. They have a Src homology 3 (SH3) domain linked by a flexible loop to a guanylate kinase (GK)-like domain (Petegem et al., 2004; Chen et al., 2004). Their interaction with  $\text{Ca}_v1.1$  is reversible (Bichet et al., 2000), and the GK domain of  $\text{Ca}_v\beta$  is free to interact with other proteins (Petegem et al., 2004; Chen et al., 2004; Hidalgo et al., 2006).

In the present study, we show that an L-type  $\text{Ca}_v$  channel  $\beta$ -subunit ( $\text{Ca}_v\beta 1$ ), physically associates with Slo1 subunits of  $\text{BK}_{\text{Ca}}$  channels at two distinct regions: a non-canonical SH3 domain-binding motif, previously shown to link  $\text{BK}_{\text{Ca}}$  channels to the actin cytoskeleton via the adaptor protein cortactin (Tian et al., 2006), and also at sites within the calcium-bowl region of Slo1. Surprisingly, the  $\text{Ca}_v\beta 1$ -Slo1 interaction does not require the presence of other channel subunits or any other proteins. Moreover, the interaction is functionally significant, as it markedly slows  $\text{BK}_{\text{Ca}}$  activation and deactivation kinetics and causes a significant decrease in  $\text{Ca}^{2+}$  sensitivity that can be readily detected in both inside-out excised patch and whole-cell recordings. Finally we show that the effects of  $\text{Ca}_v\beta 1$  on  $\text{BK}_{\text{Ca}}$  gating can be attributed to the interaction with the calcium bowl region of Slo1.

## Materials and Methods

**Plasmids and antibodies.** An expression plasmid (pCMV-Myc-Slo1) encoding N-terminal (ectofacial) Myc-tagged mouse Slo1 was provided by Dr. Min Li of Johns Hopkins University. A plasmid encoding full length human  $\text{Ca}_v\beta 1$  (Accession# NM\_000723.3) was obtained from OriGene Technologies, Inc. (Rockville, MD). Other plasmids, including pGBKT7-Slo1<sub>G785-A985</sub>, pDsRed- $\text{Ca}_v\beta 1$ , pCMV-HA- $\text{Ca}_v\beta 1$ , pGEXKG-Slo1<sub>G785-A985</sub>, pGEXKG-Slo1<sub>G785-L843</sub>, pGEXKG-Slo1<sub>Q844-I883</sub>, pGEXKG-Slo1<sub>T884-N936</sub>, pGEXKG-Slo1<sub>I937-A985</sub>, pcDNA 3.1(-)- $\text{Ca}_v\beta 1$ , pcDNA 3.1(-)- $\text{Ca}_v\beta 1_{\text{M1-D224}}$ , and pcDNA 3.1(-)- $\text{Ca}_v\beta 1_{\text{P162-R598}}$  were constructed in our laboratory using standard methods. The fidelity of all constructs was confirmed by sequencing. Mouse anti-Myc 9B11 and Mouse anti-HA 2367 (Cell Signaling, Danvers, MA) were used for immunoblot analysis, whereas FITC-conjugated goat anti-Myc ab1263 (Abcam, Cambridge, MA) was used only for labeling of intracellular stores of Slo1 channels in confocal microscopy. Immunoblot and confocal analyses of endogenously expressed channel subunits were carried out with a rabbit antibody against mouse Slo1 (AB5228, Chemicon, Temecula, CA) and a monoclonal antibody against  $\text{Ca}_v\beta 1$  (H00000782-M01, Abnova, Heidelberg, Germany), as described below.

**Yeast two-hybrid screen.** This was carried out using the Matchmaker System™ (BD Biosciences, San Jose, CA) according to the manufacturer's instructions, as described in detail previously (Kim et al., 2007c). Briefly, a cDNA library of embryonic day 9 (E9) chick ciliary ganglion (CG) prepared in our laboratory was screened using a construct encoding amino acids 785-985 of mouse Slo1 cloned into the pGBKT7 bait vector. Colonies expressing potential interacting proteins were identified by blue-white selection

carried out on a quadruple dropout medium supplemented with X- $\alpha$ -gal. After selection, the pGADT7 plasmids encoding fragments of putative interacting proteins were isolated from yeast colonies, sequenced, and subjected to BLAST search analysis.

**Co-immunoprecipitation, GST pull-down assays and *in vitro* binding assays.**

Immunoblot analyses, co-immunoprecipitation and glutathione transferase (GST) pull-down assays were carried out as described previously (Kim et al., 2007c). The *in vitro* binding assay is a slight modification of the GST-pulldown assay. Full-length Ca $_v$  $\beta$ 1 cDNA was cloned into the pcDNA3.1(-) vector for *in vitro* transcription and translation using the TNT T7 Quick Coupled Transcription/Translation System<sup>TM</sup> (Promega, Madison, WI). Biotinylated lysine residues were incorporated into the *in vitro* translated protein using Trancend tRNA<sup>TM</sup> (Promega), which allowed for chemiluminescent detection of Ca $_v$  $\beta$ 1 on nitrocellulose membranes using the Trancend<sup>TM</sup> non-radioactive detection system (Promega). Glutathione-Sepharose 4B beads carrying GST-fusion proteins prepared from Slo1 were incubated with *in vitro* translated Ca $_v$  $\beta$ 1 in PBS containing 0.2% Triton X-100 (PBST) overnight at 4° C with gentle rotation. The beads were washed repeatedly in PBST and eluted in a buffer containing 10 mM glutathione. Eluates were separated by 10% SDS-PAGE, transferred to nitrocellulose membranes, and analysed by addition of a streptavidin-HRP conjugate (1:10,000 dilution in a Tris-buffered saline containing 0.5% Tween-20) followed by detection of labeled proteins by chemiluminescence.

**Cell culture and transfection.** HEK293T (human embryonic kidney) cells were grown in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO) containing 10% heat-inactivated fetal bovine serum at 37°C in a 5% CO $_2$  incubator. Transfection procedures



are described in Kim et al., (2007c). Cells were used for physiology or biochemistry 48 h after transfection. Neurons from E9 chick ciliary ganglia were dissociated and cultured as described previously (Cameron et al., 1998; Lhuillier and Dryer, 2002).

**Immunostaining and confocal microscopy.** E9 CG neurons were maintained in culture for 3 hr or 48 hr and then fixed in 4% paraformaldehyde for 10 min, blocked, and permeabilized with PBS containing 0.5% Triton X-100. The preparations were then incubated with rabbit anti-Slo1 (1:500 dilution) and mouse anti-Ca<sub>v</sub>β1 (1:1000 dilution) overnight at 4°C. After repeated washing, cells were incubated Alexa-488-conjugated anti-rabbit and CY3-conjugated anti-mouse secondary antibodies for 1 hr at 37°, rinsed repeatedly in PBS, and mounted in Vectorshield™ (Vector Laboratories, Burlingame, CA). Images were collected on an Olympus FV-1000 confocal microscope using a Plan Apo N 60X 1.42NA oil-immersion objective. Green fluorescence was evoked using an excitation wavelength of 495 nm while monitoring emission at 519 nm. Red fluorescence was evoked by excitation at 580 nm and emission was monitored at 620 nm.

**Immunochemical analysis of cell-surface Slo1 channels in HEK293T cells.** Intact HEK293T cells expressing Myc-tagged Slo1 channels in the presence or absence of Ca<sub>v</sub>β1 were treated with mouse anti-Myc (9B11, Cell Signaling) for 20 min in normal culture medium at 37°C to label surface Slo1 channels. Cells were then rinsed in PBS, fixed in 4% paraformaldehyde for 10 min, permeabilized with PBST, blocked, and then labeled with Cy3-conjugated goat anti-mouse and FITC-conjugated goat anti-Myc (ab1263) for 1-2 hr at room temperature. These tagged antibodies allowed visualization of surface (red fluorescence) and intracellular (green fluorescence) Slo1 channels, respectively, by confocal microscopy. Cell-surface biotinylation assays were carried out

as described in detail previously (Kim et al., 2007a b, c). Briefly, intact HEK293T cells were labeled with a membrane impermeable biotinylation reagent (sulfo-*N*-hydroxy-succinimidobiotin) (Pierce Biotechnology, Rockford, IL). Cells were then lysed and biotinylated proteins from the cell surface were recovered by incubating lysates with streptavidin-agarose beads. A sample of the initial lysate was reserved to measure expression of total Slo1 proteins (surface + intracellular). All of the samples were separated by SDS-PAGE and analyzed by immunoblot using an antibody against the Myc tags on the Slo1 channels.

**Electrophysiology and statistics.** All physiological experiments were conducted at room temperature. Recordings were made from red fluorescent HEK293T cells co-expressing Slo1 channels with RFP-tagged  $\text{Ca}_v\beta 1$  or with RFP. Inside-out patches were excised using standard methods. Briefly, fire-polished glass micropipettes were filled with a solution containing (in mM): 140 KCl, 1.2  $\text{MgCl}_2$ , 14 glucose and 10 HEPES, pH 7.2, and had resistances of 2-5  $\text{M}\Omega$  after filling. Test solutions bathing the cytoplasmic face of the patch membrane contained (in mM): 140 KCl, 1.2  $\text{MgCl}_2$ , 14 glucose, 10 HEPES, pH 7.2, and < 1 nM free  $\text{Ca}^{2+}$  buffered with 10 mM EGTA, or 1, 5 or 10  $\mu\text{M}$  free  $\text{Ca}^{2+}$  buffered with 10 mM HEDTA. The free  $\text{Ca}^{2+}$  concentrations in these solutions were checked using an Orion 97-20 calcium electrode (Thermo Fisher Scientific, Waltham, MA) calibrated with commercial solution standards obtained from World Precision Instruments (Sarasota, FL). Currents in each test solution were evoked by a series of eight 450-ms depolarizing steps from a holding potential of -60 mV using an Axopatch 1D amplifier (Axon Instruments, Foster City, CA). Currents were digitized, and analyzed off-line using PClamp<sup>TM</sup> software (Axon Instruments). Because the potassium

equilibrium potential ( $E_K$ ) in these conditions is 0 mV, large inward tail-currents appear immediately after the break of the depolarizing step pulses, and some of the step commands (e.g. to -40 mV) also yield inward currents. Methods for whole-cell recordings of Slo1 currents in transiently transfected HEK293T cells have been described elsewhere (Kim et al., 2007a, b, c). Briefly, the bathing solution contained (in mM): NaCl 150, KCl 0.08, MgCl<sub>2</sub> 0.8, CaCl<sub>2</sub> 5.4, HEPES 10, pH 7.4. The pipette solution contained (in mM): NaCl 145, KCl 2, MgCl<sub>2</sub> 6.2, HEPES 10, pH 7.2, and 5  $\mu$ M free Ca<sup>2+</sup> buffered with 10 mM HEDTA. Note that with these solutions, the concentrations of K<sup>+</sup> inside and outside the cell are reduced to prevent whole-cell currents from saturating the patch clamp amplifier, but  $E_K$  is physiological. Voltage activation curves in inside-out patches were generated from tail-currents recorded at -60 mV by plotting the fractional activation ( $I_{\text{tail}}/\bar{I}_{\text{tail}}$ ) against the voltage of the step command  $V$  used to evoke the currents, and fitting the resulting curves with the Boltzmann function:

$$I_{\text{tail}}/\bar{I}_{\text{tail}} = [1 + \exp(-(V-V_{1/2}) qF/RT)]^{-1}$$

where  $I_{\text{tail}}$  is the tail-current amplitude evoked by pulse  $V$ ,  $\bar{I}_{\text{tail}}$  is the maximal tail-current (determined from the traces in the patch evoked by steps to +80 mV in 10  $\mu$ M Ca<sup>2+</sup>),  $V_{1/2}$  is the voltage of half-maximal activation,  $q$  is a slope constant, and  $F$ ,  $R$ , and  $T$  have their usual significance. A descriptive time constant related to the kinetics of channel activation ( $\tau_{\text{act}}$ ) was obtained by fitting the rising phase of evoked currents with a single-exponential using the Levenberg-Marquardt algorithms implemented in Origin™ v7.0 software (Northampton, MS). We tested the hypothesis that the presence or absence of Ca<sub>v</sub>β1 affects the Ca<sup>2+</sup>-sensitivity of the dependent variables  $V_{1/2}$  or  $\tau_{\text{act}}$  by two-way analysis of variance (ANOVA), with data from 6 patches in each group of cells. Other

quantitative data are graphically presented as mean  $\pm$  S.E.M. compiled from 10-25 cells in each group. Designs in which a single experimental group was compared to a single control group were analyzed using Student's unpaired *t*-test. All statistical analyses were performed using Statistica™ software (Statsoft, Tulsa, OK) with *P* < 0.05 regarded as significant.

Methods for whole-cell recordings and quantification of  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  currents and voltage-activated  $\text{Ca}^{2+}$  currents from chick CG neurons have been described in detail previously (e.g. Dourado and Dryer, 1992; Cameron et al., 1998). Briefly the bath solution contained (in mM): NaCl 145, KCl 5.3,  $\text{MgCl}_2$  0.8,  $\text{CaCl}_2$  5.4, HEPES 10, and 250 nM tetrodotoxin, pH 7.4. Pipette solutions contained (in mM): KCl 120,  $\text{MgCl}_2$  2, HEPES 10, EGTA 10, pH 7.2. For  $\text{Ca}^{2+}$ -free salines, all external  $\text{Ca}^{2+}$  was replaced on an equimolar basis with  $\text{MgCl}_2$ . Currents were evoked by step depolarizations from a holding potential of -40 mV, and net  $\text{Ca}^{2+}$ -dependent currents were calculated by digital subtractions (control –  $\text{Ca}^{2+}$ -free) and normalized for cell surface area as described previously (Dourado and Dryer, 1992). We have previously shown that all of the macroscopic outward currents observed under these conditions are carried by  $\text{BK}_{\text{Ca}}$  channels (Lhuillier and Dryer, 1999; Cameron and Dryer, 2000). The much smaller voltage-activated  $\text{Ca}^{2+}$  currents were measured using similar procedures, except that the bath also contained 250 nM tetrodotoxin, 10 mM tetraethylammonium, 5 mM 4-aminopyridine, and 2  $\mu\text{M}$   $\omega$ -conotoxin, and KCl in the pipette solutions was replaced with CsCl. In the presence of  $\omega$ -conotoxin, essentially all of the remaining  $\text{Ca}^{2+}$  current in ciliary neurons is carried by nifedipine-sensitive L-type channels (Wisgirda and Dryer, 1994).

## Results

**BK<sub>Ca</sub> channels interact directly with Ca<sub>v</sub>β1.** To identify proteins that regulate BK<sub>Ca</sub> channels, we conducted a yeast two-hybrid screen from an embryonic day 9 (E9) chick ciliary ganglion (CG) cDNA library, using a bait from the cytoplasmic C-terminal (G785-A985) of Slo1 that is conserved in most splice variants. This bait region includes the calcium bowl (T911-Q933) (Schreiber et al 1997) and a portion of the RCK2 domain (Xia et al., 2002) (Fig. 1a). The β1 subunit of L-type Ca<sup>2+</sup> channels (Ca<sub>v</sub>β1) emerged repeatedly in this screen. We confirmed that Slo1 binds to Ca<sub>v</sub>β1 by four independent methods. First, we observed that Ca<sub>v</sub>β1 co-immunoprecipitates with native Slo1 channels when E9 CG extracts were probed with Slo1 antibodies (Fig. 1b). This also occurred in HEK293T cells co-expressing Myc-tagged Slo1 channels and HA-tagged Ca<sub>v</sub>β1 subunits (Fig. 1c). HEK293T cells do not express endogenous voltage-activated Ca<sup>2+</sup> channels, and this result therefore suggests that the interaction does not require the pore-forming subunits of voltage-activated Ca<sup>2+</sup> channels. In addition, we carried out pull-down experiments to confirm the interaction. To do this, we prepared a glutathione transferase (GST) fusion protein encoding the bait (GST-Slo1<sub>G785-A985</sub>) and incubated it with chick CG extracts. We observed that GST-Slo1<sub>G785-A985</sub> could precipitate Ca<sub>v</sub>β1 subunits from chick CG extracts that could be detected by immunoblot analysis, whereas GST was ineffective (Fig. 1d). In addition, we observed co-localization of endogenous Ca<sub>v</sub>β1 and Slo1 subunits in cultured E9 CG neurons by confocal microscopy. Extensive co-localization of Slo1 (green fluorescence) and Ca<sub>v</sub>β1 (red fluorescence) was readily detectable in merged images, and this appeared to occur in intracellular pools as well as on the plasma membrane (Fig. 1e, *top, middle*) in CG neurons processed 3 hr after

dissociation. Co-localization of Slo1 and Ca<sub>v</sub>β1 also occurred in neurites that extended from neuronal somata in cells maintained in culture for longer periods of time (Fig. 1e, *bottom*).

**Ca<sub>v</sub>β1 physically associates with Slo1 at two distinct regions.** To identify specific regions of Slo1 that interact with Ca<sub>v</sub>β1, we made four smaller GST fusion-proteins comprised of mutually exclusive regions within our initial Slo1 bait, shown schematically in Fig. 2a. We observed that GST-Slo1<sub>T884-N936</sub>, which includes the calcium bowl (T911-Q933), was able to precipitate Ca<sub>v</sub>β1 from CG extracts, whereas GST, GST-Slo1<sub>G785-L843</sub>, GST-Slo1<sub>Q844-I883</sub> and GST-Slo1<sub>I937-A985</sub> were ineffective (Fig. 2b). Recall that the calcium bowl region of Slo1 is required for a portion of high-affinity (micromolar) Ca<sup>2+</sup> binding and activation of BK<sub>Ca</sub> channels (Bao et al., 2002; Zeng et al., 2005). We also looked for potential Ca<sub>v</sub>β1 binding sites outside the portions of Slo1 that we used for our bait. Since Ca<sub>v</sub>β1 has a functional SH3 domain (Tian et al., 2006), we prepared a GST fusion protein that includes the non-canonical SH3-binding motifs of Slo1 (GST-Slo1<sub>E637-D677</sub>). This fusion protein was also able to precipitate Ca<sub>v</sub>β1 from chick CG extracts (Fig. 2b). These interactions were observed in extracts of neuronal cells that express a full complement of voltage- and ligand-gated channels, and they cannot exclude Slo1-Ca<sub>v</sub>β1 interactions that occur as part of a larger complex. To address that issue, we placed human Ca<sub>v</sub>β1 cDNA into pcDNA3.1 (-) vector and translated it *in vitro* with incorporated biotinylated lysine residues to facilitate detection. The purified product was then added to various purified GST-Slo1 fusion proteins as binary mixtures. We observed that Ca<sub>v</sub>β1 interacted directly with GST-Slo1<sub>T884-N936</sub> and GST-Slo1<sub>E637-D677</sub> but not with the other

GST-Slo1 fusion proteins (Fig. 2c). From these results we conclude that Slo1-Ca<sub>v</sub>β1 interactions do not require contributions from other proteins.

**Ca<sub>v</sub>β1 affects BK<sub>Ca</sub> gating by reducing its sensitivity to Ca<sup>2+</sup>.** The functional significance of Slo1-Ca<sub>v</sub>β1 interactions was examined in inside-out patches excised from HEK293T cells expressing one or both of these channel subunits. For these experiments, we placed the cDNA encoding human Ca<sub>v</sub>β1 subunit into the pDsRed2-N1 expression vector, which allows for expression of Ca<sub>v</sub>β1 with a C-terminal red fluorescent protein (RFP) tag. Control cells expressed RFP. Voltage-evoked currents in symmetrical 140 mM KCl were recorded in patches excised from HEK293T cells expressing Slo1 in the presence or absence of Ca<sub>v</sub>β1-RFP. When either Ca<sub>v</sub>β1-RFP or RFP were expressed by themselves in HEK293T cells, the RFP tag was found throughout the cell and we did not detect voltage-evoked currents in excised inside-out patches (data not shown). Co-expression of Slo1 with RFP yielded robust Ca<sup>2+</sup>- and voltage-dependent currents similar to those observed by others (Fig. 3). However, co-expression of Slo1 and Ca<sub>v</sub>β1-RFP resulted in families of currents with substantially slower activation kinetics than those observed when Slo1 was expressed by itself (Fig. 3a, b, c). This was analyzed by fitting the rising phase of currents recorded at +80 mV as a single exponential, and then comparing the resulting activation time constants ( $\tau_{act}$ ) in patches from control and co-transfected HEK293T cells exposed to three different concentrations of Ca<sup>2+</sup> (Fig. 3c). These data were analyzed by two-way ANOVA. We observed significant effects of Ca<sub>v</sub>β1-RFP ( $F_{30} = 66.44215$ ,  $P < 0.0001$ ) and Ca<sup>2+</sup> ( $F_{30} = 32.47008$ ,  $P < 0.0001$ ) on the kinetics of activation. More importantly, there was a statistically significant interaction effect between the effects Ca<sup>2+</sup> concentration and channel stoichiometry on the resulting

activation kinetics ( $F_{30} = 8.62344$ ,  $P < 0.01$ ), suggesting that the  $\text{Ca}_v\beta 1$  subunit regulates the sensitivity of Slo1 channels to  $\text{Ca}^{2+}$ . A similar conclusion emerged from analyses of activation curves constructed from measurements of tail current amplitudes evoked by families of voltage steps applied in the presence of different bath  $\text{Ca}^{2+}$  concentrations (Fig. 3 d, e). Increasing bath  $\text{Ca}^{2+}$  caused a significant ( $F_{30} = 17.53$ ,  $P < 0.0003$ ) shift in the voltage of half-maximal activation ( $V_{1/2}$ ) to more negative membrane potentials in control cells expressing Slo1 alone. There was also a significant effect of  $\text{Ca}^{2+}$  on the  $V_{1/2}$  for activation in cells co-expressing  $\text{Ca}_v\beta 1$  ( $F_{30} = 60.836$ ,  $P < 0.00001$ ). However, the resulting  $\text{BK}_{\text{Ca}}$  channels appeared to be less sensitive to  $\text{Ca}^{2+}$  than control channels, and there was a statistically significant interaction effect between the response to  $\text{Ca}^{2+}$  and the presence of  $\text{Ca}_v\beta 1$  ( $F_{30} = 6.159$ ,  $P < .0057$ ). This can be readily seen by comparing the mean  $V_{1/2}$  for channel activation obtained at 5  $\mu\text{M}$   $\text{Ca}^{2+}$  in the two groups, which is statistically significant ( $P < 0.05$ ) by Scheffé's post hoc test (Fig. 3e). This pattern suggests a reduction in the sensitivity of Slo1 to  $\text{Ca}^{2+}$  in the presence of  $\text{Ca}_v\beta 1$ , possibly because this subunit binds close to or within the calcium bowl domains of Slo1.

We observed similar effects of  $\text{Ca}_v\beta 1$  on the amplitudes and kinetics of whole-cell currents recorded with pipettes containing 5  $\mu\text{M}$  free  $\text{Ca}^{2+}$  from transfected HEK293T cells using methods described previously. The voltage-evoked currents recorded from cells co-expressing Slo1 and  $\text{Ca}_v\beta 1$ -RFP were markedly different from those observed in cells co-expressing Slo1 and RFP (Fig. 4a). Specifically, currents were markedly reduced in amplitude and were significantly slower in  $\text{Ca}_v\beta 1$ -RFP co-transfected cells (Fig. 4b, c), much as was seen with inside-out patches exposed to the same concentration  $\text{Ca}^{2+}$ .



**Over-expression of  $\text{Ca}_v\beta 1$  inhibits  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  currents and potentiates L-type**

**$\text{Ca}^{2+}$  currents in native neurons.** In order to study the implications of Slo1- $\text{Ca}_v\beta 1$  interactions in native conditions, we examined the effects of over-expressing  $\text{Ca}_v\beta 1$  chick CG neurons placed in culture on E9. Stabilized cultures were transiently transfected with either  $\text{Ca}_v\beta 1$ -RFP or RFP, and whole-cell currents were recorded from fluorescent cells 48 hr later using methods that we have used for many years to isolate  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  currents or voltage-activated  $\text{Ca}^{2+}$  currents (Cameron et al., 1998). The effects of  $\text{Ca}_v\beta 1$  were similar to those seen in HEK293T cells. Specifically, there was a statistically significant reduction in the mean amplitude of  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  currents in CG neurons transfected with  $\text{Ca}_v\beta 1$ -RFP compared to those observed in cells transfected with RFP (Fig 5a, b). The rising phase of macroscopic  $\text{Ca}^{2+}$ -dependent  $\text{K}^+$  currents was also substantially slower, much as we observed in HEK293T cells expressing  $\text{Ca}_v\beta 1$ . By contrast, we observed significant potentiation of endogenous voltage-activated  $\text{Ca}^{2+}$  currents in  $\text{Ca}_v\beta 1$ -RFP transfected CG neurons compared to those seen in cells expressing RFP (Fig. 5c, d). We observed that  $\text{Ca}_v\beta 1$  expression also caused a slight left shift in the voltage-dependence of  $\text{Ca}^{2+}$  currents (Fig 5e).

**$\text{Ca}_v\beta 1$  has no effect on surface expression of Slo1.** Although the above experiments establish an effect of  $\text{Ca}_v\beta 1$  on Slo1 gating, they do not address the possibility that this subunit influences the steady-state surface expression of Slo1. We addressed this issue in three different types of experiments. First, there was no significant difference in average maximal outward currents evoked by voltage steps to + 60 mV in the presence of 20  $\mu\text{M}$  bath  $\text{Ca}^{2+}$  in inside-out patches excised from HEK293T cells expressing Slo1 in combination with either RFP or  $\text{Ca}_v\beta 1$ -RFP (Fig. 6a). Current differences in those

recording conditions are likely to reflect the number of functional channels in the plasma membrane. In addition, we examined the surface expression of Slo1 channels in HEK293T cells in the presence and absence of  $\text{Ca}_v\beta 1$  by labeling surface Slo1 channels in live cells with FITC-conjugated anti-Myc antibody (green). The cells were then fixed, blocked, permeabilized and labeled with a non-conjugated anti-Myc raised in a different species to obtain signal from intracellular Slo1 channels (red). The distribution of channels was then determined by confocal microscopy, using the same laser intensities for all measurements. The resulting images suggest that co-expression of  $\text{Ca}_v\beta 1$  had no effect on the surface expression of Slo1 (Fig. 6b). In addition, we carried out cell surface biotinylation assays in HEK293T cells expressing Myc-tagged Slo1 channels in the presence or absence of HA tagged  $\text{Ca}_v\beta 1$  and observed no quantitative difference in the amounts of total or cell-surface Slo1 (Fig 6c). From these data we conclude that  $\text{Ca}_v\beta 1$  primarily affects the gating properties of  $\text{BK}_{\text{Ca}}$  channels, and that the effects on macroscopic current amplitude in native cells and in heterologous expression systems are not caused by significant effects on steady-state surface expression of Slo1.

### **Interactions with the GK domain of $\text{Ca}_v\beta 1$ are sufficient to modulate $\text{BK}_{\text{Ca}}$ gating.**

Recent structural analyses of the family of  $\text{Ca}_v\beta$  subunits have demonstrated that an SH3 domain and a guanylate kinase (GK) domain form a bi-dentate interaction with the pore-forming subunits of L-type  $\text{Ca}^{2+}$  channels, resulting in changes in their gating, trafficking and stability in the plasma membrane (Takahashi et al., 2005). The non-catalytic GK domain is a structural signature of the membrane-associated guanylate kinase (MAGUK) family of proteins, and it is a potential protein-protein interaction motif (Petegem et al., 2004; Chen et al., 2004) (Fig. 7a). In order to identify the regions in  $\text{Ca}_v\beta 1$  that interact

with Slo1, we carried out in vitro binding assays in binary mixtures of GST-Slo1 fusion proteins and two different purified  $\text{Ca}_v\beta 1$  fragments. The latter include  $\text{Ca}_v\beta 1_{\text{M1-D224}}$  and  $\text{Ca}_v\beta 1_{\text{P162-R598}}$ , which comprise the SH3 and GK domains, respectively. We observed that  $\text{Ca}_v\beta 1_{\text{M1-D224}}$  binds to GST-Slo1<sub>E637-D677</sub>, a region within Slo1 that contains a non-canonical SH3-binding motif (Tian et al., 2006). We also observed that  $\text{Ca}_v\beta 1_{\text{P162-R598}}$  binds to GST-Slo1<sub>T884-N936</sub>, which includes the calcium bowl domain (Fig. 7b). To examine the functional significance of these interactions, we expressed the  $\text{Ca}_v\beta 1$  fragments as RFP fusion proteins in HEK293T cells, along with full-length Slo1. We then examined Slo1 gating in red fluorescent cells using whole-cell recordings with pipettes containing 5  $\mu\text{M}$   $\text{Ca}^{2+}$ , as described above. We observed that co-expression of RFP- $\text{Ca}_v\beta 1_{\text{P162-R598}}$  caused slowing and inhibition of Slo1 currents similar to that observed with full-length RFP- $\text{Ca}_v\beta 1$ , whereas RFP- $\text{Ca}_v\beta 1_{\text{M1-D224}}$  had no apparent effect on these parameters of Slo1 gating (Fig. 7c, d). This suggests that interactions with the GK domain region of  $\text{Ca}_v\beta 1$  are sufficient to modulate  $\text{BK}_{\text{Ca}}$  gating.

## Discussion

In many excitable cells, BK<sub>Ca</sub> channels are components of a feedback system that limits the amount of Ca<sup>2+</sup> influx evoked by membrane depolarization. Because of these dynamics, BK<sub>Ca</sub> channels can contribute to spike repolarization, membrane potential oscillations, and the control of muscle contractility (Salkoff et al., 2006). Several investigators have addressed the question of how BK<sub>Ca</sub> channels can be selectively regulated by Ca<sup>2+</sup> influx through Ca<sub>v</sub> channels given that BK<sub>Ca</sub> channels typically require micromolar concentrations of free Ca<sup>2+</sup> for robust activation at physiological membrane potentials (Grunnet & Kaufmann, 2004; Berkefeld et al., 2006; Loane et al., 2007). These studies have shown that complexes comprised of the pore-forming  $\alpha$ -subunits of BK<sub>Ca</sub> channels (Slo1) and voltage-activated Ca<sup>2+</sup> channels (Ca<sub>v</sub>1.2, Ca<sub>v</sub>2.1 and Ca<sub>v</sub>2.2) can be co-purified from mammalian brain. Although these complexes can include auxiliary (non pore-forming) subunits of BK<sub>Ca</sub> channels ( $\beta$ 2 and  $\beta$ 4) and Ca<sub>v</sub> channels (Ca<sub>v</sub> $\beta$ 1, Ca<sub>v</sub> $\beta$ 2, Ca<sub>v</sub> $\beta$ 3, and Ca<sub>v</sub> $\beta$ 4) (Berkefeld et al., 2006), the auxiliary subunits are not necessary for BK<sub>Ca</sub> channels to reside within the local (~ 20 nm) and steep Ca<sup>2+</sup> concentration gradient that builds up around an open Ca<sub>v</sub> channel pore in the presence of mobile buffers (Loane et al., 2007). Moreover, not all cell-surface BK<sub>Ca</sub> channels in neural cells occur in a complex with functional Ca<sub>v</sub> channels (Gola and Crest, 1993; Marrion and Tavalin, 1998) and the potential consequences of formation Slo1-Ca<sub>v</sub> $\beta$  complexes in which the pore-forming  $\alpha$ -subunits of Ca<sup>2+</sup> channels are missing have not been examined previously.

In the present study we have observed a functionally significant interaction between Slo1 (BK<sub>Ca</sub> channel) and Ca<sub>v</sub>β1 subunits that can occur in the absence of other proteins and that has a profound effect on the gating properties of BK<sub>Ca</sub> channels. Formation of a complex with Ca<sub>v</sub>β1 markedly slows voltage-evoked activation of BK<sub>Ca</sub> channels and reduces their apparent Ca<sup>2+</sup>-sensitivity, leading to net inhibition of gating at moderate concentrations of cytoplasmic Ca<sup>2+</sup>. It is important to note that this can occur in the absence of any other Ca<sub>v</sub> channel subunits. In addition, we observed that co-expression of Ca<sub>v</sub>β1 subunits did not have a marked effect on the steady-state surface expression of Slo1 subunits as detected by electrophysiology or biochemistry in HEK293T cells.

Previous studies have shown that Ca<sub>v</sub>β subunits interact with a conserved domain in the pore-forming α-subunits of Ca<sub>v</sub> channels known as the alpha-interaction domain (AID) (Chen et al., 2004). The AID interacts primarily with the GK domains of Ca<sub>v</sub>β subunits (Chen et al., 2004), and co-expression of a fragment containing only a portion of the GK domain is sufficient to cause shifts in the voltage-dependence and kinetics of Ca<sub>v</sub> channels (Chen et al., 2004). However, complete reconstitution of all of the modulatory activities of Ca<sub>v</sub>β subunits, including stimulation of trafficking, occurred only when fragments separately comprised of SH3 and GK domains were simultaneously co-expressed with Ca<sub>v</sub> α-subunits in HEK293 cells (Takahashi et al., 2004). The situation with Slo1 channels is somewhat different, as both of the protein-interaction domains of Ca<sub>v</sub>β1 can bind to Slo1 channels, although they do so at different sites in the cytoplasmic C-terminal of the later. Moreover, expression of a fragment that contained only the GK

domain was sufficient to reproduce the modulatory effects of full-length  $\text{Ca}_v\beta 1$  on the  $\text{Ca}^{2+}$ -dependent gating properties of  $\text{BK}_{\text{Ca}}$  channels. Interestingly, the GK domain of  $\text{Ca}_v\beta 1$  interacts at a site close to or within the calcium bowl domain of Slo1, a region necessary for at least a portion of high-affinity activation of  $\text{BK}_{\text{Ca}}$  channels by  $\text{Ca}^{2+}$  (Bao et al., 2002; Zeng et al., 2005). The calcium bowl domain appears to be especially important in regulating the kinetics of  $\text{Ca}^{2+}$ -dependent Slo1 channel activation at low (1-10  $\mu\text{M}$ )  $\text{Ca}^{2+}$  concentrations (Zeng et al., 2005), and it is notable that one of the largest effects of  $\text{Ca}_v\beta 1$  is to slow activation of co-expressed Slo1 channels over that same concentration range. It is possible that binding of the  $\text{Ca}_v\beta 1$  GK domain to Slo1 modulates  $\text{Ca}^{2+}$  activation by simple steric hindrance within the calcium bowl, or through an allosteric mechanism that entails conformational changes in the overall  $\text{Ca}^{2+}$  binding pocket in that part of the Slo1 channel. Expression of the SH3 domain of  $\text{Ca}_v\beta 1$  by itself did not affect  $\text{Ca}^{2+}$ -dependent gating of Slo1 channels, but we cannot exclude that the SH3 domain plays a role in modulating other aspects of Slo1 function, possibly by allowing formation of a larger complex. In this regard, Slo1 channels have a non-canonical SH3-binding motif that has been implicated in cytoskeletal interactions that contribute to stretch-sensitive gating (Tian et al., 2006). It is possible that  $\text{Ca}_v\beta 1$  forms a bridge that allows mechanical coupling between SH3-binding motif and the calcium bowl, thereby contributing to the process of stretch-sensitive gating.

One question that emerges is whether  $\text{Ca}_v\beta 1$  subunits are independent targets of physiological regulation. As already noted, the portions of  $\text{Ca}_v\beta 1$  that produce functional effects upon binding to Slo1 are located within the GK domain, and the available

crystallographic data suggests that these sites may be occluded in those  $\text{Ca}_v\beta 1$  subunits that are already bound to  $\text{Ca}_v$   $\alpha$ -subunits (Chen et al., 2004). This suggests that under most conditions, interactions between  $\text{Ca}_v\beta$  and  $\text{Ca}_v$   $\alpha$ -subunits predominate, and this is supported by the observation that Slo1 channels expressed in the presence of a combination of  $\text{Ca}_v\beta 1$  and  $\text{Ca}_v$   $\alpha$ -subunits have fast kinetics, similar to those observed when Slo1 is expressed by itself (Berkefeld et al., 2006), an observation that we have confirmed (S. Jha and S. E. Dryer, unpublished observations). Moreover, the fact that  $\text{Ca}_v\beta 1$  subunits can stimulate the surface expression of  $\text{Ca}_v$   $\alpha$ -subunits suggests that all high threshold voltage-activated  $\text{Ca}^{2+}$  channels are assembled with a  $\beta$ -subunit prior to insertion into the plasma membrane. However, it is possible that these dynamics can be altered by changes in the relative abundance of the various subunits, and as yet, very little is known about factors that regulate  $\text{Ca}_v\beta$  at the transcriptional or translational level. However, it is worth noting that dynamin, a protein involved in endocytotic regulation of membrane-associated proteins, can bind to the SH3 domains of  $\text{Ca}_v\beta 1$  even in the absence of other subunits (Gonzalez-Gutierrez et al., 2007). In addition, several members of the RGK family of Ras-like GTPases can also interact directly with the GK domains of  $\text{Ca}_v\beta 1$  subunits leading to down regulation of their steady-state expression on the cell surface (Béguin et al., 2001). Thus, it is possible that the amounts of  $\text{Ca}_v\beta 1$  at the cell surface are regulated independently of other subunits, and that their removal from the plasma membrane exposes protein interaction motifs that allow them to form new interactions, e.g. with Slo1.

The present data argue against a role for  $\text{Ca}_v\beta 1$  in regulation of the trafficking of Slo1 channels, although it should be noted that this process is sometimes cell type-dependent, and HEK293T cells may not have provided the appropriate context in which to observe such an effect. On the other hand, increases in the surface expression of  $\text{Ca}_v\beta 1$  could increase voltage-evoked  $\text{Ca}^{2+}$  influx by potentiation of  $\text{Ca}_v$  channels, and by independently slowing and inhibiting  $\text{BK}_{\text{Ca}}$  channels, thereby suppressing the main negative feedback to voltage-dependent  $\text{Ca}^{2+}$  influx. In this regard, the effect of  $\text{Ca}_v\beta 1$  subunits on  $\text{BK}_{\text{Ca}}$  channels is not saturated under normal physiological conditions, as over-expression of  $\text{Ca}_v\beta 1$  in native ciliary ganglion neurons caused slowing and reduction in mean densities of macroscopic  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels, along with a simultaneous increase in L-type  $\text{Ca}^{2+}$  currents. Finally, we should note that all of our experiments have been carried out on  $\text{Ca}_v\beta 1$  subunits, which normally contribute to formation of L-type  $\text{Ca}^{2+}$  channels. The question arises as to whether other  $\text{Ca}_v\beta$  subunits can produce a similar effect on Slo1. Although we have no data to address this issue, the GK domains of this group of proteins are very highly conserved (Dolphin, 2003), and therefore it is quite possible that this is a general property of all of them. This is notable because interactions between other  $\text{Ca}_v\beta$  subunits and Slo1 have been described in brain (Berkefeld et al., 2006; Loane et al., 2007).

In summary, we have identified a functionally significant interaction between  $\text{Ca}_v\beta 1$ , an auxiliary subunit of a voltage-activated  $\text{Ca}^{2+}$  channel, and Slo1 the principle pore-forming subunit of large-conductance  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels. Binding of  $\text{Ca}_v\beta 1$  to Slo1 can occur in the absence of any other channel subunits, and the interaction



produced profound affects on the  $\text{Ca}^{2+}$ -dependent gating of  $\text{BK}_{\text{Ca}}$  channels. This interaction may comprise part of a mechanism to modulate negative feedback control of voltage-dependent  $\text{Ca}^{2+}$  influx in excitable cells.

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### Footnotes

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## Figure Legends

**Figure 1** Direct interaction between  $\text{Ca}_v\beta 1$  and the calcium bowl and SH3 domain-binding motifs of Slo1. A, Schematic representation of Slo1. The bait fragment used in the yeast-two hybrid screen is highlighted in blue. The calcium bowl and a non-canonical SH3-binding domain are indicated in yellow and magenta, respectively. B, Co-immunoprecipitation of  $\text{Ca}_v\beta 1$  and Slo1 from chick ciliary ganglion extracts. C, Co-immunoprecipitation of  $\text{Ca}_v\beta 1$  and Slo1 from extracts of HEK293T cells expressing Myc-Slo1 and HA- $\text{Ca}_v\beta 1$  after transient transfection. D, GST-pull down assays carried out on chick ciliary ganglion lysates show that GST-Slo1<sub>G785-A985</sub> binds to  $\text{Ca}_v\beta 1$ . E, Co-localization of  $\text{Ca}_v\beta 1$  and Slo1 in E9 chick ciliary ganglion neurons kept in dissociated cell culture for 3 hr (*top*) or two days (*middle*). Regions of neurites outlined by white boxes are shown at a higher magnification on the bottom panels.

**Figure 2** Interactions between  $\text{Ca}_v\beta 1$  and Slo1 do not require the presence of additional proteins. A, Schematic representation of GST fusion-protein constructs encoding GST-Slo1<sub>G785-A985</sub>, GST-Slo1<sub>G785-L843</sub>, GST-Slo1<sub>Q844-I883</sub>, GST-Slo1<sub>T884-N936</sub>, GST-Slo1<sub>I937-A985</sub> and GST-Slo1<sub>E637-D677</sub>. B, GST pull-down assays show that GST-Slo1<sub>I937-A985</sub> and GST-Slo1<sub>E637-D677</sub> bind to  $\text{Ca}_v\beta 1$  in chick ciliary ganglion extracts. C, A full-length biotin- $\text{Ca}_v\beta 1$  binds directly to GST-Slo1<sub>I937-A985</sub> and GST-Slo1<sub>E637-D677</sub> in a binary mixture. No other proteins were present in the assay.

**Figure 3**  $\text{Ca}_v\beta 1$  modulates the gating properties of  $\text{BK}_{\text{Ca}}$  channels. Inside-out patch were excised from HEK293T cells expressing Slo1 in the presence or absence of  $\text{Ca}_v\beta 1$ . A, Representative families of currents evoked by voltage steps (-80 mV to +80 mV) in

inside-out patches at different bath  $\text{Ca}^{2+}$  concentrations, as indicated. No currents were detected in  $\text{Ca}^{2+}$ -free bath solutions (data not shown). Note slower kinetics in presence of  $\text{Ca}_v\beta 1$ . B, The rising phase of currents evoked by a voltage step to +80 mV in the presence of 10  $\mu\text{M}$   $\text{Ca}^{2+}$ . These are plotted so as to facilitate comparisons of activation kinetics. C, The rising phase of these types of evoked currents were fitted with single-exponential curves and the resulting time constants plotted against bath  $\text{Ca}^{2+}$ . Increasing bath  $\text{Ca}^{2+}$  causes channels to activate more rapidly. Two-way ANOVA of this data set reveals a significant ( $P < 0.0001$ ) interaction effect, indicating that the presence of  $\text{Ca}_v\beta 1$  affects the  $\text{Ca}^{2+}$ -sensitivity of the resulting activation kinetics. Error bars are S.E.M. D, Voltage-activation curves of Slo1 and Slo1+ $\text{Ca}_v\beta 1$  channels obtained at different bath  $\text{Ca}^{2+}$  concentrations constructed from the tail currents recorded from inside-out patches, readily seen in panel A. Data points (mean  $\pm$  S.E.M.) are shown with superimposed fitted Boltzmann curves. Bath  $\text{Ca}^{2+}$  concentrations (in  $\mu\text{M}$ ) are indicated next to the corresponding curves. E, Plot of the  $V_{1/2}$  values derived from Boltzmann fits at different bath  $\text{Ca}^{2+}$  concentrations for Slo1 and Slo1+ $\text{Ca}_v\beta 1$  channels. These data were analyzed by two-way ANOVA, which revealed a significant interaction effect between the response to  $\text{Ca}^{2+}$  and the presence of  $\text{Ca}_v\beta 1$  ( $P < .0057$ ).

**Figure 4** Whole cell currents from HEK293T cells expressing Slo1 in the presence or absence of RFP-tagged  $\text{Ca}_v\beta 1$ . A, Families of voltage-evoked whole-cell currents in transfected HEK293T cells co-expressing Slo1 and RFP (*top*) or Slo1 and  $\text{Ca}_v\beta 1$ -RFP (*bottom*). The recording pipettes contained 5  $\mu\text{M}$   $\text{Ca}^{2+}$ . Note smaller and slower currents in presence of  $\text{Ca}_v\beta 1$ -RFP. B, The bar graph shows mean  $\pm$  S.E.M. of the maximal whole-cell currents evoked by steps to +80 mV. Cells co-expressing RFP- $\text{Ca}_v\beta 1$  have

significantly smaller currents ( $P < 0.01$  by student's unpaired  $t$ -test). C, The effect of  $\text{Ca}_v\beta 1$  on the rising phase of representative whole-cell currents evoked by a step to +80mV plotted so as to facilitate comparison of activation kinetics.

**Figure 5** Over-expression of  $\text{Ca}_v\beta 1$  affects macroscopic  $\text{Ca}^{2+}$ -dependent  $\text{K}^+$  currents and voltage-activated  $\text{Ca}^{2+}$  currents in chick CG neurons. Whole-cell currents were evoked by a series of depolarizing voltage steps in the presence and absence of external  $\text{Ca}^{2+}$ , and net  $\text{Ca}^{2+}$ -dependent currents were obtained by digital subtraction. A, Families of  $\text{Ca}^{2+}$ -dependent voltage-evoked  $\text{K}^+$  currents in CG neurons over-expressing RFP or  $\text{Ca}_v\beta 1$ -RFP. Note smaller and slower currents in  $\text{Ca}_v\beta 1$ -RFP transfected neurons. B, Mean  $\pm$  S.E.M. of the maximal  $\text{Ca}^{2+}$ -dependent outward currents evoked by steps to +80 mV from a holding potential of -40 mV. Current densities were significantly smaller when  $\text{Ca}_v\beta 1$ -RFP was present ( $P < 0.01$  by student's unpaired  $t$ -test). C, Comparison of voltage-evoked maximal whole cell L-type calcium currents recorded from neurons expressing RFP or  $\text{Ca}_v\beta 1$ -RFP, as indicated. The recording electrodes contained 150 mM CsCl and external solutions contained 0.1  $\mu\text{M}$  tetrodotoxin, 10 mM tetraethylammonium, and 2  $\mu\text{M}$   $\omega$ -conotoxin. D, Mean  $\pm$  S.E.M. of the maximal L-type current densities evoked from a holding potential of -40 mV. Current densities were significantly smaller in cells over-expressing  $\text{Ca}_v\beta 1$ -RFP compared to cells over-expressing RFP ( $P < 0.01$  by student's unpaired  $t$ -test) E, Normalized current-voltage relationship of L-type  $\text{Ca}^{2+}$  currents in CG neurons over-expressing RFP (black) or  $\text{Ca}_v\beta 1$ -RFP (grey). Data points are mean  $\pm$  S.E.M. from 8 cells in each group, and the curves are spline fits with no theoretical significance.

**Figure 6** Co-expression of  $\text{Ca}_v\beta 1$  does not alter steady-state levels of Slo1 on the surface

of HEK293T cells. A, Mean maximal currents ( $+60$  mV and  $10 \mu\text{M Ca}^{2+}$ ) in inside-out patches excised from HEK293T cells co-expressing Slo1 and RFP or Slo1 and  $\text{Ca}_v\beta 1$ -RFP are not significantly different. B, Confocal images of the distribution of Slo1 channels in transfected HEK293T cells obtained using different primary antibodies against ectofacial Myc tags on Slo1 applied before and after cell permeabilization. Note that  $\text{Ca}_v\beta 1$  has no effect on surface expression of Slo1 (*green fluorescence*) or on Slo1 channels in intracellular pools (*red fluorescence*). The same laser intensities were used to obtain these images. C, Cell-surface biotinylation assay using antibodies against the Myc tags on Slo1 shows no significant effect of  $\text{Ca}_v\beta 1$  on the surface expression of Slo1.

**Figure 7** The guanylate kinase (GK) and SH3 domains of  $\text{Ca}_v\beta 1$  interact with Slo1 and the GK domain is necessary and sufficient to affect Slo1 gating. A, Schematic diagram of  $\text{Ca}_v\beta 1$ . The SH3 and GK domains are shown in grey and black, respectively. B, *In vitro* binding assay showing that the  $\text{Ca}_v\beta 1_{\text{M1-D224}}$  fragment, which includes the SH3 domain, binds to a non-canonical SH3-binding motif in Slo1 (Tian et al., 2006). A fragment comprised of  $\text{Ca}_v\beta 1_{\text{P162-R598}}$ , which includes the GK domain, binds to the calcium bowl region of Slo1. C, Whole-cell recordings from HEK293T cells made with recording pipettes containing  $5 \mu\text{M Ca}^{2+}$  show that the  $\text{Ca}_v\beta 1_{\text{P162-R598}}$  fragment inhibits and slows macroscopic currents carried by Slo1. However, the  $\text{Ca}_v\beta 1_{\text{M1-D224}}$  fragment does not. Bar graphs represent mean  $\pm$  S.E.M. Inset shows representative traces from HEK293T cells co-expressing the SH3-domain fragment.

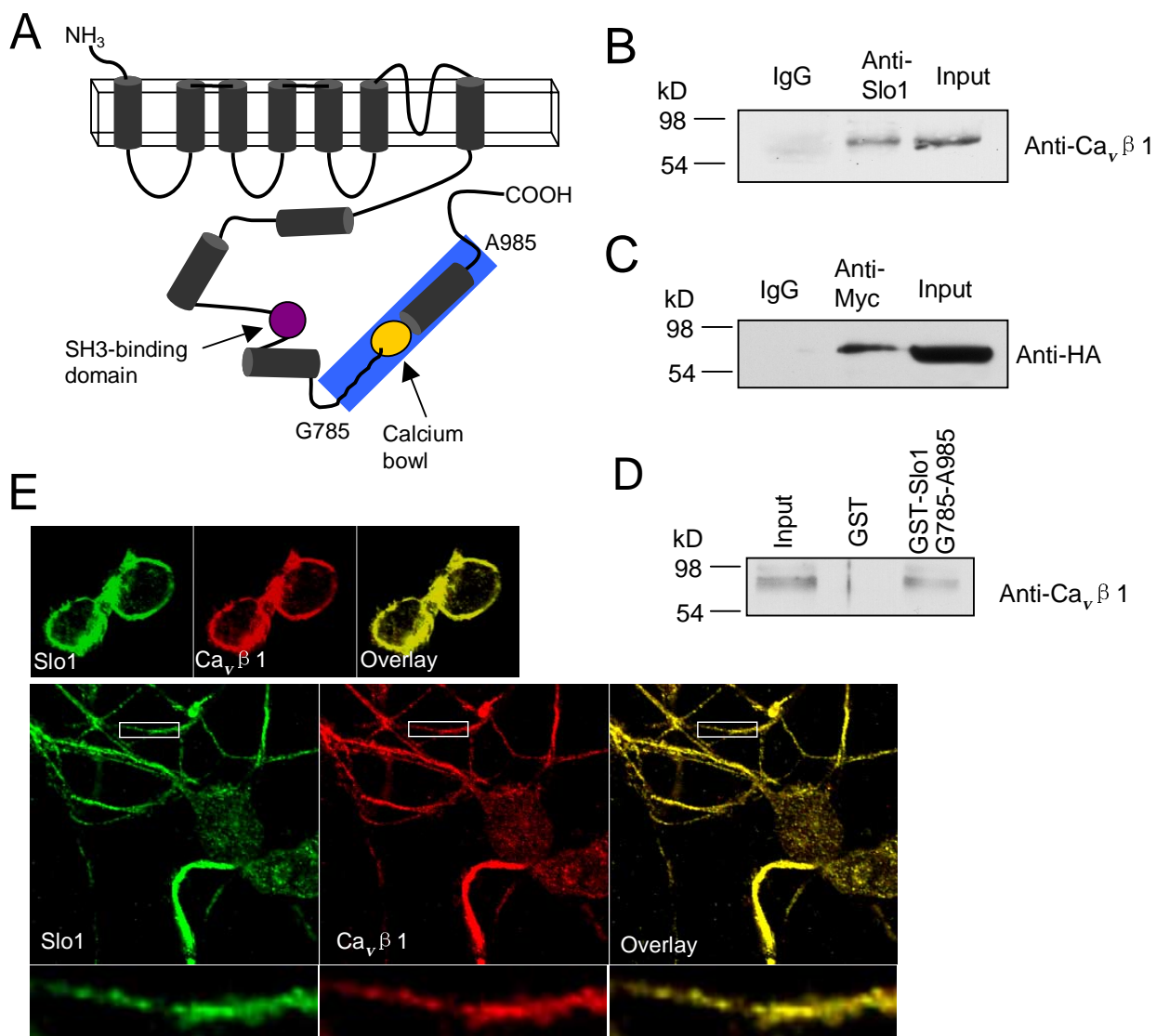


Fig 1

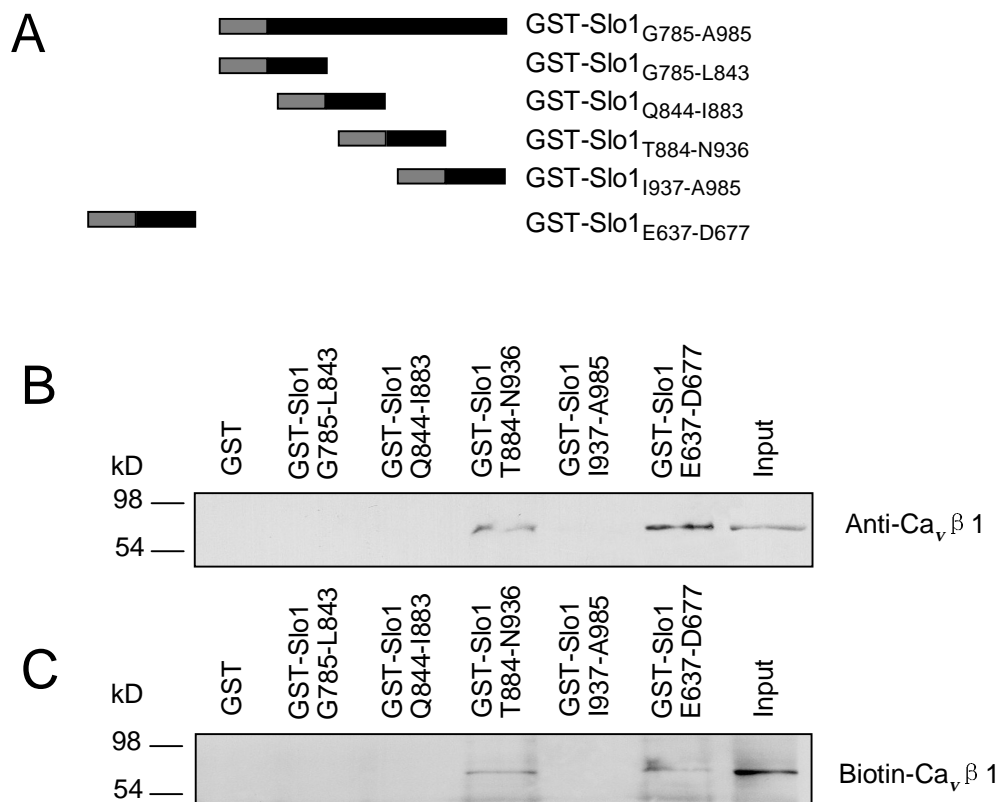


Fig 2

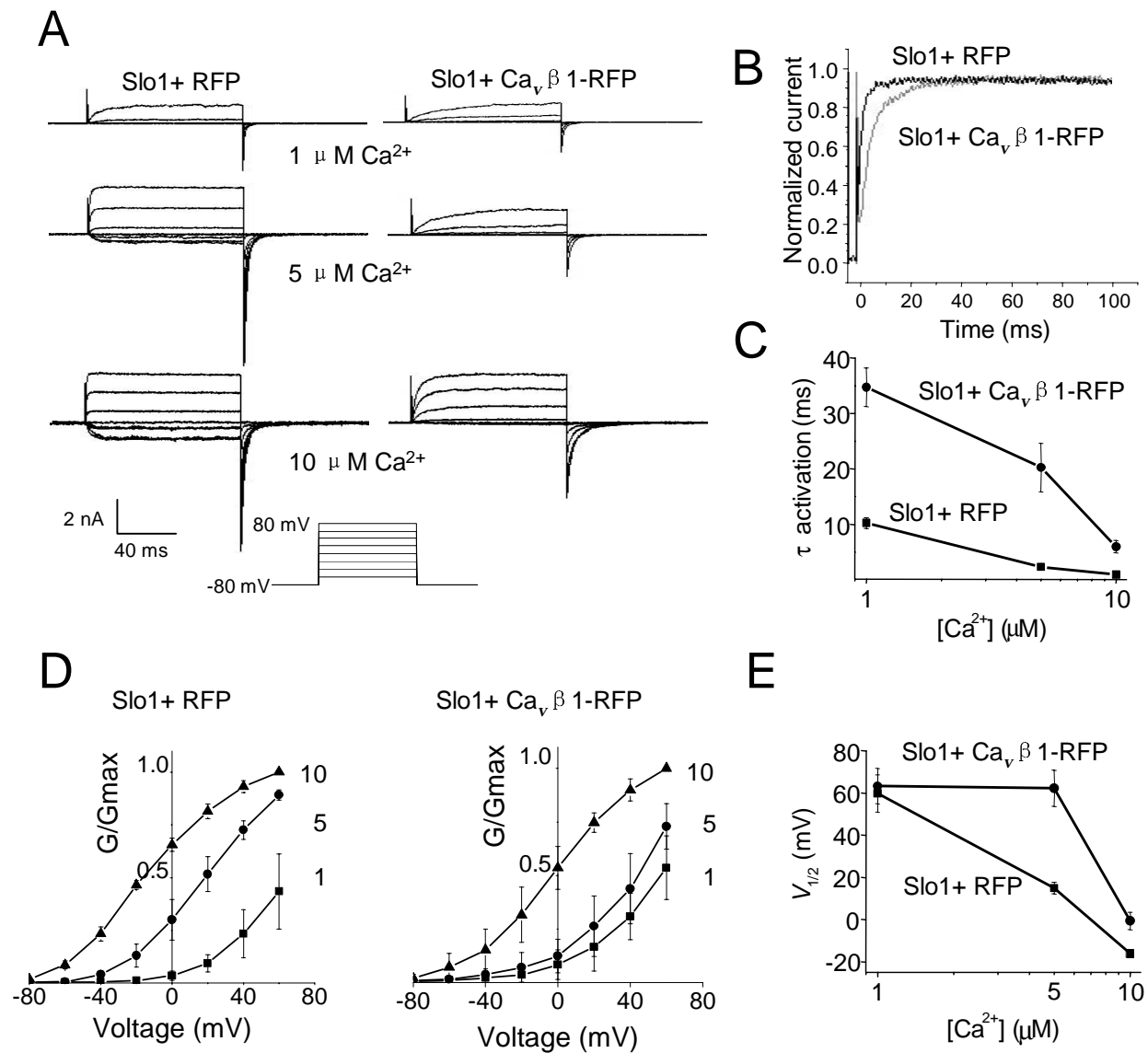


Fig 3

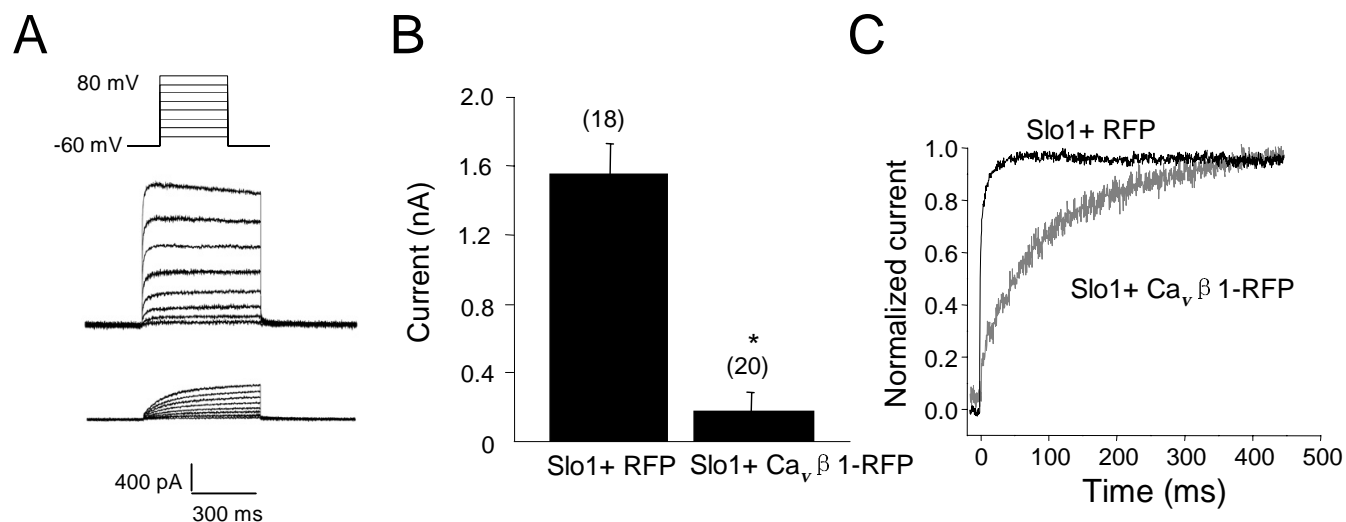


Fig 4



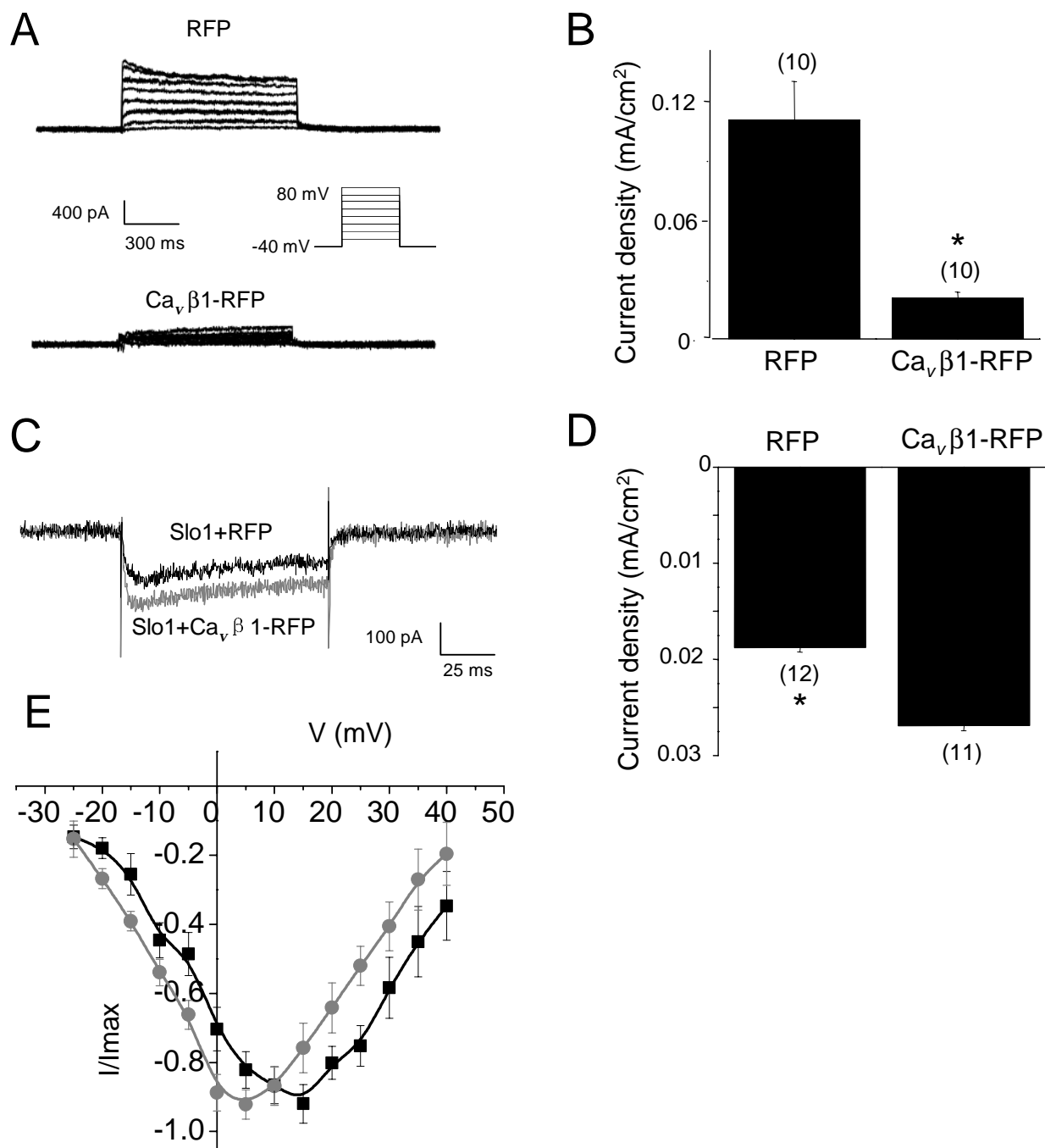


Fig 5

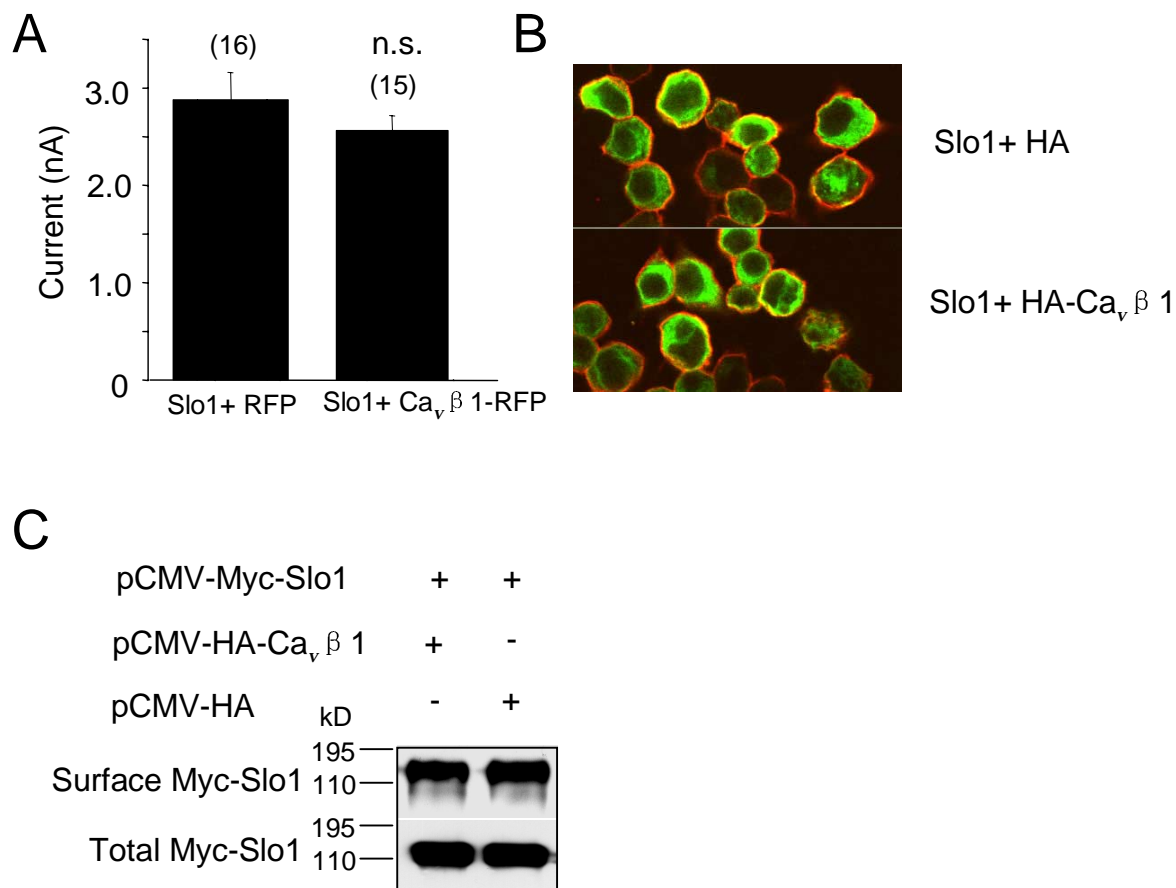


Fig. 6

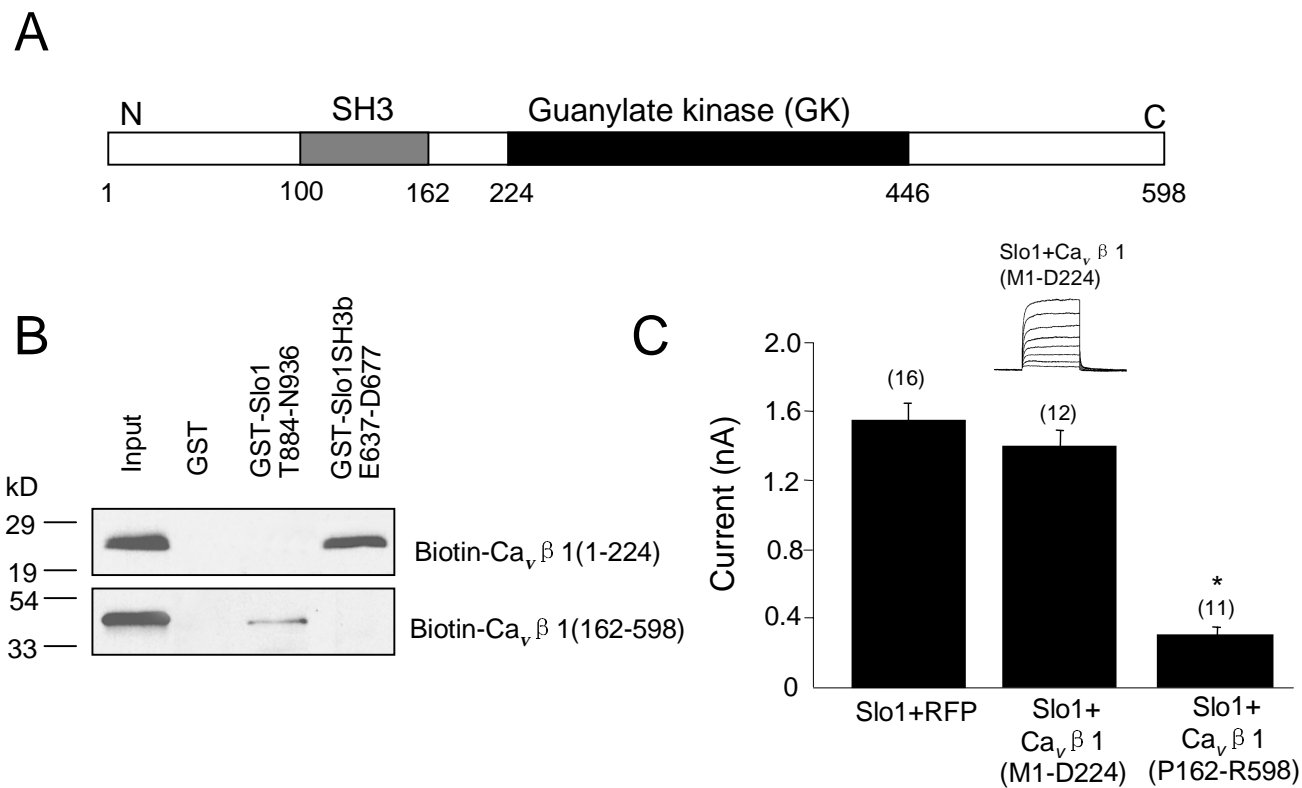


Fig. 7