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Heneghan, John F.; Ganguli, Tora Mitra; Stanish, Lee F.; Liu, Liwang; Zhao, Rubing; and Rittenhouse, Ann R., "The Ca2+ channel beta subunit determines whether stimulation of Gq-coupled receptors enhances or inhibits N current" (2009). GSBS Student Publications. 1709.  
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Citation: J Gen Physiol. 2009 Nov;134(5):369-84. Link to article on publisher's site

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The Ca\(^{2+}\) channel \(\beta\) subunit determines whether stimulation of G\(_q\)-coupled receptors enhances or inhibits N current

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In superior cervical ganglion (SCG) neurons, stimulation of \(M_1\) receptors (\(M_1\)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 M1Rs. In addition, techniques that diminish AA's concentration during \(M_1\)R stimulation minimize N-current modulation. However, other studies suggest depletion of phosphatidylinositol-4,5-bisphosphate during \(M_1\)R stimulation suffices to elicit modulation. In this study, we used an expression system to examine the physiological mechanisms regulating modulation. We found the \(\beta\) subunit (CaV\(_{\beta}\)) acts as a molecular switch regulating whether modulation results in enhancement or inhibition. In human embryonic kidney 293 cells, stimulation of \(M_1\)Rs or neurokinin-1 receptors (NK-1Rs) inhibited activity of N channels formed by CaV\(_{2.2}\) and coexpressed with CaV\(_{\beta 1b}\), CaV\(_{\beta 3}\), or CaV\(_{\beta 4}\) but enhanced activity of N channels containing CaV\(_{\beta 2a}\). Exogenously applied AA produced the same pattern of modulation. Coexpression of CaV\(_{\beta 2a}\), CaV\(_{\beta 3}\), and CaV\(_{\beta 4}\) recapitulated the modulatory response previously seen in SCG neurons, implying heterogeneous association of CaV\(_{\beta}\) with CaV\(_{2.2}\). Further experiments with mutated, chimeric CaV\(_{\beta}\) subunits and free palmitic acid revealed that palmitoylation of CaV\(_{\beta 2a}\) is essential for loss of inhibition. The data presented here fit a model in which CaV\(_{\beta 2a}\) blocks inhibition, thus unmasking enhancement. Our discovery that the presence or absence of palmitoylated CaV\(_{\beta 2a}\) toggles \(M_1\)-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 \(M_1\)Rs or NK-1Rs will fluctuate between enhancement and inhibition based on the presence of palmitoylated CaV\(_{\beta 2a}\).

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\(^{2+}\) 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 (Wigsga 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\(_q\) 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 \(M_1\) receptor (\(M_1\)R), a G\(_q\)-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 \(G_q\) (Macdonald et al., 1996), suggesting that multiple G\(_q\)PCRs converge on this pathway (Suh and Hille, 2005). Recent studies propose that a reduction in phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)\(_P_2\)) levels during G\(_q\)PCR stimulation suffices for inhibition (Wu et al., 2002; Gamper et al., 2004; Michailidis et al., 2005).
In this model, channels are available to open when PtdIns(4,5)P₂ associates with them. During muscarinic stimulation, PtdIns(4,5)P₂ is depleted from the membrane, creating a concentration gradient that favors PtdIns(4,5)P₂ dissociating and diffusing away from channels. This model appeals to many in part because PtdIns(4,5)P₂ 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 M₁Rs requires events downstream of PtdIns(4,5)P₂ 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, M₁R 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 PLA₂ (Liu et al., 2006). Consistent with the need for PtdIns(4,5)P₂ metabolites, inhibiting AA release from phospholipids by antagonizing PLA₂ activity or including BSA, a scavenger of AA (Spector, 1975), in the bath solution minimizes N-current modulation by Oxo-M (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, M₁R 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 PLA₂ (Liu et al., 2006). Consistent with the need for PtdIns(4,5)P₂ metabolites, inhibiting AA release from phospholipids by antagonizing PLA₂ activity or including BSA, a scavenger of AA (Spector, 1975), in the bath solution minimizes N-current modulation by Oxo-M (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).

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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, α2δ-1, and CaV3.

(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 of the membrane (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. Data were curve fit using the Boltzmann equation function in Origin 7.0, yielding a curve fitting the equation G/G_max = G_max + (G_min - G_max)/(1 + exp[(V_m - V_m1/2)/k]), where G_max is the maximal conductance, G_min is the minimal conductance, V_m is the test potential, and k is the slope parameter.

(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, α2δ-1, and CaV3 and transiently transfected with 500–1,000 ng/well of M1Rs. eGFP is shown in the time course and representative sweeps. The charge carrier was increased to 20 mM Ba2+ to improve the signal to noise ratio, and accordingly the test potential was adjusted to 10 mV.

(G and H) HEK-M1 cells were transiently transfected with N-channel subunits CaV2.2, α2δ-1, and CaV3β2a. 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 ni-modipine 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 currents. 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) vs. voltage (V_m), were plotted for whole-cell currents before and after AA. Data were curve fit using the Boltzmann equation function in Origin 7.0, yielding a curve fitting the equation G/G_max = G_max + (G_min - G_max)/(1 + exp[(V_m - V_m1/2)/k]), where G_max is the maximal conductance, G_min is the minimal conductance, V_m is the test potential, and k is the slope parameter.
RT-PCR of CaVβ 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 deoxyribonucleotide 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 transcription, and RNase-free water to make up a total volume of 10 µl (all reagents were obtained from Invitrogen). Images of immunofluorescence were obtained at room temperature using a custom-built, video-rate confocal microscope (Sanderson and Parker, 2005) with a 40× objective lens. An excitation wavelength of 488 nm was used, and emission spectra were collected with long-pass filters (OG10S) at 515 nm (Perez and Sanderson, 2005).

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 CaV2.2, αβδ-1, and CaVβ3, which most commonly associates with CaV2.2 (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.

CaVβ subunit controls N-current modulation by GqPCRs
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 GqPCR modulation, recombinant N current exhibited robust, fast inactivation (Fig. 1, D and F, and Fig. 2 C) as previously observed with channels containing CaVβ3 (Olcese 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 CaVβ2a (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 CaVβ2 mRNA (Lin et al., 1996), we hypothesized that the majority of N current arises from CaVβ2a-containing channels. If true, these channels might exhibit both inhibition and enhancement. We found that Oxo-M rapidly inhibited N current from cells expressing CaVβ2a. However, unlike CaVβ3, initial inhibition gave way to stable enhancement (Fig. 1, G and H). Moreover, comparison of I-V plots revealed

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-eicosatetraenoic 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 CaV2.2, αβδ-1, CaVβ2a, and CaVβ3, 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.
Figure 2. Caβ determines N-current modulation by GqPCRs and AA. (A–D) HEK-M1 cells were transiently transfected with CaV2.2, α2δ-1, and various Caβ subunits. Currents, measured in 5 mM [Ba2+] 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 Caβ2a, 20 mM [Ba2+] 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 µM Oxo-M (gray bars) and that Oxo-M no longer inhibited current at any potential but enhanced current at negative test potentials (Fig. 2 B). Although we hypothesized that expression of Caβ2a would elicit enhancement at negative potentials, separation of enhancement from inhibition based on Caβ expression was unanticipated. Therefore, we tested whether other Caβs would dually modulate N current by repeating the aforementioned experiments with two other neuronal Caβs: Caβ1b and Caβ4. As with Caβ3, when either Caβ1b (Fig. 2 A) or Caβ4 (Fig. 2 D) was expressed, Oxo-M inhibited current at all test potentials, indicating that channels containing Caβ2a exhibit unique N-current modulation by M1Rs.

To determine whether M1Rs uniquely influence the profile of modulation, we examined whether N-current modulation by another GqPCR depends on Caβ. We tested NK-1Rs because in SCG neurons, stimulation of M1Rs and NK-1Rs inhibits N current through converging signaling pathways that require Gαq and downstream activation of PLC (Shapiro et al., 1994; Kammermeier et al., 2000; Liu and Rittenhouse, 2003a). We examined current modulation that occurred after exposure to SP. As with Oxo-M, an initial transient inhibition progressed to a stable current enhancement when Caβ2a was expressed along with CaV2.2 and α2δ-1 (Fig. 3, A and C). In contrast, SP elicited sustained inhibition with Caβ3 expression (Fig. 2 E and Fig. 3, B and C). These data indicate that control of modulation by Caβ is a mechanism used by other GqPCRs rather than being unique to M1Rs (Fig. 1 A).

AA elicits a profile of N-current modulation similar to GqPCR stimulation

AA mimics N-current modulation by M1Rs in SCG neurons and may serve as a downstream messenger of this pathway (Liu and Rittenhouse, 2003a). If so, AA also should inhibit or enhance recombinant N current similarly to M1R or NK-1R stimulation. When this hypothesis was tested, AA’s actions recapitulated the pattern of modulation observed with M1R or NK-1R stimulation: enhancement and inhibition separated based on Caβ expression (Fig. 2, F–J). If elevated AA levels mediate enhancement and inhibition, both should reverse after washout of exogenous AA. As with SCG neurons, washing with copious amounts of bath solution or with 1 mg/ml BSA reversed modulation (Fig. S2, A–F). Moreover, enhancement recurred with a second application of AA after washing with BSA (Fig. S2 A). These findings

5 nM SP (black bars). Percent change in current amplitude was highly significant between Caβ2a and Caβ1b, Caβ3, or Caβ4 irrespective of whether Oxo-M or SP was applied (**, P < 0.005; one-way ANOVA). (F–J) Modulation of N current by 10 µM 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 Ca<sub>v</sub> 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 Ca<sub>v</sub>2.2 coexpression with different Ca<sub>v</sub>β isoforms within individual neurons (Scott et al., 1996). Similarly, SCG neurons may exhibit both forms of modulation because Ca<sub>v</sub>2.2 coassembles with different Ca<sub>v</sub>βs. If this hypothesis is correct, the pattern of modulation observed in SCG neurons should recapitulate in HEK-M1 cells transfected with multiple Ca<sub>v</sub>βs.

When cells were transfected with Ca<sub>v</sub>β2a and Ca<sub>v</sub>β3, 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 Ca<sub>v</sub>β2a, Ca<sub>v</sub>β3, and Ca<sub>v</sub>β4 mRNA with trace amounts of Ca<sub>v</sub>β1b mRNA (Lin et al., 1996). We verified the presence of Ca<sub>v</sub>β2, Ca<sub>v</sub>β3, and Ca<sub>v</sub>β4 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 Ca<sub>v</sub>β2a, Ca<sub>v</sub>β3-, and Ca<sub>v</sub>β4-transformed plasmid. Transfection with a mixture of cDNAs in the molar ratio of 10:1:1 for Ca<sub>v</sub>β2a/Ca<sub>v</sub>β3/Ca<sub>v</sub>β4 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 Ca<sub>v</sub>β isoforms with Ca<sub>v</sub>2.2.

The antibody used in the immunocytochemistry experiments recognizes the peptide sequence RSPKP-SANSVTSPHSKE (NeuroMab data sheet N8b_1). This sequence exists in Ca<sub>v</sub>β1a, 2b, 2c, 2d, and 2e but not 2f, 2g, or 2h. Because the presence of Ca<sub>v</sub>β2a variant has never been confirmed in rat neonatal SCG neurons, we looked for its presence by RT-PCR using primers designed to recognize the peptide sequence RSPKP-SANSVTSPHSKE (NeuroMab data sheet N8b_1). This sequence exists in Ca<sub>v</sub>β1a, 2b, 2c, 2d, and 2e but not 2f, 2g, or 2h. Because the presence of Ca<sub>v</sub>β2a variant has never been confirmed in rat neonatal SCG neurons, we looked for its presence by RT-PCR using primers designed to recognize the peptide sequence RSPKP-SANSVTSPHSKE (NeuroMab data sheet N8b_1). This sequence exists in Ca<sub>v</sub>β1a, 2b, 2c, 2d, and 2e but not 2f, 2g, or 2h. Because the presence of Ca<sub>v</sub>β2a variant has never been confirmed in rat neonatal SCG neurons, we looked for its presence by RT-PCR using primers designed to recognize the peptide sequence RSPKP-SANSVTSPHSKE (NeuroMab data sheet N8b_1). This sequence exists in Ca<sub>v</sub>β1a, 2b, 2c, 2d, and 2e but not 2f, 2g, or 2h. Because the presence of Ca<sub>v</sub>β2a variant has never been confirmed in rat neonatal SCG neurons, we looked for its presence by RT-PCR using primers designed to recognize the peptide sequence RSPKP-SANSVTSPHSKE (NeuroMab data sheet N8b_1). This sequence exists in Ca<sub>v</sub>β1a, 2b, 2c, 2d, and 2e but not 2f, 2g, or 2h. Because the presence of Ca<sub>v</sub>β2a variant has never been confirmed in rat neonatal SCG neurons, we looked for its presence by RT-PCR using primers designed to recognize the peptide sequence RSPKP-SANSVTSPHSKE (NeuroMab data sheet N8b_1). This sequence exists in Ca<sub>v</sub>β1a, 2b, 2c, 2d, and 2e but not 2f, 2g, or 2h. Because the presence of Ca<sub>v</sub>β2a variant has never been confirmed in rat neonatal SCG neurons, we looked for its presence by RT-PCR using primers designed to recognize the peptide sequence RSPKP-SANSVTSPHSKE (NeuroMab data sheet N8b_1).
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-
CaV recorded using 20 mM Ba2+. *, P < 0.05; paired 
nOxo-M application (n = 6 cells). All currents for the figure were run parallel experiments of CaV
SCG. As a positive control, we used the same three animals CaV (HC) homogenates from three different 7-d-old rats comparing
sent SEM. Bars, 0.4 nA. (D) RT-PCR of SCG and hippocampal = 4 neurons). Error bars repre-
were exposed to no antibodies, (A, top) Confocal micrographs of individual SCG neurons. Start-
ing from the left, the neurons were exposed to no antibodies, secondary antibody (anti–mouse Alexa Fluor 488) only, mouse anti-CaVβ2 followed by secondary rabbit anti–mouse Alexa Fluor 488, anti-CaVβ3 followed by anti–rabbit Alexa Fluor 488, and mouse anti-CaVβ2 followed by secondary antibody. (bottom) Corresponding view shown in bright field. Bar, 10 µm. (B) Representa-
tive current traces and mean I-V plots taken from HEK-M1 cells transiently transfected with a 12:12:10:1:1 ratio of CaV2.2/αβ-1/
CaVβ2/CaVβ3/CaVβ4; taken before (black) and after (red) 10µM Oxo-M application (n = 6 cells). All currents for the figure were recorded using 20 mM Ba2+. *, P < 0.05; paired t test at each test potential. (C) Current traces and mean I-V plots taken from freshly dissociated SCG neurons before (black) and after (red) application of 10 µM Oxo-M (n = 4 neurons). Error bars repre-
SEM. Bars, 0.4 nA. (D) RT-PCR of SCG and hippocampal (HC) homogenates from three different 7-d-old rats comparing CaVβ2a and CaVβ3 expression. RNA from animal #1 generated lanes 1, 4, 7, and 10. RNA from animal #2 generated lanes 2, 5, 8, and 11. RNA from animal #3 generated lanes 3, 6, 9, and 12. Lane 13 represents a negative control: RT-PCR reaction without isolated RNA.

Figure 4. Expression of multiple CaVβ subunits in SCG neurons. specifically for CaVβ2a. Using tissue from three different animals, we assessed the presence of CaVβ2a and CaVβ3 in SCG. As a positive control, we used the same three animals to run parallel experiments of CaVβ isofrom expression in the hippocampus, a region reported to be enriched in CaVβ2 (Day et al., 1998). We found CaVβ2a expression in three of three hippocampal samples and in three of three SCG samples. We also found CaVβ3 expression in two of three SCG samples but found negligible CaVβ3 expression in hippocampal samples.

A model for dual modulation of N current
We established a working model for how CaVβ2a controls modulation based on previous studies that examined AA’s mechanism of inhibition of different Ca2+ channels (Barrett et al., 2001; Liu et al., 2001). Our experiments with SCG neurons demonstrated that current inhibition by AA occurs intracellularly or within the inner leaflet of the membrane. In contrast, enhancement appears located extracellularly or within the outer leaflet of the bilayer (Barrett et al., 2001; Liu et al., 2001). CaVβ subunits bind to cytoplasmic loops of CaV2.2 (Pragnell et al., 1994). Thus, we hypothesized that the unique interactions of CaVβ2a with CaV2.2 somehow attenuate inhibition. In SCG neurons (Barrett et al., 2001; Liu et al., 2001) and with recombinant channels containing either CaVβ2a or CaVβ3 (Fig. 5, A and B), AA induces initial N-current enhancement, which is sustained with CaV2.2a but for CaVβ3 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 CaVβ2a expression. Both CaVβ2a- and CaVβ3-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 CaVβ subunit expression. These data suggest that CaVβ2a’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, CaVβ2a unmasks latent enhancement.

Loss of CaVβ2a palmitoylation restores partial inhibition of N current by Oxo-M or AA
Having established that CaVβ2a uniquely blocks N-current inhibition by GPCR stimulation or exogenous AA, we investigated the structural aspects of CaVβ2a to determine its mechanism of action. A conspicuous feature of CaVβ2a is its unique palmitoylation of two cysteine residues near the N terminus (Chien et al., 1996). Our
observation that palmitoylated CaVβ2a interferes with a fatty acid–mediated inhibition of CaV2.2 raises the possibility of direct antagonism between palmitic acid and AA (Fig. 5 E). Therefore, we tested a depalmitoylated mutant to determine whether CaVβ2a must be palmitoylated to minimize inhibition and reveal enhancement. Currents from channels containing depalmitoylated CaVβ2a (CaVβ2a[C3,4S]; Chien et al., 1996) exhibited an

Figure 5. CaVβ2a blocks N-current inhibition, revealing latent enhancement. (A and B) Time courses of cells expressing either CaVβ2a (A) or CaVβ3 (B) exposed to AA both exhibit enhancement during the initial phase of time course. However, when CaVβ3 is present, inhibition eventually dominates, indicating that both inhibitory and enhancement sites are available for AA to bind. When CaVβ2a is present, inhibition no longer occurs, suggesting the palmitoylated CaVβ2a 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 CaVβ2a (C) or CaVβ3 (D), indicating that an increased sensitivity to voltage is masked by a dominating inhibition in cells expressing CaVβ3. (E) Schematic representation of working model. Upon GqPCR stimulation, the released AA binds to both inhibitory and enhancement sites on the channel. When CaVβ3 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 CaVβ2a is present, inhibition no longer occurs because the palmitoylated CaVβ2a 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 CaVβ2a(C3,4S) (A and B), CaVβ2bβ1b (C and D), or CaVβ2aβ3 (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.
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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 CaVβ should convert current inhibition to enhancement. Therefore, we tested a chimera in which the variable N terminus of CaVβ1b was replaced with the palmitoylated 16-amino acid N terminus of CaVβ2a (CaVβ2aβ1b; Chien et al., 1998). In cells expressing CaVβ2aβ1b, 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 CaVβ1b (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 CaVβ3 was replaced with the N terminus of CaVβ2a (CaVβ2aβ3; Chien et al., 1998) did not block inhibition as effectively. In cells expressing CaVβ2aβ3, inhibition by Oxo-M was diminished compared with wild-type CaVβ3, 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 CaVβ2a’s palmitoylated N terminus to CaVβ3 only partially reproduced the stable enhancement by Oxo-M or AA normally observed with wild-type CaVβ2a. Because CaVβ2a protein shares highest sequence homology to CaVβ1b (Birnbaumer et al., 1998), the simplest interpretation for the varied results with the different chimeras is that CaVβ2aβ1b positions the palmitoylated N terminus so that it minimizes N-current inhibition similarly to wild-type CaVβ2a. In contrast, the more divergent CaVβ2aβ3 positions the palmitoylated N terminus in a different position relative to CaV2.2 where it is less effective in minimizing inhibition (Fig. 6 H). In support of this notion, CaVβ2aβ3-containing cells exhibited varied kinetics, from nonactivating currents resembling CaVβ2a-containing channel activity (Fig. 2 B) to rapidly inactivating currents resembling depalmitoylated CaVβ2a-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

Figure 7. Palmitic acid antagonizes inhibition of N current by GqPCR stimulation. HEK-M1 cells were transiently transfected with NK-1R, CaV2.2, αβδ-1, and CaVβ3. (A) Diagram showing that preincubation of HEK-M1 cells with exogenously applied palmitic acid blocks inhibition of N channels by fatty acids, such as AA, released after NK-1R or M1R activation. (B) Summary showing preincubation of cells with 10 µM palmitic acid (PA) significantly attenuates inhibition by SP or Oxo-M (*, P < 0.05 compared with percent change in the absence of palmitic acid). Error bars represent SEM. (C) Modulation of N current by SP from cells expressing CaVβ3 is shown in a representative time course, current traces taken before and 3 min after application of SP, and I-V plots (n = 5). (D) Preincubation of cells with palmitic acid causes loss of inhibition by SP as shown in a time course, current traces, and I-V plot (n = 6). (E) Preincubation of HEK-M1 cells with palmitic acid also causes loss of inhibition by Oxo-M, as shown in a representative time course, current traces, and I-V plots (n = 4). Bars, 0.4 nA.
suggests that Ca\(3\beta\)2a\(3\beta\)3 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 CaV\(3\beta\)3-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 \(\mu M\) 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 CaV\(3\beta\)3-containing channels in HEK-M1 cells by Oxo-M after a 10-min preincubation with 10 \(\mu M\) 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 CaV\(3\beta\)3-containing N channels. More importantly, these results indicate palmitic acid is both necessary and sufficient to antagonize N-current inhibition during G\(q\)PCR stimulation (Fig. 8).

**DISCUSSION**

In this study, we used a recombinant system to investigate the enhancement and inhibition of N current that occurs during M\(1\)R stimulation and AA application. In so doing, we made the discovery that the N-channel’s \(\beta\) subunit directs opposite effects of N-current modulation. Specifically, M\(1\)R stimulation or exogenous AA uniquely enhanced whole-cell N current from cells expressing CaV\(3\beta\)2a but inhibited N current from cells expressing CaV\(3\beta\)1b, CaV\(3\beta\)3, or CaV\(3\beta\)4. This finding allowed us to further examine the modulation of N current and reach several additional conclusions. First, the striking recapitulation of M\(1\)R-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 G\(q\)PCRs. Third, cells expressing multiple forms of CaV\(3\beta\) 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 CaV2.2 coupling with different CaV\(3\beta\) isoforms. This finding provides an explanation of how stimulation of M\(1\)Rs 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 CaV constructs, we demonstrated that palmitoylation of CaVβ2a 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 GqGPCRs, suggesting that CaVβ2a’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; Delmas et al., 2005; Suh and Hille, 2005); 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, PLA2 antibodies dialyzed internally, and neurons harvested from PLA2−/− knockout mice (Liu and Rittenhouse, 2003a; Liu et al., 2004, 2006). Here we have taken a molecular approach in which expression of palmitoylated CaVβ2a provides a highly specific agent to block inhibition by AA, whereas nonpalmitoylated wild-type CaVβ 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, 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 CaVβ subunits are cytoplasmic, we hypothesized that CaVβ2a 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 CaVβ2a 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, CaVβ2a 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 CaVβ2 is highly expressed (Day et al., 1998). Finally, using HEK-293 cells transiently transfected with recombinant M1Rs and N channels containing CaVβ3, an earlier study showed that carbachol inhibited Ca2+ 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 CaVβ2a’s capacity to toggle CaV2.2 modulation from inhibition to enhancement after stimulation of GqPCRs. Here and in other systems, palmitoylation targets or anchors proteins to specific membrane domains (Chien et al., 1996; Resh, 2006). Although dynamic palmitoylation of CaVβ2a has been shown to alter the inactivation kinetics of voltage-gated CaV2.1 channels in bovine chromaffin cells, the assumed mechanism of action is restriction of movement of channel domains by anchoring the CaVβ2a 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, consequently 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 GqPCRs, the palmitoyl groups of one protein, CaVβ2a, block inhibition of a second protein, CaV2.2.

Our finding that palmitoylation antagonizes an inhibition mediated by PtdIns(4,5)P2 breakdown and increased free AA (Liu and Rittenhouse, 2003a; Gamper et al., 2004) raises the possibility that dual palmitoyl groups interact directly with CaV2.2 to antagonize AA’s interaction with CaV2.2 and subsequent inhibition of N-channel activity. The idea that AA may interact directly with CaV2.2 channels is supported by the finding that M1R stimulation or application of exogenous AA also inhibits recombinant CaV3 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 CaV3 (T type) channels do not require coexpression of CaVβ or αδ 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 CaV channels because native and recombinant CaV1, CaV2, and CaV3 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 CaVβ2a may serve as a phospholipid mimic by competing with PtdIns(4,5)P2 and free AA for interaction with CaV2.2

Under basal conditions, AA normally resides in the sn-2 position of PtdIns(4,5)P2. Because palmitoylated CaVβ2a appears to antagonize the actions of free AA on CaV2.2, it may also compete with PtdIns(4,5)P2 for interaction with CaV2.2, the two palmitic acids of CaVβ2a residing in sites normally occupied by the two fatty acid tails of PtdIns(4,5)P2. Consistent with its ability to block N-current inhibition by free AA, palmitoylated CaVβ2a 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)P2 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)P2 or by remaining associated with channels after phospholipid breakdown (Roberts-Crowley et al., 2009).

Our findings that free palmitic acid blocks inhibition by SP of CaVβ3-containing channels or depalmitoylated CaVβ2a-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)P2 as well as AA compete for the same site of interaction with CaV2.2 as the palmitoyl groups of CaVβ2a and where the location of that site is awaits future investigation. However, we have observed that whole-cell recombinant current from CaVβ2a-containing channels, in contrast to CaVβ3-containing channels (Gamper et al., 2004), run down only minimally over time (unpublished data), consistent with palmitoylated CaVβ2a functionally substituting for PtdIns(4,5)P2. Thus, when taken together, our findings and the results of other laboratories best fit a model in which palmitoylated CaVβ2a, PtdIns(4,5)P2, and free AA compete for an overlapping interaction site on CaV2.2. Given the extent that lipids such as PtdIns(4,5)P2 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 CaVβ2a may underlie novel forms of synaptic plasticity

In particular, the presence or absence of palmitoylated CaVβ2a may have far-reaching consequences for synaptic plasticity. CaVβ2 expression displays an overlapping distribution with GqPCRs (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 Ca\textsuperscript{2+} channel activity may specifically affect postsynaptic membrane excitability. In support of this notion, increased current amplitude and/or kinetic changes associated with Ca\textsuperscript{2+} 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 Ca\textsubscript{V}2 and Ca\textsubscript{V}3 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 Ca\textsubscript{V}1b, Ca\textsubscript{V}3, or Ca\textsubscript{V}4 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 Ca\textsubscript{V}2\textsubscript{2} 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 Ca\textsubscript{V}2 expression in damaged hippocampal regions. In contrast, patients that underwent surgical lesioning exhibit hippocampal Ca\textsubscript{V}2 levels indistinguishable from control patients (Lie et al., 1999), suggesting Ca\textsubscript{V}2 expression is dynamically regulated by electrical activity. Moreover, it has been reported that although Ca\textsubscript{V}2 expression appears necessary for surface expression of the N channel, once the channel reaches the cell surface, the Ca\textsubscript{V}2 subunit can be exchanged (Restituito et al., 2001; Hidalgo et al., 2006). Thus, changes in current modulation caused by changes in Ca\textsubscript{V}2 expression should not require a turnover of the entire channel complex.

Whether up-regulation of Ca\textsubscript{V}2 is a response to counteract hyperexcitability or whether increased Ca\textsubscript{V}2 levels contribute to excitotoxic neurodegeneration has not been determined. Nevertheless, these findings document in vivo plasticity of Ca\textsubscript{V}2 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 Ca\textsubscript{V}2\textsubscript{a} in postsynaptic regions should create a previously unrealized level of plasticity in which the response to a transmitter, acting on its G\textsubscript{PCR} from moment to moment, may switch from inhibitory to excitatory depending on whether Ca\textsubscript{V}2\textsubscript{a}’s palmitoyl groups interact with Ca\textsubscript{V}2\textsubscript{2}.

A role for multiple Ca\textsubscript{V}3 isoforms in neurons

In summary, we have shown that multiple Ca\textsubscript{V}3 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 Ca\textsubscript{V}3 subunits in the control of modulation and indicate a means by which G\textsubscript{PCR} 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.

We thank E. Liman for HEK-M1 cells, D. Lipscombe for tsA cells and Ca\textsubscript{V}2, Ca\textsubscript{V}1b, and Ca\textsubscript{V}4 plasmids, N. Nathanson for M, R plasmid, E. Perez-Reyes for Ca\textsubscript{V}1b, Ca\textsubscript{V}2a, and Ca\textsubscript{V}4 plasmids, A. Fox for Ca\textsubscript{V}2a(C3,4S) plasmid, and R. Ten Eick for Ca\textsubscript{V}2a/Ca\textsubscript{V}3 and Ca\textsubscript{V}2a/Ca\textsubscript{V}3 plasmids. We thank Y. Bai and M. Sanderson for helping capture fluorescent images of SCG neurons. We also thank M.L. Roberts-Crowley for help with transfection and sharing unpublished data. We thank A. Carruthers, P. Charnet, H. Colecraft, H. Florman, J. Glaven, L. Hayward, J.R. Lemos, J. Marshall, W.R. Kocert, H.-S. Li, S.R. Ikeda, J. Jonassen, and M.L. Roberts-Crowley for reading various versions of the manuscript. We thank T.F. Chung (University of Massachusetts Medical School [UMMS] tissue culture facility) for help growing HEK-M1 cells.

This project was funded by National Institutes of Health grant NS34195 (to A.R. Rittenhouse) and support from UMMS.

Edward N. Pugh, Jr. served as editor.

Submitted: 29 January 2009
Accepted: 2 October 2009

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Published October 26, 2009


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Caβ2a toggles N-current modulation by GqPCRs

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