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The Ca\textsuperscript{2+} channel β subunit determines whether stimulation of G\textsubscript{q}-coupled receptors enhances or inhibits N current

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In superior cervical ganglion (SCG) neurons, stimulation of M\textsubscript{1} receptors (M\textsubscript{1}Rs) produces a distinct pattern of modulation of N-type calcium (N-) channel activity, enhancing currents elicited with negative test potentials and inhibiting currents elicited with positive test potentials. Exogenously applied arachidonic acid (AA) reproduces this profile of modulation, suggesting AA functions as a downstream messenger of M\textsubscript{1}Rs. In addition, techniques that diminish AA's concentration during M\textsubscript{1}R stimulation minimize N-current modulation. However, other studies suggest depletion of phosphatidylinositol-4,5-bisphosphate during M\textsubscript{1}R stimulation suffices to elicit modulation. In this study, we used an expression system to examine the physiological mechanisms regulating modulation. We found the β subunit (Ca\textsubscript{V}2.2) acts as a molecular switch regulating whether modulation results in enhancement or inhibition. In human embryonic kidney 293 cells, stimulation of M\textsubscript{1}Rs or neurokinin-1 receptors (NK-1Rs) inhibited activity of N channels formed by Ca\textsubscript{V}2.2 and coexpressed with Ca\textsubscript{V}2.1b, Ca\textsubscript{V}3.3, or Ca\textsubscript{V}3.4 but enhanced activity of N channels containing Ca\textsubscript{V}2.2a. Exogenously applied AA produced the same pattern of modulation. Coexpression of Ca\textsubscript{V}2.2a, Ca\textsubscript{V}3.3, and Ca\textsubscript{V}3.4 recapitulated the modulatory response previously seen in SCG neurons, implying heterogeneous association of Ca\textsubscript{V}2.2 with Ca\textsubscript{V}2.2. Further experiments with mutated, chimeric Ca\textsubscript{V}β subunits and free palmitic acid revealed that palmitoylation of Ca\textsubscript{V}2.2a is essential for loss of inhibition. The data presented here fit a model in which Ca\textsubscript{V}2.2a blocks inhibition, thus unmasking enhancement. Our discovery that the presence or absence of palmitoylated Ca\textsubscript{V}2.2a toggles M\textsubscript{1}R- or NK-1R–mediated modulation of N current between enhancement and inhibition identifies a novel role for palmitoylation. Moreover, these findings predict that at synapses, modulation of N-channel activity by M\textsubscript{1}Rs or NK-1Rs will fluctuate between enhancement and inhibition based on the presence of palmitoylated Ca\textsubscript{V}2.2a.

INTRODUCTION

All neural function results from a series of electrical and chemical signals. The two realms of signaling are often bridged within neurons by voltage-gated Ca\textsuperscript{2+} channels, such as N channels (West et al., 2001). With depolarization of postsynaptic sites, N-channel activity (N current) triggers biochemical changes, including modulation of certain ion channels (Wigsgird and Dryer, 1994), enzyme activation (Rittenhouse and Zigmund, 1999), and gene transcription (Brosenitsch and Katz, 2001; West et al., 2001; Zhao et al., 2007). In turn, G\textsubscript{q} protein-coupled receptors (PCRs) converge on several signal transduction cascades to modulate the N channel’s response to changing membrane potential. Some of these pathways are well described, so both signaling molecules and sites of modulation on N channels are known (Suh and Hille, 2005).

A notable exception is the M\textsubscript{1} receptor (M\textsubscript{1}R), a G\textsubscript{q}PCR that modulates postsynaptic N current by an incompletely described signaling cascade referred to as the slow pathway (Beech et al., 1992; Mathie et al., 1992). Other transmitters such as substance P (SP), the natural ligand for the neurokinin-1 receptor (NK-1R), also modulate N current by a slow pathway (Shapiro and Hille, 1993; Kammermeier et al., 2000) that couples to G\textsubscript{q} (Macdonald et al., 1996), suggesting that multiple G\textsubscript{q}PCRs converge on this pathway (Suh and Hille, 2005). Recent studies propose that a reduction in phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)\textsubscript{P}_2) levels during G\textsubscript{q}PCR stimulation suffices for inhibition (Wu et al., 2002; Gamper et al., 2004; Michailidis et al., 2009).
2007). In this model, channels are available to open when PtdIns(4,5)P$_2$ associates with them. During muscarinic stimulation, PtdIns(4,5)P$_2$ is depleted from the membrane, creating a concentration gradient that favors PtdIns(4,5)P$_2$ dissociating and diffusing away from channels. This model appeals to many in part because PtdIns(4,5)P$_2$ dissociation and depletion have been established as the mechanism that inhibits M current, a voltage-gated K$^+$ current (Suh and Hille, 2002, 2007; Ford et al., 2003; Suh et al., 2006).

However, our previous studies with superior cervical ganglion (SCG) neurons indicate that modulation of native N current by M$_1$Rs requires events downstream of PtdIns(4,5)P$_2$ hydrolysis (Liu and Rittenhouse, 2003a; Liu et al., 2004, 2008). We found that the muscarinic agonist oxotremorine-M (Oxo-M) and arachidonic acid (AA) elicit the same distinct pattern of modulation (Liu et al., 2001; Liu and Rittenhouse, 2003a), not only inhibiting N current at positive test potentials but also enhancing N current at negative test potentials (Fig. 1 A). Enhancement and inhibition by AA occur at distinct sites and exhibit different biophysical characteristics (Barrett et al., 2001; Liu et al., 2001). Moreover, M$_1$R stimulation releases AA in central and SCG neurons (Tencé et al., 1994; Liu et al., 2006). In contrast, only minimal fatty acid release occurs after muscarinic stimulation in SCG neurons lacking group IVa PLA$_2$ (Liu et al., 2006). Consistent with the need for PtdIns(4,5)P$_2$ metabolites, inhibiting AA release from phospholipids by antagonizing PLA$_2$ activity or including BSA, a scavenger of AA (Spector, 1975), in the bath solution minimizes N-current modulation by Oxo-M (Liu and Rittenhouse, 2003a; Liu et al., 2003, 2004), suggesting that AA may be a second messenger of the slow pathway.

At present, no binding site for either PtdIns(4,5)P$_2$ or AA on N channels has been determined. Moreover, whether the sites that confer AA sensitivity reside in the same channel or whether two distinct channel populations mediate enhancement and inhibition remains untested. Therefore, we attempted to use the power of recombinant M$_1$Rs and N channels. N channels are multimeric complexes defined by a large pore-forming subunit (Ca$_V$2.2) that associates with ancillary $\alpha_2\delta$ and Ca$_V$3$\beta$ subunits (Catterall, 2000). We made the serendipitous discovery that enhancement and inhibition separate without mutagenesis simply by expressing different wild-type Ca$_V$3$\beta$s.

**MATERIALS AND METHODS**

**Transfection of HEK-M1 cells**

HEK-293 cells with a stably transfected M$_1$R (HEK-M1; Peralta et al., 1988), a gift from E. Liman (University of Southern California, Los Angeles, CA), were cultured in Dulbecco’s modified Eagle’s medium/F12 (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), 1% G418 (geneticin-selective antibiotic, nonsterile; Invitrogen), and 1% HT supplement (Invitrogen). Cells were transfected into 12-well plates for transfection. Recombinant cells at 50–80% confluence were transfected for 1 h with Ca$_V$2.2 e($\Delta$24a, +31a, +37b), the N-channel variant found in SCG neurons (Lin et al., 1997); $\alpha_2\delta$-1, which is also expressed in SCG (Lin et al., 2004); and various Ca$_V$3$\beta$s at a 1:1:1 or 1:1:2 molar ratio along with enhanced green fluorescent protein (eGFP) at $\sim$10% of the total DNA using Lipofectamine and PLUS reagent (Invitrogen) as per the manufacturer’s instructions. Where noted, the transfection mixture contained expression plasmids encoding 0.028 µg/well NK-1R (UMR cDNA Resource Center, University of Missouri, Rolla, MO). 500–1,000 ng total DNA was transfected per well. 24–48 h after transfection, cells were replated on poly-L-lysine-coated coverslips and allowed to settle for at least 1 h before recording. tsA 201 cells stably transfected with Ca$_V$2.2, $\alpha_2\delta_1$, and Ca$_V$3$\beta$3 were provided by D. Lipscombe (Brown University, Providence, RI). These cells were transfected with 500–1,000 ng per well of the M$_1$R (a gift from N. Nathanson, University of Washington, Seattle, WA) along with eGFP at 10% of the total DNA and processed as described above for current recordings.

Ca$_V$3$\beta$1b (GenBank/EMBL/DDB accession no. X61394), Ca$_V$3$\beta$2a (GenBank accession no. M80545), and Ca$_V$3$\beta$4 (GenBank accession no. L02515) were provided by E. Perez-Reyes (University of Virginia, Charlottesville, VA); Ca$_V$2.2 (GenBank accession no. AF055477), $\alpha_2\delta$-1 (GenBank accession no. AF286488), and Ca$_V$3$\beta$3 (GenBank accession no. M88751) were provided by D. Lipscombe. The Ca$_V$3$\beta$2a(C3,4S) mutant was provided by A. Fox (University of Chicago, Chicago, IL); Ca$_V$3$\beta$2a$\beta$3 and Ca$_V$3$\beta$2a$\beta$1b chimeras were provided by R. Ten Eick (Northwestern University, Evanston, IL). The mutant and chimeric Ca$_V$3$\beta$s were originally made in M. Hosey’s laboratory (Northwestern University; Chien et al., 1996, 1998; Chien and Hosey, 1998). Recordings from untransfected HEK-M1 cells yielded whole-cell currents of 9.0 ± 1.3 pA in 20 mM Ba$^+$ (Roberts-Crowley, M., personal communication). To avoid spurious results from endogenous currents, any transfected cells having less than $\sim$150 pA were discarded.

**Preparation of neonatal rat SCG neurons for electrophysiology**

Dissociated sympathetic neurons were obtained from SCG of 1–4-d-old Sprague-Dawley rats (Charles River) according to the methods of Liu and co-workers (Liu et al., 2001; Liu and Rittenhouse, 2003a). To isolate the actions of the slow pathway on N-type current, cells were incubated for at least 5 h with 500 ng/ml pertussis toxin (Biological Laboratories, Inc.) before initiating whole-cell recording experiments (Bernheim et al., 1992). Also, bath solutions included 1 µM of the L-type Ca$_V$2.2 channel antagonist nimodipine to minimize the small amount of L-type current present in SCG neurons. Cells were used within 12 h to avoid recording from cells with processes.

**Electrophysiology**

Ba$^{2+}$ currents were recorded at room temperature (20–24°C) using the whole-cell configuration of a model 3900a (Dagan), Axon 200A, or Axon 200B (MDS Analytical Technologies) patch-clamp amplifier using methods that were described previously (Barrett et al., 2001; Liu et al., 2001). Currents were filtered at 5 kHz using the amplifier’s four-pole low-pass Bessel filter and were digitized at 20 kHz with a micro1401 interface (Cambridge Electronic Design [CED]). Data were collected using Patch 6.4 or Signal 2.15 software suites (CED) and stored on a personal computer. Before analysis, capacitive and leak currents were subtracted using a scaled-up current elicited with a test pulse to $\sim$100 mV. Pipette resistance ranged from 2.5 to 5 M$\Omega$. Ba$^{2+}$ currents were elicited every 4 s by stepping from −90 to 0 mV for 100 ms unless...
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Figure 1.

M1R-induced inhibition of recombinant N current is blocked by a PLA2 antagonist. HEK-M1 cells were transiently transfected with N-channel subunits CaV2.2, \( \alpha_2\delta -1 \), and CaV3.2.2.2.

(A) Working model of the slow pathway in which AA release, catalyzed by PLA2, is a necessary component to elicit N-current modulation. AA enhances current (green arrow) by acting at a distinct site that may be extracellular or in the outer leaflet of the membrane (Barrett et al., 2001). AA inhibits N current by acting at an intracellular site or a site within the inner leaflet (Barrett et al., 2001; Liu et al., 2001). (B) Summary of the percent change in mean current amplitude in tsA 201 cells after 1-min exposure to 10 µM Oxo-M or in HEK-M1 cells in the presence or absence of 10 µM OPC, a PLA2 antagonist. Error bars represent SEM. (C) Time course of peak inward current using 5 mM Ba2+ as charge carrier before, during, and after bath application of 10 µM Oxo-M. (D) Representative whole-cell current traces selected from the respective time course before (black) and 1 min after (red) Oxo-M application. Currents were elicited every 4 s by stepping to a test potential of 0 mV for 100 ms. (E and F) Modulation of N current from tsA 201 cells stably transfected with CaV2.2, \( \alpha_2\delta -1 \), and CaV3.2. Modulation of N current by M1R stimulation illustrated in a time course (G) and current traces (H). (I and J) N-current modulation from cells expressing HEK-M1 cells transfected as for C. Cells were exposed to OPC for at least 3 min before application of Oxo-M. Bars, 0.4 nA.

**Pharmacological agents**

Oxo-M (Tocris Biosciences, Inc.) was prepared as a stock solution in double-distilled water and was diluted 1,000 times with bath solution. SP was prepared as a stock solution in 0.05 M acetic acid and diluted at least 2,000 times with bath solution. AA (Nu-Chek Prep), oleyloxyethyl phosphorylcholine (OPC; EMD), and nifedipine were prepared as stock solutions made up in 100% ethanol and diluted 1,000 times with bath solution. All chemicals were obtained from Sigma-Aldrich except where noted.

**Data analysis**

The Patch 6.4 and Signal 2.15 software were used to measure peak inward current of whole-cell traces. A trough-seeking function was used to determine peak current where indicated. Data were further analyzed using Excel (Microsoft) and Origin 7.0 (OriginLab). Percent inhibition was calculated as \([I - I']/I\] × 100, where I is the control current amplitude determined by a mean of five whole-cell current measurements before application of a particular agent and I’ is the mean of five current measurements at the time specified for the determination. Conductance was calculated from a modified Ohm’s law equation, \( G = I/(V_m - V_{rev}) \), where I is the peak current at each test potential, \( V_m \) is the test potential, and \( V_{rev} \) is the apparent reversal potential. Relative conductance curves, (\( G/G_{max} \)) – voltage (\( V_m \)), were plotted for whole-cell currents before and after AA. Data were curve fit using the Boltzmann function equation in Origin 7.0, yielding a curve fitting the equation \( G/G_{max} = G_{max} + (G_{min} - G_{max})/(1 + \exp[(V_m - V_{1/2})/k]) \), where \( G_{max} \) is the maximal conductance, \( G_{min} \) is the minimal conductance, \( G_m \) is the test potential of 0 mV for 100 ms. (E and F) Modulation of N current from tsA 201 cells stably transfected with CaV2.2, \( \alpha_2\delta -1 \), and CaV3. Modulation of N current by M1R stimulation illustrated in a time course (G) and current traces (H). (I and J) N-current modulation from cells expressing HEK-M1 cells transfected as for C. Cells were exposed to OPC for at least 3 min before application of Oxo-M. Bars, 0.4 nA.
potential, \( V_{m}/2 \) is the voltage at half-maximal conductance, and \( k \) is the slope factor.

**Immunocytochemistry of CaV1\( \beta \) subunits**

Adult SCG neurons were plated on chamber slides (Nagle Nune International), treated with poly-L-lysine, and cultured overnight. Cells were washed with PBS twice for 5 min and fixed with 100% acetone for 10 min. The fixative was washed away with PBS at room temperature three times for 5 min. Cells were then exposed to PBS containing 10% normal goat serum for 60 min at room temperature followed by incubation with primary antibody for 60 min at room temperature. Mouse anti-CaV1\( \beta \) (NeuroMab), rabbit anti-CaV1\( \beta \) (Sigma-Aldrich), and mouse anti-Cav5 (NeuroMab) were diluted 1:1,000 in Antibody Diluent (Dako). Thereafter, cells were washed with PBS three times for 5 min and were incubated for 60 min in the dark at room temperature with Alexa Fluor 488 anti-mouse antibody or Alexa Fluor 488 anti-rabbit antibody (Invitrogen) diluted 1:200 in Antibody Diluent. After incubation with secondary antibodies, cells were washed with PBS three times for 5 min. A second fixation was performed. Slides were then washed with distilled water and covered with the aqueous mounting medium Prolong Gold Antifade reagent (Invitrogen). Images of immunofluorescence were obtained at room temperature using a custom-built, video-rate confocal microscope (Sanderson and Parker, 2003) with a 40× objective lens. An excitation wavelength of 488 nm was used, and emission spectra were collected with long-pass filters (OGSIS) at 515 nm (Perez and Sanderson, 2005).

**RT-PCR of CaV\( \beta \) subunits**

Homogenized tissue samples were lysed in 0.8 ml TRIZOL reagent (Invitrogen), and DNA was removed from RNA using phenol-chloroform phase separation. Next, RNA was precipitated using isopropyl alcohol and washed with 75% ethanol. After drying, the RNA pellet was dissolved in RNase-free water. Subsequently, reverse transcription was performed using 1.0 µl of 10× buffer reverse transcriptase, 1.0 µl deoxycytidinenucleotide triphosphate (dNTP) mix (5 mM each of dNTP), 1.0 µl oligo-dT primer (0.5 mg/ml), 0.125 µl RNase inhibitor (40 U/µl; Promega), 0.5 µl Omniscript Reverse transcriptase, and RNase-free water to make up a total volume of 10 µl (all reagents were obtained from Qiagen unless otherwise noted). The mixture was incubated at 37°C for 1 h and heated at 93°C for 5 min followed by cooling on ice to rapidly inactivate the transcriptase. Aliquots of reverse transcription PCR product were amplified by PCR using the primers CaV1\( \beta \)2a (5'-ATAACCACAGAGAGAGGAGGCACA-3' and 5'-TATACATCCCTGTTCCACTCGCCA-3'; 368 bp; Lin et al., 1996). Reaction mixes for PCR contained 2 µl of template cDNA, 2.5 µl of 10× buffer, 0.5 µl dNTP mix, and 2 µl each of forward and reverse primer and were made up in distilled water to a final volume of 25 µl. The protocol for amplification was 94°C for 4 min followed by 33 cycles of 94°C for 45 s, 60°C for 45 s, 72°C for 2 min, and holding at 72°C for 10 min for last extension. PCR products were run on 1.8% agarose gel stained with ethidium bromide along with a 1,000-bp DNA ladder (Fermentas Life Sciences).

**Statistical analysis**

Summary data are presented as means ± SEM. A two-tailed paired \( t \) test was used to determine differences between control and agonist. For comparisons of two different groups, data were analyzed by a two-tailed Student’s \( t \) test for two means. For multiple comparisons, statistical significance was determined using Origin 7.0 by a one-way ANOVA followed by a Tukey multiple comparison posthoc test. Statistical significance was set at \( P \leq 0.05 \).

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**Online supplemental material**

Fig. S1 shows that exogenous application of AA inhibits N-type current in SCG neurons. The L-type channel agonist FPL 64176 was used to distinguish effects of AA on N current from L current. The AA analogue ETYA (5,8,11,14-eicosatetraynoic acid) was used to block metabolism of AA. Fig. S2 demonstrates that the enhancing and inhibiting effects of AA are reversible using an application of fatty acid–free BSA subsequent to AA application. Fig. S3 shows that in HEK-M1 cells transfected with CaV1\( \beta \)2, \( \alpha \)6-1, CaV1\( \beta \)2a, and CaV1\( \beta \)3, both enhancement and inhibition of N current occurs during application of AA. Online supplemental material is available at http://www.jgp.org/cgi/content/full/jgp.200910203/DC1.

**RESULTS**

To test whether a homogeneous population of N channels recapitulates both enhancement and inhibition, N currents were screened for modulation by Oxo-M. Recombinant currents were evoked from HEK-M1 cells transiently transfected with N-channel subunits CaV1\( \beta \)2, \( \alpha \)6-1, and CaV1\( \beta \)3, which most commonly associates with CaV1\( \beta \)2 (Witcher et al., 1993). Oxo-M rapidly inhibited peak inward current, reaching a stable inhibition within 90 s (Fig. 1, B–D). Because N-current enhancement is voltage dependent (Liu and Rittenhouse, 2003a), we compared current–voltage (I–V) plots measured before and after Oxo-M, expecting to observe current enhancement at negative test potentials. However, Oxo-M inhibited current at virtually all voltages (Fig. 2 C). tsA 201 cells stably transfected with the same subunits exhibited a similar profile of inhibition after exposure to Oxo-M (Fig. 1, B, E, and F), ruling out a system-specific effect.

CaV1\( \beta \) subunit controls N-current modulation by \( \alpha \)6PCRs

Lack of enhancement at any potential indicated the recombinant system was fundamentally different from N-current modulation observed in SCG neurons. In addition to incomplete recapitulation of \( \alpha \)6PCR modulation, recombinant N current exhibited robust, fast inactivation (Fig. 1, D and F, and Fig. 2 C) as previously observed with channels containing CaV1\( \beta \)3 (O Alecse et al., 1994). In contrast, native N current in SCG neurons exhibits kinetics with little inactivation (Plummer et al., 1989), similar to recombinant N current from channels containing CaV1\( \beta \)2a (Hurley et al., 2000; Yasuda et al., 2004). Moreover, AA robustly inhibits noninactivating current in SCG neurons evoked with a test pulse duration ranging from 20 (Fig. S1) to 700 ms (Liu and Rittenhouse, 2000; Liu et al., 2001). Because SCG neurons express CaV1\( \beta \)2 mRNA (Lin et al., 1996), we hypothesized that the majority of N current arises from CaV1\( \beta \)2a-containing channels. If true, these channels might exhibit both inhibition and enhancement. We found that Oxo-M rapidly inhibited N current from cells expressing CaV1\( \beta \)2a. However, unlike CaV1\( \beta \)3, initial inhibition gave way to stable enhancement (Fig. 1, G and H). Moreover, comparison of I–V plots revealed...
CA\(\beta\) determines N-current modulation by GqPCRs and AA. (A–D) HEK-M1 cells were transiently transfected with CA2.2, \(\alpha_2\delta-1\), and various CA\(\beta\) subunits. Currents, measured in 5 mM [Ba\(^{2+}\)], were elicited every 4 s by stepping from \(-90\) to 0 mV for 100 ms unless otherwise noted. Representative whole-cell current traces were always taken before (black) and 3 min after (red) drug application. Averaged normalized I-V plots were taken before (black closed circles) and 90 s after (red open circles) agonist application. \(n = 3–6\) cells per group. For cells expressing CA\(\beta_{2a}\), 20 mM [Ba\(^{2+}\)] was used as charge carrier to improve the signal to noise ratio. The test potential was adjusted to 10 mV to correct for the shift in peak inward current. (E) Histogram summarizing N-current modulation after 3 min of 10 \(\mu\)M Oxo-M (gray bars) and 5 nM SP (black bars). Percent change in current amplitude was highly significant between CA\(\beta_{2a}\) and CA\(\beta_{1b}\), CA\(\beta_{3}\), or CA\(\beta_{4}\) irrespective of whether Oxo-M or SP was applied (\***, P < 0.005; one-way ANOVA). (F–J) Modulation of N current by 10 \(\mu\)M AA shown as current traces and averaged I-V plots (\(n = 4–5\) cells per group). (J) Histogram summarizing N-current modulation after 3 min of AA (\***, P < 0.005; one-way ANOVA). Error bars represent SEM. Bars, 0.4 nA.
Expression of multiple $\text{Ca}_2\beta$ isoforms produces heterogeneous N currents

Our findings yield a possible explanation for why SCG neurons exhibit both enhancement and inhibition of whole-cell N current (Barrett et al., 2001; Liu and Rittenhouse, 2003a; Liu et al., 2004). N current’s diverse biophysical properties in different neurons have been attributed to $\text{Ca}_2.2$ coexpression with different $\text{Ca}_2\beta$ isoforms within individual neurons (Scott et al., 1996). Similarly, SCG neurons may exhibit both forms of modulation because $\text{Ca}_2.2$ coassembles with different $\text{Ca}_2\beta$s. If this hypothesis is correct, the pattern of modulation observed in SCG neurons should recapitulate in HEK-M1 cells transfected with multiple $\text{Ca}_2\beta$s. When cells were transfected with $\text{Ca}_2\beta_2a$ and $\text{Ca}_2\beta_3$, the I-V plots recapitulated I-V plots from SCG neurons (Liu and Rittenhouse, 2003a), with enhancement occurring at negative potentials and inhibition at positive potentials after exogenously applied AA (Fig. S3). However, unlike activity from SCG neurons (Liu et al., 2001; Liu and Rittenhouse, 2003a), the currents rapidly inactivated. It has been reported that SCG neurons from 4-d-old rat pups express $\text{Ca}_2\beta_2a$, $\text{Ca}_2\beta_3$, and $\text{Ca}_2\beta_4$ mRNA with trace amounts of $\text{Ca}_2\beta_1b$ mRNA (Lin et al., 1996). We verified the presence of $\text{Ca}_2\beta_2$, $\text{Ca}_2\beta_3$, and $\text{Ca}_2\beta_4$ protein in freshly dissociated SCG neurons by immunocytochemistry (Fig. 4 A). Therefore, we retested for the modulatory response in HEK-M1 cells transfected with varying ratios of $\text{Ca}_2\beta_2a$, $\text{Ca}_2\beta_3$, and $\text{Ca}_2\beta_4$-transformed plasmid. Transfection with a mixture of cDNAs in the molar ratio of 10:1:1 for $\text{Ca}_2\beta_2a/\text{Ca}_2\beta_3/\text{Ca}_2\beta_4$ in a HEK-M1 cell closely recapitulated the N-current kinetics we had previously observed in SCG neurons (Barrett and Rittenhouse, 2000; Liu and Rittenhouse, 2003a). Application of 10 µM Oxo-M resulted in significant inhibition of currents elicited at test potentials 10–50 mV, with no significant inhibition at lower potentials (Fig. 4 B). To ensure the transfection mixture is an accurate recapitulation of the SCG neuron, we harvested fresh SCG neurons from neonatal rat pups and tested them using the same experimental conditions as we used for the HEK-M1 cells. We found that application of 10 µM Oxo-M also resulted in significant inhibition of native currents elicited at test potentials 10–50 mV, with no significant change at lower potentials (Fig. 4 C). These findings suggest that the N current observed in SCG neurons results from heterogeneous expression of different $\text{Ca}_2\beta$ isoforms with $\text{Ca}_2.2$.

The antibody used in the immunocytochemistry experiments recognizes the peptide sequence RSPKP-SANSVTSPHSKE (NeuroMab data sheet N8b_1). This sequence exists in $\text{Ca}_2\beta_2a$, $\text{Ca}_2\beta_2b$, $\text{Ca}_2\beta_2c$, $\text{Ca}_2\beta_2d$, and $\text{Ca}_2\beta_2e$ but not $\text{Ca}_2\beta_2f$, $\text{Ca}_2\beta_2g$, or $\text{Ca}_2\beta_2h$. Because the presence of $\text{Ca}_2\beta_2a$ variant has never been confirmed in rat neonatal SCG neurons, we looked for its presence by RT-PCR using primers designed
corroborate our assertion that AA mediates N-current modulation by the slow pathway (Liu and Rittenhouse, 2003a,b; Liu et al., 2004).

As a final test of AA’s role in the slow pathway, we antagonized endogenous AA’s release from membrane phospholipids using the PLA2 antagonist OPC. Exposure to Oxo-M for 60 s significantly inhibited N current from $\text{Ca}_2\beta_3$-containing channels ($P < 0.05$; $n = 16$). In contrast, OPC eliminated significant current inhibition by Oxo-M ($P > 0.05$; $n = 7$; Fig. 1, B, I, and J). A similar profile was observed with SP (not depicted). The striking recapitulation by AA of $\text{G}_q\text{PCR}$-induced N-current enhancement and inhibition, coupled with the loss of modulation when antagonizing AA release or sequestering free AA with BSA, advance our previous hypothesis that PLA2 and AA participate in the slow pathway (Liu and Rittenhouse, 2003a,b; Liu et al., 2004).

Figure 3.  NK-1R stimulation elicits a similar profile of N-current modulation as $M_1$s. (A and B) HEK-M1 cells were transiently transfected with the NK-1R along with $\text{Ca}_2.2$, $\alpha_2\delta-1$, and either $\text{Ca}_2\beta_2a$ (A) or $\text{Ca}_2\beta_3$ (B). Using 5 nM SP as the agonist, current modulation is shown in a time course of peak current (left), representative whole-cell current traces (middle), and averaged I-V plots (right; $n = 4$). Bars, 0.4 nA. (C) Summary of the modulatory effects of SP on currents from cells expressing either $\text{Ca}_2\beta_2a$ or $\text{Ca}_2\beta_3$ ($***$, $P < 0.005$; one-way ANOVA). No significant difference in current inhibition was observed from cells expressing $\text{Ca}_2\beta_3$ with 5 and 250 nM SP ($P > 0.26$). Error bars represent SEM.
transiently transfected with a 12:12:10:1:1 ratio of CaV2.2/tative current traces and mean I-V plots taken from HEK-M1 cells responding view shown in bright field. Bar, 10 µm. (B) Represen-

currents recorded using 20 mM Ba2+. *, P < 0.05; paired

n = 6 cells). All currents for the figure were

Oxo-M application (Fig. 5, A and B), AA induces initial N-current enhancement, which is sustained with CaV2.2a but for CaV3β3 is followed by a more slowly progressing inhibition. This observation supports the model. Thus, we postulated that every

CaV2.2 exhibits current enhancement that becomes masked by subsequent, more dominant inhibition.

Because our previous work indicated that enhancement by AA arises from increased voltage sensitivity (Barrett et al., 2001), we compared normalized conductance–voltage (G-V) curves to determine whether increased voltage sensitivity occurs independently of CaV2.2a expression. Both CaV2.2a- and CaV3-containing cells exhibited a negative shift in conductance in response to AA (Fig. 5, C and D), demonstrating that AA increases N-channel voltage sensitivity independently of CaV3β subunit expression. These data suggest that CaV2.2a’s unique ability to attenuate inhibition reveals sustained enhancement. In contrast, inhibition results from increased stability of a closed state (Liu and Rittenhouse, 2000), essentially reducing the number of channels available to open. This type of inhibition should dominate an increase in voltage sensitivity; if channels will not open at any voltage, increasing their voltage sensitivity will have little effect on currents. Thus, by blocking a dominating inhibition, CaV2.2a unmasks latent enhancement.

Loss of CaV2.2a palmitoylation restores partial inhibition of N current by Oxo-M or AA

Having established that CaV2.2a uniquely blocks N-current inhibition by GPCR stimulation or exogenous AA, we investigated the structural aspects of CaV2.2a to determine its mechanism of action. A conspicuous feature of CaV2.2a is its unique palmitoylation of two cysteine residues near the N terminus (Chien et al., 1996). Our
Caβ2a toggles N-current modulation by GqPCRs

The observation that palmitoylated Caβ2a interferes with a fatty acid–mediated inhibition of CaV2.2 raises the possibility of direct antagonism between palmitic acid and AA (Fig. 5E). Therefore, we tested a depalmitoylated mutant to determine whether Caβ2a must be palmitoylated to minimize inhibition and reveal enhancement. Currents from channels containing depalmitoylated Caβ2a (Caβ2a[C3,4S]; Chien et al., 1996) exhibited an

Figure 5. Caβ2a blocks N-current inhibition, revealing latent enhancement. (A and B) Time courses of cells expressing either Caβ2a (A) or Caβ3 (B) exposed to AA both exhibit enhancement during the initial phase of time course. However, when Caβ3 is present, inhibition eventually dominates, indicating that both inhibitory and enhancement sites are available for AA to bind. When Caβ2a is present, inhibition no longer occurs, suggesting the palmitoylated Caβ2a occupies the inhibitory site without conferring inhibition. (C and D) Normalized conductance–voltage plots generated from I-V data show a negative shift in G/Gmax for cells expressing either Caβ2a (C) or Caβ3 (D), indicating that an increased sensitivity to voltage is masked by a dominating inhibition in cells expressing Caβ3. (E) Schematic representation of working model. Upon GqPCR stimulation, the released AA binds to both inhibitory and enhancement sites on the channel. When Caβ3 is present, both inhibitory and enhancement sites are available for AA to bind. However, because inhibition dominates, the resultant modulation observed is current inhibition. When Caβ2a is present, inhibition no longer occurs because the palmitoylated Caβ2a occupies the inhibitory site without conferring inhibition. With inhibition antagonized, sustained current enhancement is observed.

Figure 6. Palmitoylation determines which form of modulation is observed. (A–F) Using 20 mM Ba2+, modulation by Oxo-M or AA of cells expressing mutant Caβ2a(C3,4S) (A and B), Caβ2aβ1b (C and D), or Caβ2aβ3 (E and F) is shown in current traces and I-V plots. Bars, 0.4 nA. (G) Summary of the modulatory effects on currents after application of Oxo-M or AA for 3 min (n = 4–7). Error bars represent SEM. (H) Schematic representation of working model.
initial transient inhibition after Oxo-M that relaxed, yielding a small but insignificant inhibition (Fig. 6, A and G). AA initially enhanced current amplitude, followed by significant inhibition (Fig. 6, B and G). The loss of enhancement and appearance of inhibition, although differing in magnitude with Oxo-M or AA, support the idea that palmitoylation blocks inhibition.

If palmitoylation is necessary for antagonizing inhibition, palmitoylating another CaV\(\beta\) should convert current inhibition to enhancement. Therefore, we tested a chimera in which the variable N terminus of CaV\(\beta\)1b was replaced with the palmitoylated 16-amino acid N terminus of CaV\(\beta\)2a (CaV\(\beta\)2a-b1b; Chien et al., 1998).

In cells expressing CaV\(\beta\)2a-b1b, application of Oxo-M enhanced current with no inhibition (Fig. 6 G). Moreover, AA no longer inhibited these currents significantly (Fig. 6 G) compared with CaV\(\beta\)1b (\(n = 4\); Fig. 2 J). The difference in modulation by AA and Oxo-M may be attributed to an exaggerated response to exogenous application of AA compared with the response to physiological concentrations of AA released upon M1R stimulation. I-V plots after either Oxo-M or AA exhibited enhancement of current at negative test potentials and no inhibition at positive test potentials (Fig. 6, C and D), suggesting that the palmitoylated N terminus is both necessary and sufficient to block inhibition.

However, a second chimera in which the N terminus of CaV\(\beta\)3 was replaced with the N terminus of CaV\(\beta\)2a (CaV\(\beta\)2a-b3; Chien et al., 1998) did not block inhibition as effectively. In cells expressing CaV\(\beta\)2a-b3, inhibition by Oxo-M was diminished compared with wild-type CaV\(\beta\)3, whereas AA reversibly inhibited currents similarly to wild-type levels (Fig. 6, E–G). I-V plots taken before and 90 s after application of Oxo-M or AA exhibited inhibition at positive test potentials (Fig. 6, E and F). These results indicate that addition of CaV\(\beta\)2a's palmitoylated N terminus to CaV\(\beta\)3 only partially reproduced the stable enhancement by Oxo-M or AA normally observed with wild-type CaV\(\beta\)2a. Because CaV\(\beta\)2a protein shares highest sequence homology to CaV\(\beta\)1b (Birnbaumer et al., 1998), the simplest interpretation for the varied results with the different chimeras is that CaV\(\beta\)2a positions the palmitoylated N terminus so that it minimizes N-current inhibition similarly to wild-type CaV\(\beta\)2a. In contrast, the more divergent CaV\(\beta\)2a-b3 positions the palmitoylated N terminus in a different position relative to CaV\(\beta\)2.2 where it is less effective in minimizing inhibition (Fig. 6 H). In support of this notion, CaV\(\beta\)2a-b3-containing cells exhibited varied kinetics, from nonactivating currents resembling CaV\(\beta\)2a-containing channel activity (Fig. 2 B) to rapidly inactivating currents resembling depalmitoylated CaV\(\beta\)2a-containing channel activity (Fig. 6, A and B). Moreover, the kinetics varied within individual recordings and from cell to cell from a rapidly inactivating to a nonactivating kinetic profile (unpublished data). The unstable inactivation kinetics...
suggests that CaVβ2αβ3 cannot dock properly to CaV2.2, thus destabilizing and changing the location of the chimera’s palmitoyl groups.

**Free palmitic acid minimizes N-current inhibition**

If our interpretation that the palmitoyl groups antagonize inhibition is correct, freeing the palmitoyl groups from protein constraints might allow the fatty acids to find and assume their optimal position for blocking inhibition (Fig. 7 A). Alternatively, if the palmitic acids do not interact directly with CaV2.2, introducing free palmitic acid should not alter N-channel modulation by agonist. We tested this prediction first by preincubating cells expressing NK-1Rs and CaVβ3-containing channels with free palmitic acid for at least 8 min. Under these conditions, the sustained inhibition normally observed with SP (Fig. 7, B and C) was replaced by an initial enhancement that relaxed, resulting in no significant change (P > 0.24) in current amplitude over time (Fig. 7, B and D). Moreover, compared with control conditions, little inhibition by SP was detected in I-V plots from cells preincubated with 10 µM palmitic acid (Fig. 7, C and D). To ensure the block of inhibition was not specific to NK-1Rs, we tested N-current modulation of CaVβ3-containing channels in HEK-M1 cells by Oxo-M after a 10-min preincubation with 10 µM palmitic acid. As with SP, free palmitic acid prevented the sustained inhibition observed in its absence, as demonstrated by time courses and I-V plots (compare Fig. 7, B and E with Fig. 1, B–F and Fig. 2, C and E). Bath application of palmitic acid for 8 min did not significantly affect N current (not depicted) when compared with currents recorded under control conditions (Fig. 7 C), indicating that palmitic acid itself has no modulatory effect. These findings demonstrate that exogenous application of free palmitic acid suffices to block current inhibition of CaVβ3-containing N channels. More importantly, these results indicate palmitic acid is both necessary and sufficient to antagonize N-current inhibition during GqPCR stimulation (Fig. 8).

**DISCUSSION**

In this study, we used a recombinant system to investigate the enhancement and inhibition of N current that occurs during M1R stimulation and AA application. In so doing, we made the discovery that the N-channel’s β subunit directs opposite effects of N-current modulation. Specifically, M1R stimulation or exogenous AA uniquely enhanced whole-cell N current from cells expressing CaVβ2α but inhibited N current from cells expressing CaVβ1b, CaVβ3, or CaVβ4. This finding allowed us to further examine the modulation of N current and reach several additional conclusions. First, the striking recapitulation of M1R-induced modulation by AA, coupled with the loss of modulation when AA release by OPC is blocked, is strong evidence that AA is an integral component of the slow pathway. Second, NK-1R stimulation exhibited a similar dual effect, indicating this unique profile of modulation may be conserved among GqPCRs. Third, cells expressing multiple forms of CaVβ subunits matched the modulatory profile of SCG neurons under the same recording conditions, implying that the characteristic pattern of modulation observed in SCG neurons results from CaVβ2.2 coupling with different CaVβ isoforms. This finding provides an explanation of how stimulation of M1Rs or exogenous AA can elicit both enhancement and inhibition.
of N current in individual sympathetic neurons (Liu and Rittenhouse, 2003a). Fourth, using mutated and chimeric CaV₂β constructs, we demonstrated that palmitoylation of CaV₂β₂a is a key feature in its capacity to toggle modulation from inhibition to enhancement. Finally, exogenous application of palmitic acid prevented the characteristic N-current modulation by GαPCRs, suggesting that CaV₂β₂a’s palmitoyl groups interfere with a mechanism or mechanisms within the slow pathway.

Increased free AA appears necessary for N-current inhibition by the slow pathway

It is well documented that PtdIns(4,5)P₂ breakdown by PLC is necessary for slow pathway inhibition (Wu et al., 2002; Liu and Rittenhouse, 2003a; Gamper et al., 2004; Liu et al., 2004, 2006), however, whether its breakdown is sufficient for N-current inhibition remains controversial (Wu et al., 2002; Gamper et al., 2004; Suh and Hille, 2005). Our data indicate that fatty acid liberation is both necessary and sufficient to elicit inhibition of CaV₂.2 (Barrett et al., 2001; Liu and Rittenhouse, 2003a; Liu et al., 2004, 2007). To demonstrate a role for free AA in N-current inhibition, we used pharmacological agents, the AA scavenger BSA, PLα₂ₐ antibodies dialyzed internally, and neurons harvested from PLα₂⁻/⁻ knockout mice (Liu and Rittenhouse, 2003a; Liu et al., 2004, 2006). Here we have taken a molecular approach in which expression of palmitoylated CaV₂β2a provides a highly specific agent to block inhibition by AA, whereas nonpalmitoylated wild-type CaV₂β subunits conveniently act as negative controls. It must be stressed that none of the findings reported here minimizes PtdIns(4,5)P₂’s role but rather documents a requirement for AA in N-current inhibition.

A resolution to the paradox would entail a role for both PtdIns(4,5)P₂ and AA acting either directly or indirectly on the channel. A model for PtdIns(4,5)P₂ and AA opposing each other in modulation of K⁺ channels has been published (Oliver et al., 2004) in which PtdIns(4,5)P₂ and AA act at different sites of the channel. Direct competition is possible because AA is an integral piece of the PtdIns(4,5)P₂ molecule, normally residing in the sn-2 position, and thus could compete for binding to CaV₂.2. Although indirect actions of PtdIns(4,5)P₂ and AA must be considered, as both are bioactive molecules with multiple downstream effects, no requirement for downstream enzymes such as phosphatases or kinases has been found. Moreover, antagonizing enzymes that further metabolize AA have no effect on modulation (Liu et al., 2001; Liu and Rittenhouse, 2003a). Nevertheless, increased levels of PtdIns(4,5)P₂ increase N-channel availability to open, whereas increased levels of AA stabilize N channels in closed and/or inactivated states (Liu and Rittenhouse, 2000; Gamper et al., 2004). These opposing effects suggest competition.

A working model for enhancement and inhibition

By exposing SCG neurons to exogenous AA, we characterized N-current enhancement and inhibition as distinct molecular events acting at different sites on N channels. Restriction of AA movement across the cell membrane suggested that enhancement requires AA on the extracellular side or within the outer leaflet, whereas inhibition requires AA on the intracellular side or within the inner leaflet (Barrett et al., 2001). These two sites presumably would be equally accessible to AA generated internally because movement of long-chain fatty acids across cell membranes occurs in <1 s (Hamilton et al., 2002). Because CaV₂β subunits are cytoplasmic, we hypothesized that CaV₂β₂a attenuates inhibition without affecting enhancement. In dissociated neurons, AA induces an initial current enhancement followed by a slower progressing inhibition (Barrett et al., 2001; Liu et al., 2001). The simplest explanation for these data is that the initial enhancement becomes masked by the subsequent, more dominant, inhibition. Because previous work indicated that enhancement stems from increased voltage sensitivity (Barrett et al., 2001), we used normalized G-V curves (Fig. 5) to show that an increase in voltage sensitivity occurs independently of CaV₂β₂ expression. In contrast, inhibition results from an increase in a slow form of inactivation most likely as a result of increased dwell time in one or more intermediate closed states (Liu and Rittenhouse, 2000). This type of inhibition would essentially reduce the available number of channels providing calcium influx, dominating an enhancement produced by a shift in voltage sensitivity. Thus, by blocking a dominant inhibition, CaV₂β₂a unmasks a latent enhancement. The masking of enhancement offers some explanation for the finding that the majority of studies on N-current modulation have observed inhibition (Elmslie, 2003).

Nevertheless, enhancement and inhibition have been reported from experimental systems similar to the one used here. For example, AA both shifts activation toward negative potentials and simultaneously increases steady-state inactivation of T channels (Talavera et al., 2004). Also, in SCG neurons, the amide of AA, anandamide, elicited enhancement at negative potentials and inhibition at positive potentials in I-V plots (Guo and Ikeda, 2004). Enhancement marked by a leftward shift in voltage sensitivity was reported for R-type channels in the hippocampus (Tai et al., 2006), a region in which CaV₂β2 is highly expressed (Day et al., 1998). Finally, using HEK-293 cells transiently transfected with recombinant M₁Rs and N channels containing CaV₂β₃, an earlier study showed that carbachol inhibited CaV₂β₃ current with no obvious enhancement (Melliti et al., 2001). However, I-V curves showed a discernible leftward shift in voltage sensitivity after carbachol.
A new role for palmitoyl groups of palmitoylated proteins

Our data identify a previously unrecognized role for protein palmitoylation in which it serves as the key feature of Ca_v2.2a’s capacity to toggle Ca_v2.2 modulation from inhibition to enhancement after stimulation of G_qPCRs. Here and in other systems, palmitoylation targets or anchors proteins to specific membrane domains (Chien et al., 1996; Resh, 2006). Although dynamic palmitoylation of Ca_v2.2a has been shown to alter the inactivation kinetics of voltage-gated Ca^{2+} channels in bovine chromaffin cells, the assumed mechanism of action is restriction of movement of channel domains by anchoring the Ca_v2.2a subunit to the membrane (Hurley et al., 2000). This anchoring in some way is thought to impede current inactivation (Restituito et al., 2001). Thus, palmitoylation increases protein stability and efficiency of action but rarely alters the functional properties of proteins. One exception to this generalization is retinal epithelial protein 65 (RPE65), a chaperone protein for all-trans-retinyl esters. In this case, palmitoylation not only targets RPE65 to the plasma membrane but also reverses RPE65’s binding specificity for vitamin A to all-trans-retinyl ester, consequentially affecting how rapidly photoreceptors respond to light (Xue et al., 2004). Thus, palmitoylation qualitatively alters the substrate specificity of the same protein that is reversibly palmitoylated. Here, our data extend the functions of palmitoylation in a new direction by revealing that after stimulation of G_qPCRs, the palmitoyl groups of one protein, Ca_v2.2a, block inhibition of a second protein, Ca_v2.2.

Our finding that palmitoylation antagonizes an inhibition mediated by PtdIns(4,5)P_2 breakdown and increased free AA (Liu and Rittenhouse, 2003a; Gamper et al., 2004) raises the possibility that dual palmitoyl groups interact directly with Ca_v2.2 to antagonize AA’s interaction with Ca_v2.2 and subsequent inhibition of N-channel activity. The idea that AA may interact directly with Ca^{2+} channels is supported by the finding that M_1R stimulation or application of exogenous AA also inhibits recombinant Ca_v3 currents in whole-cell (Zhang et al., 2000; Talavera et al., 2004; Hildebrand et al., 2007) and ripped-off patch configurations (Chemin et al., 2007). Because Ca_v3 (T type) channels do not require coexpression of Ca_v3 or ailitation to open, T-channel inhibition must occur at a site on the pore-forming subunit. Moreover, AA inhibits T current with a Hill coefficient of 1.6, indicating cooperative binding of at least two AA molecules to T channels (Talavera et al., 2004). Collectively, these findings are consistent with a direct interaction between AA and T channels. AA likely confers N-current inhibition by acting at a homologous site found on many Ca_v channels because native and recombinant Ca_v1, Ca_v2, and Ca_v 3 channels exhibit similar changes in gating after AA (Shimada and Somlyo, 1992; Petit-Jacques and Hartzell, 1996; Liu and Rittenhouse, 2000; Vellani et al., 2000; Talavera et al., 2004; Chemin et al., 2007; Liu, 2007; Roberts-Crowley and Rittenhouse, 2009).

Palmitoylated Ca_v2a may serve as a phospholipid mimic by competing with PtdIns(4,5)P_2 and free AA for interaction with Ca_v2.2

Under basal conditions, AA normally resides in the sn-2 position of PtdIns(4,5)P_2. Because palmitoylated Ca_v2a appears to antagonize the actions of free AA on Ca_v2.2, it may also compete with PtdIns(4,5)P_2 for interaction with Ca_v2.2. The two palmitic acids of Ca_v2a residing in sites normally occupied by the two fatty acid tails of PtdIns(4,5)P_2. Consistent with its ability to block N-current inhibition by free AA, palmitoylated Ca_v2a acts as a phospholipid mimic to maintain normal channel activity (Fig. 8). This model is attractive in that it incorporates previous models, which propose that PtdIns(4,5)P_2 associates with channels, increasing their availability to open (Wu et al., 2002; Gamper et al., 2004). The model also supports our previous findings (Liu and Rittenhouse, 2003a) that increased free AA confers current inhibition either by displacing PtdIns(4,5)P_2 or by remaining associated with channels after phospholipid breakdown (Roberts-Crowley et al., 2009).

Our findings that free palmitic acids blocks inhibition by SP of Ca_v3-containing channels or depalmitoylated Ca_v2a-containing channels (see Mitra-Ganguli et al. in this issue) is consistent with the idea that the palmitic acids occupy the same sites recognized by AA. Whether PtdIns(4,5)P_2 as well as AA compete for the same site of interaction with Ca_v2.2 as the palmitoyl groups of Ca_v2a and where the location of that site is awaits future investigation. However, we have observed that whole-cell recombinant current from Ca_v2a-containing channels, in contrast to Ca_v3-containing channels (Gamper et al., 2004), run down only minimally over time (unpublished data), consistent with palmitoylated Ca_v2a functionally substituting for PtdIns(4,5)P_2. Thus, when taken together, our findings and the results of other laboratories best fit a model in which palmitoylated Ca_v2a, PtdIns(4,5)P_2, and free AA compete for an overlapping interaction site on Ca_v2.2. Given the extent that lipids such as PtdIns(4,5)P_2 and AA associate with membrane protein complexes (Piomelli, 1993; Doughman et al., 2003), the interference of such interactions by palmitoylated proteins is predicted to occur in other protein complexes, yielding broad importance beyond ion channel functioning.

Expression of palmitoylated Ca_v2a may underlie novel forms of synaptic plasticity

In particular, the presence or absence of palmitoylated Ca_v2a may have far-reaching consequences for synaptic plasticity. Ca_v2 expression displays an overlapping distribution with G_qPCRs (Tencé et al., 1994; Liu and Rittenhouse, 2003a) throughout the brain and is
primarily localized postsynaptically in dendrites and cell bodies (Lie et al., 1999), indicating that enhancement of Ca²⁺ channel activity may specifically affect postsynaptic membrane excitability. In support of this notion, increased current amplitude and/or kinetic changes associated with Ca²⁺ current enhancement (Zhang et al., 2000; Bannister et al., 2004; Guo and Ikeda, 2004; Talavera et al., 2004; Tai et al., 2006; Chemin et al., 2007; Mitra-Ganguli et al., 2009) occur in different neurons and recombinant channels from the CaV2 and CaV3 families (Keyser and Alger, 1990; Melliti et al., 2001; Chemin et al., 2007; Meza et al., 2007). In contrast, native or recombinant channels associated with CaVβ1b, CaVβ3, or CaVβ4 exhibit inhibition after similar stimulation (Keyser and Alger, 1990; Liu and Rittenhouse, 2003a; Gamper et al., 2004; Guo and Ikeda, 2004; Meza et al., 2007). Because CaVβ2 expression changes developmentally (Tanaka et al., 1995) and with activity (Lie et al., 1999), postsynaptic responses also may change over time. Patients with temporal lobe epilepsy exhibit increased postsynaptic CaVβ2 expression in damaged hippocampal regions. In contrast, patients that underwent surgical lesioning exhibit hippocampal CaVβ levels indistinguishable from control patients (Lie et al., 1999), suggesting CaVβ2 expression is dynamically regulated by electrical activity. Moreover, it has been reported that although CaVβ expression appears necessary for surface expression of the N channel, once the channel reaches the cell surface, the CaVβ subunit can be exchanged (Restituito et al., 2001; Hidalgo et al., 2006). Thus, changes in current modulation caused by changes in CaVβ expression should not require a turnover of the entire channel complex.

Whether up-regulation of CaVβ2 is a response to counteract hyperexcitability or whether increased CaVβ2 levels contribute to excitotoxic neurodegeneration has not been determined. Nevertheless, these findings document in vivo plasticity of CaVβ2 expression. In the short term, palmitoyl acyl transferases dynamically regulate protein palmitoylation in hippocampal dendrites and cell bodies to control synaptic function (El-Husseini et al., 2002; Fukata et al., 2004). Pulse–chase experiments using tritiated palmitate indicate that palmitate turns over multiple times during the lifetime of most palmitoylated proteins, and recent experiments suggest that the pulse–chase experiments may underestimate the turnover rate by 15–20-fold as a result of reacylation of proteins by the radiolabeled palmitate within a cell (for review see Baekkeskov and Kanaani, 2009). Thus, expression of CaVβ2a in postsynaptic regions should create a previously unrealized level of plasticity in which the response to a transmitter, acting on its GqPCR from moment to moment, may switch from inhibitory to excitatory depending on whether CaVβ2a’s palmitoyl groups interact with CaVβ2.

A role for multiple CaVβ isoforms in neurons

In summary, we have shown that multiple CaVβ isoforms expressed in a recombinant system can recapitulate native N-current modulation by the slow pathway. Given the diversity of neuronal functions for calcium influx, there must be localized control of a channel’s response to modulation. Our findings provide a previously unrecognized role for CaVβ subunits in the control of modulation and indicate a means by which GqPCR stimulation can simultaneously up- and down-regulate calcium flux at distinct sites within a single cell. Moreover, our experiments and analyses identify the sites that confer sensitivity to inhibition by AA as a likely site of plasticity.

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