Voltage-dependent regulation of CaV2.2 channels by Gq-coupled receptor is facilitated by membrane-localized β subunit

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G protein–coupled receptors (GPCRs) signal through molecular messengers, such as Gβγ, CaV2, and phosphatidylinositol 4,5-bisphosphate (PIP2), to modulate N-type voltage-gated CaV2 (CaV2.2) channels, playing a crucial role in regulating synaptic transmission. However, the cellular pathways through which GqPCRs inhibit CaV2 channel current are not completely understood. Here, we report that the location of CaV2 β subunits is key to determining the voltage dependence of CaV2.2 channel modulation by GqPCRs. Application of the muscarinic agonist oxotremorine-M to tsA-201 cells expressing M1 receptors, together with CaV2 N-terminal α1B, α2δ1, and membrane-localized β2a subunits, shifted the current-voltage relationship for CaV2.2 activation 5 mV to the right and slowed current activation. Muscarinic suppression of CaV2.2 activity was relieved by strong depolarizing prepulses. Moreover, when the C terminus of β-adrenergic receptor kinase (which binds Gβγ) was coexpressed with N-type channels, inhibition of CaV2.2 current after M1 receptor activation was markedly reduced and delayed, whereas the delay between PIP2 hydrolysis and inhibition of CaV2.2 current was decreased. When the Gβγ-insensitive CaV2.2 α1C-1B chimera was expressed, voltage-dependent inhibition of calcium current was virtually abolished, suggesting that M1 receptors act through Gβγ to inhibit CaV2.2 channels bearing membrane-localized CaV2 β2a subunits. Expression of cytosolic β subunits such as β2b and β3, as well as the palmitoylation-negative mutant β2a(C3,4S), reduced the voltage dependence of M1, muscarinic inhibition of CaV2.2 channels, whereas it increased inhibition mediated by PIP2 depletion. Together, our results indicate that, with membrane-localized CaV2 β subunits, CaV2.2 channels are subject to Gβγ-mediated voltage-dependent inhibition, whereas cytosol-localized β subunits confer more effective PIP2-mediated voltage-independent regulation. Thus, the voltage dependence of GqPCR regulation of calcium channels can be determined by the location of isotype-specific CaV2 β subunits.

INTRODUCTION

Voltage-gated calcium (CaV) channels play fundamental roles in mediating calcium influx upon depolarization (Hille, 2001). They regulate many physiological responses ranging from neurotransmission to muscle contraction. Dysfunction in CaV channels is associated with many pathological conditions such as pain, epilepsy, migraine, and autism (Catterall, 2011). A CaV channel consists of three protein subunits, CaV α1, α2δ, and β (Hofmann et al., 1999; Catterall, 2000). CaV α1 and α2δ subunits are transmembrane proteins responsible for forming the voltage-sensitive pore of the channel and promoting CaV α1 subunit stabilization at the plasma membrane, respectively. CaV β subunits are intracellular components that play an essential role in regulating gating properties and receptor modulation of CaV channels. The CaV β subunit sets the sensitivity of CaV channels to the membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PIP2). Lipidation (palmitoylation) of the β subunit results in a plasma membrane localization and a decrease in current inactivation and PIP2 sensitivity of CaV2.2 channels (Hurley et al., 2000; Suh et al., 2012).

As CaV channels are critical in virtually all excitable cells, they are also intensely and dynamically modulated by an array of receptor-dependent signals. This includes regulation by G proteins after G protein–coupled receptor (GPCR) activation (Zamponi and Currie, 2013). For the GPCRs coupled to pertussis toxin (PTX)–sensitive Gq/o protein, it is the Gβγ subunit that acts at the cytoplasmic surface of the membrane to bind directly to the CaV2.2 α subunit after Gq/11PCR activation, consequently inhibiting CaV current (Herlitze et al., 1996; Ikeda, 1996). This is the most extensively studied mechanism, characterized by its fast and membrane-delimited inhibition (Bernheim et al., 1991), slowed activation kinetics, and a positive shift in the voltage dependence of the channel (Bean, 1989; Elmslie et al., 1990). This inhibition can be transiently relieved by large-step depolarizations that elicit dissociation of Gβγ from the channel (Boland and Bean, 1993) and is thus also known as the voltage-dependent pathway (Dolphin,
2003; Currie, 2010). Contrastingly, a slow, voltage-independent inhibition occurs mostly through the activation of $G\_i/PCRs$. In this case, the $G\_i/PLC$-mediated depletion of PIP$_2$ and/or arachidonic acid generation is an important signaling messenger (Wu et al., 2002; Liu and Rittenhouse, 2003; Gamper et al., 2004; Suh et al., 2010). However, it seems that, depending on the receptor type, the voltage dependency of channel suppression would be determined by different messengers and showed different shape of regulation (see review, Tedford and Zamponi, 2006; Kisilevsky et al., 2008).

Although $G\_i/PCRs$ are widespread in the presynaptic neurons, $G\_i/PCRs$ are known to inhibit Ca$_{\text{v}}$2.2 in somata of sensory and sympathetic neurons (Filippov et al., 1998; Haley et al., 2000; Liu et al., 2004). Muscarinic acetylcholine receptor stimulation inhibits Ca$_{\text{v}}$ channels through both of the described pathways (Hille, 1994). $M_2$ and $M_4$ receptor subtypes are coupled to $G_{\alpha_j}$ and engage the voltage-dependent pathway to inhibit Ca$_{\text{v}}$ channels, whereas the $M_1$, $M_3$, and $M_5$ subtypes are coupled to $G_{\alpha_i}$ and modulate Ca$_{\text{v}}$ channels through the voltage-independent, second-messenger pathway. However, the molecular mechanism underlying the latter $G\_i/PCR$ modulation has been questioned and needs further clarification.

Recent studies have developed and implemented useful techniques to further dissect the different modes of Ca$_{\text{v}}$ channel modulation. Through the use of zebrafish voltage-sensitive phosphatase (Dr-VSP), reversible depletion of membrane PIP$_2$ became possible by applying a large depolarizing pulse that activates the enzyme (Murata et al., 2005; Suh et al., 2010). This allows exclusive analysis of PIP$_2$ depletion effects on channel modulation without any other production of second messengers or the activation of receptors (Okamura et al., 2009). Furthermore, genetically expressible inhibitors and real-time indicators have helped identify the molecular mechanisms by which inhibition of Ca$_{\text{v}}$2.2 occurs after muscarinic receptor activation (Koch et al., 1994; van der Wal et al., 2001; Jensen et al., 2009).

Using these techniques, we continued our mechanistic study of $G_{\text{q}}$ protein inhibition of N-type Ca$_{\text{v}}$2.2 channels. In superior cervical ganglion (SCG) neurons of the rat, it has been established that $G_{\text{q}}{11}/PLC$ activation and subsequent PIP$_2$ hydrolysis produce the major voltage-independent regulation of Ca$_{\text{v}}$2.2 channel after $M_1$ muscarinic receptor activation; however, there also is some voltage-dependent regulation, raising the possibility of a role for G$\beta$y as well (Kammermeier et al., 2000; Melliti et al., 2001; Gamper et al., 2004; Suh et al., 2010; Vivas et al., 2013). Also arguing for a second signaling pathway, Suh et al. (2012) found much less current inhibition after direct depletion of PIP$_2$ through the use of Dr-VSP than was seen by activation of $M_1$ muscarinic receptors. In the present study, we propose that inhibition of N-type Ca$_{\text{v}}$ channels after $G\_i/PCR$ activation occurs not only through the familiar PIP$_2$-dependent and voltage-independent pathway, but also through the phospholipid-independent, G$\beta$y-dependent pathway. Furthermore, we find that the relative predominance of these two pathways changes according to the Ca$_{\text{v}}$ $\beta$ subunit present.

MATERIALS AND METHODS

Cell culture and transfection

Human embryonic kidney tsA-201 cells were maintained in DMEM supplemented with 10% FBS and 0.2% penicillin/streptomycin in 100-mm culture dishes. Subculture was accomplished every 7 d as cell density reached 75–80% using Ca$^{2+}$-free DPBS for detaching and suspending the cells. For transfection, Lipofectamine 2000 (Invitrogen) was used when the confluency of cells reached 40–70%. In all experiments on Ca$_{\text{v}}$ channels, cells were cotransfected with $\alpha$1B, $\alpha$2B, and various $\beta$ subunits in a 1:1:1 molar ratio. Transfected cells were detached by trypsin and then moved onto poly-$\gamma$-lysine–coated chips of coverslip 24 h after transfection, 12–24 h before the experiments. The cDNAs used were the channel subunits $\alpha$1B of rat Ca$_{\text{v}}$2.2e[37b], $\beta$3, and $\alpha$2B1 (from D. Lipscombe, Brown University, Providence, RI), rat $\beta$2A (from W.A. Catterall, University of Washington, Seattle, WA), chimeric rat $\beta$2A(C3,4S) (from J. Hurley, Indiana University, Bloomington, IN), rat $\beta$2A(C3,4S)-GFP (subcloned by D. Kim), chimeric Ca$_{\text{v}}$2.2(1C1-1B) (from D. Yue, Johns Hopkins University, Baltimore, MD), Dr-VSP with IRES EGFP (from Y. Okamura, Osaka University, Osaka, Japan), human ECFP-PH(PLC$\alpha$1B) and EYFP-PH(PLC$\alpha$1B) (from K. Jalink, The Netherlands Cancer Institute, Amsterdam, Netherlands), $M_4$ muscarinic receptor (from N. Nathanson, University of Washington), human $M_5$ muscarinic receptor (from Guthrie Resource Center, Rolla, MO), and $C$ terminus of $\beta$-adrenergic receptor kinase (\betaARK-c; from R. Lefkowitz, Duke University, Durham, NC).

Current recording

The whole-cell configuration was used to record currents carried by Ba$^{2+}$ in transfected tsA-201 cells using patch clamp amplifier EPC-9 or EPC-10 USB (HEKA) at room temperature (22–25°C). Pipette resistance was 1–4 M$\Omega$, and a series resistance of 2–6 M$\Omega$ was compensated by 60%. Ba$^{2+}$ currents were measured with p/5 subtraction with a membrane holding potential of −80 mV, followed by 10-ms step depolarization to 10 mV. For full experiments, voltage pulses were repeated every 2 or 4 s. Application of step depolarization to 120 mV for 1 s induced full activation of Dr-VSP. A conditioning depolarizing prepulse was used to test the involvement of G$\beta$y.

Fürster resonance energy transfer (FRET)

Regular pulses of indigo light (438 ± 12 nm) from a monochromator (Polychrome V; TILL Photonics) excited the fluorescent proteins. Emission, which passed through a 40×, NA 0.95 dry immersion objective lens (Olympus), was separated into short (460–500 nm) and long (520–550 nm) wavelengths by appropriate filters and then acquired by two photomultipliers. Donor and acceptor signals obtained by photometry (TILL Photonics) were transferred to the data acquisition board (PCI-6221; National Instruments). Signal acquisition and real-time calculation of FRET ratio were conducted by a homemade program. To correct bleedthrough of emission of CFP into the YFP detector, cells expressing only CFP were used to obtain the ratio of the detected signal in short and long wavelength emission channels (Jensen et al., 2009). The calculated ratio ($c$Factor = CFP/YFP = 0.55) was used.
to correct the raw YFP emission signal. The bleed-through of YFP light into the CFP detector was only 0.02 and was neglected. The FRET ratio was thus calculated as follows:

\[ \text{FRETr} = \frac{\left( \text{YFP}_R - \text{cFactor} \times \text{CFP}_R \right)}{\text{CFP}_C}, \]

where YFP\(_R\) is the signal from YFP excited as result of FRET (YFP emission by CFP excitation), CFP\(_R\) is CFP emission detected by the short wavelength photomultiplier, and YFP\(_C\) is YFP emission detected by long wavelength photomultiplier.

Confocal imaging

TsA-201 cells were transfected on poly-l-lysine–coated coverslips and imaged within the next 24–48 h. The bath solution contained 160 mM NaCl, 2.5 mM KCl, 2 mM CaCl\(_2\), 1 mM MgCl\(_2\), 10 mM HEPES, and 8 mM glucose adjusted to pH 7.4 with NaOH. Images were taken with a confocal microscope (LSM 700; Carl Zeiss) at room temperature every 5 s and processed with ZEN 2009 Light Edition (Carl Zeiss) and Igor Pro (WaveMetrics).

Solutions and materials

The bath solution used for recording Ba\(^{2+}\) current contained 150 mM NaCl, 10 mM BaCl\(_2\)-2H\(_2\)O, 1 mM MgCl\(_2\), 10 mM HEPES, and 8 mM glucose and was titrated to pH 7.4 with NaOH. The pipette solution contained 160 mM CsCl, 5 mM MgCl\(_2\), 5 mM HEPES, 0.1 mM 1,2-bis(2-aminophenoxy)ethane-N,N,N,N-tetraacetic acid (BAPTA), 3 mM Na\(_2\)ATP, and 0.1 mM Na\(_3\)GTP and was titrated to pH 7.4 with CsOH. Reagents used were oxotremorine-M (Oxo-M; Research Biochemicals); BAPTA, DMEM, FBS, Lipofectamine 2000, and penicillin/streptomycin (Invitrogen); and ATP, GTP, and other chemicals (Sigma-Aldrich).

Data analysis

Pulse/Pulse Fit 8.11 software and the patch clamp amplifier (HEKA) were used for data acquisition and analysis. Supplementary data processing used Excel (Microsoft) and Igor Pro. Exponential fits were used to measure the time constants. All quantitative data were expressed as the mean ± SEM. Comparison between two groups was analyzed using the Student’s \(t\) test, and differences were considered significant at the P < 0.05 level. Comparison among more than two groups was analyzed using one-way ANOVA followed by a post hoc test.

**RESULTS**

**M\(_1\)** muscarinic receptors may suppress N-type Ca\(_{\text{V}}\) through two modulatory pathways

To dissect pathways by which Ca\(_{\text{V}2.2}\) channels are modulated by muscarinic receptor activation, tsA-201 cells were transfected with Ca\(_{\text{V}}\) subunits \(\alpha\)\(_{1B}\), \(\alpha\)\(_{2\delta}\)\(_1\), and \(\beta\)\(_3\)

![Figure 1.](https://example.com/figure1.png)

**Figure 1.** Differential modulation of Ca\(_{\text{V}2.2}\) channels by muscarinic receptors depends on the Ca\(_{\text{V}}\) \(\beta\) subunit. (A and B) Cells transfected with \(\alpha\)\(_{1B}\) (Ca\(_{\text{V}2.2}\)), \(\alpha\)\(_{2\delta}\)\(_1\), and \(\beta\)\(_3\) (A) or \(\beta\)\(_2a\) (B) subunits were cultured in the presence or absence of PTX or heat-inactivated PTX (iPTX) for 12 h. The cells were stimulated with 10 µM Oxo-M to activate muscarinic receptors or depolarized to 120 mV for 1 s to activate the coexpressed Dr-VSP. (A) Ca\(_{\text{V}2.2}\) currents before and after the stimulation of M\(_1\) (left) and M\(_4\) (right) muscarinic receptors or the activation of Dr-VSP. (B) Ca\(_{\text{V}2.2}\) currents before and after the stimulation of M\(_1\) (left) and M\(_4\) (right) muscarinic receptors or the activation of Dr-VSP. (C) Diagram of inhibitory signaling to Ca\(_{\text{V}2.2}\) channels by M\(_1\) and M\(_4\) muscarinic receptors. VD, voltage-dependent inhibition; VI, voltage-independent inhibition.
and either M₁ or M₂ receptors. Barium currents were evoked by depolarizing voltage steps. Perfusion of the muscarinic agonist Oxo-M inhibited the current by 68 ± 3% for M₁R and by 72 ± 5% for M₂R (Fig. 1 A). The differential modulatory pathways of these receptors were first isolated with the use of PTX, which inactivates Gᵢ/o by ADP ribosylation. As expected, preincubation in PTX (300 ng/ml, 12 h) strongly reduced Oxo-M–mediated current inhibition in cells expressing the Gᵢ/o-coupled M₁R (9 ± 2%; Fig. 1 A, right). Denatured PTX did not reduce the current. Furthermore, coexpressing βARK-ct, which chelates free Gβγ subunits, prevented Caᵥ2.2 current inhibition in a manner similar to PTX (Koch et al., 1994). These experiments confirmed that the primary mechanism by which M₂R inhibits Caᵥ2.2 is through Gβγ subunits released after activation of Gᵢ/o (Fig. 1 C, bottom).

In contrast, the GqPCR M₁R is thought to inhibit Caᵥ2.2 mainly via PLC and depletion of PIP₂ (Fig. 1 C, top; Gamper et al., 2004; Suh et al., 2010; Vivas et al., 2013). Preincubation with PTX did not change the inhibition of current by M₁ muscarinic receptor stimulation (Fig. 1 A [left] and B). Thus M₁R function does not involve Gᵢ/o. The PIP₂-dependent pathway can be studied in isolation using activation of the voltage-sensitive lipid phosphatase Dr-VSP, which can deplete PIP₂ from the plasma membrane quickly during a strong depolarizing pulse. The cotransfected Dr-VSP showed different extents of Caᵥ2.2 current inhibition depending on the type of Caᵥ β subunit used, as reported in our previous study (Suh et al., 2012). Depolarization with the expressed Dr-VSP inhibited current more strongly in cells cotransfected with β3 subunits (Fig. 1 A, left) than in those cotransfected with β2a subunits (Fig. 1 B). Such observations led to the main hypothesis of this study: if Dr-VSP depletes PIP₂ from the membrane yet sometimes leads to only weak current inhibition through this phospholipid-dependent pathway, other signals must contribute to the remaining significant portion of current inhibition by M₁ muscarinic stimulation. A summary of the known signaling pathways is given in Fig. 1 C, with a question mark designating this presumed additional pathway from M₁Rs.

**Figure 2.** βARK-ct attenuates M₁ muscarinic receptor–induced inhibition of Caᵥ2.2(β2a) currents. (A) Cells transfected with Caᵥ2.2, α2δ1, and β2a in the presence and absence of βARK-ct were stimulated with Oxo-M, and the Caᵥ2.2(β2a) current suppression was measured. (B) Voltage dependence of activation of the Caᵥ2.2(β2a) channel before and during M₁ receptor stimulation with Oxo-M. Dashed line is the I-V relation during Oxo-M application, which is scaled to the peak amplitude of the control. (C) Superimposed Caᵥ2.2(β2a) current traces a and b from A. In control, the b’ dashed trace is a scaled version of b. (D) Superimposed Caᵥ2.2(β2a) and Caᵥ2.2(β3) current traces for control and during the stimulation of M₁ receptor. Blue line in right panel shows the scaled trace of Caᵥ2.2(β3) current after Oxo-M application (red). Note that during the Oxo-M application, activation of Caᵥ2.2(β2a) channels (left) but not Caᵥ2.2(β3) channels (right) is dramatically slowed.
Gβγ scavenger attenuates M₁ muscarinic receptor–induced Ca²⁺ current inhibition

A series of studies was performed to determine whether Gβγ subunits might play a role in Ca²⁺.2.2 modulation by M₁ muscarinic receptor. Several results were consistent with this hypothesis, but as we eventually show, the outcomes depended on which Ca²⁺ β subunit was used. Fig. 2 A compares Ca²⁺.2 (β2a) current inhibition in control cells with that in cells expressing the Gβγ scavenger βARK-ct. The scavenger attenuates inhibition strongly, as if Gβγ is needed for the M₁ muscarinic inhibition, when the Ca²⁺ β2a channel subunit is present. There are additional hallmarks of inhibition by Gβγ subunits. The voltage-dependent activation curves showed a shift to the right by ~5 mV during the M₁ receptor activation (Fig. 2 B). Furthermore, comparison of single traces of current recorded before and after Oxo-M treatment also revealed differences in the activation kinetics of control cells but not in cells expressing βARK-ct (Fig. 2 C). The control cells transfected with α1B and β2a subunits displayed slowing of activation during Oxo-M (also see Fig. 2 D, left), whereas cells cotransfected with βARK-ct showed little change of activation. This observation makes it seem as if the inhibition of Ca²⁺.2 (β2a) by Oxo-M involves Gβγ. In contrast, for cells expressing β3 subunits, the Gβγ hallmark changes in current activation were much more prominent (Fig. 2 D, right).

We next tested the effects of Gβγ on channel inhibition by depleting PIP₂ by means of Dr-VSP (Okamura et al., 2009), which is appropriate for experimental designs involving reversible PIP₂ depletion after an activating depolarization. The standardized voltage protocol to deplete PIP₂ from the membrane by activating Dr-VSP was applied to cells expressing βARK-ct (Fig. 3 A, top). Compared with control cells, the expression of βARK-ct did not diminish the current inhibition mediated by Dr-VSP (Fig. 3 A). Thus, we conclude that βARK-ct does not impede the PIP₂-dependent pathway of Ca²⁺.2 (β2a) inhibition, but it does block the Gβγ-dependent pathway. As is summarized in Fig. 3 B, M₁ muscarinic current inhibition in β2a-expressing cells is significantly decreased by coexpressing βARK-ct, again as if Gβγ plays an important role in Ca²⁺.2 (β2a) modulation. In contrast, the smaller current inhibition upon activation of Dr-VSP was not changed by coexpressing βARK-ct. This suggests that the M₁R-induced inhibition of Ca²⁺.2 (β2a) could involve a direct action of Gβγ on the channel itself rather than an action through the phospholipid-sensitive pathway.

Single-cell assay reveals separation of fast and slow pathways in M₁R-induced current modulation

We simultaneously measured the current modulation and PIP₂ hydrolysis in single control and βARK-ct–expressing cells. Plasma membrane PIP₂ was measured by FRET between CFP- and YFP-labeled probes that selectively bind to membrane PIP₂ (van der Wal et al., 2001; Jensen et al., 2009; Suh et al., 2010; Falkenburger et al., 2013). A decrease of their FRET interaction indicates depletion of PIP₂ that releases the probe from the membrane. Fig. 4 A plots representative time courses of Ca²⁺.2 current and the FRET change in single control and βARK-ct–expressing cells. After perfusion of Oxo-M, the decrease of FRET (blue trace) was comparable in the two cells, whereas the current inhibition (red trace) showed several differences. On average, current inhibition was 46 ± 4% in control cells (n = 7) and only 25 ± 5% in βARK-ct-expressing cells (n = 6; Fig. 4 C). Furthermore, the latency for initiation of current inhibition was less than that for the FRET decrease (Fig. 4 A [right] and B). The mean lag time between the initiation of Ca²⁺ current inhibition and PIP₂ hydrolysis was 4.1 ± 0.6 s in control cells and 0.7 ± 0.3 s in βARK-ct–expressing cells (Fig. 4 D). The variability of fluorescent protein expression was compensated by normalizing the FRET change between 0 and 1 and averaging the traces (Fig. 4 B). As expected, the time constant of FRET change (PIP₂ hydrolysis) was not affected by βARK-ct (Fig. 4 E, τ = 8.7 ± 1.5 s for control and τ = 8.9 ± 1.1 s for βARK-ct–expressing cells). Fig. 5 summarizes the main results of Fig. 4. First, we estimated a putative component of current inhibition by G protein βγ subunits by subtracting the averaged current of the two groups (Fig. 5 A, dashed green line). Then we mimicked the observed time courses with

Figure 3. Differential effects of βARK-ct on M₁R- and Dr-VSP–induced inhibition of Ca²⁺.2 (β2a) currents. (A) Ca²⁺.2 (β2a) current inhibition by Dr-VSP activation in control or cells expressing βARK-ct. Cells received a 10-ms test pulse (a) and then a 1-s depolarization to 120 mV for activating the expressed VSP, followed by the second 10-ms test pulse (b). Note that current inhibition by Dr-VSP activation was not significantly different between control and βARK-ct–expressing cells. (B) Summary of the current inhibition (percentage) after the activation of M₁ receptors (Fig. 2 A) or Dr-VSP in control and βARK-ct–expressing cells. Data are mean ± SEM (n = 5–7).
exponential curves Fig. 5 B. This model showed that, as would be appropriate for direct G protein action, the difference component is fast with an exponential time constant of 1.6 s (Fig. 5 B, dashed green line). The remaining component, attributed to PIP2 signaling, has a slow time course like the FRET change.

**Gβγ-dependent, but not PIP2-dependent, modulation is absent in a chimeric N-type channel**

The effects of Gβγ on Cav2.2 regulation were investigated through a more direct approach. We used a mutated CaV2.2 α subunit that does not bind Gβγ subunits. In this chimera, called CaV2.2 α1C-1B, the N terminus of the CaV2.2(α1B) subunit was replaced by the N terminus of the CaV1.2(α1C) subunit, which lacks the N-terminal Gβγ-binding site of α1B (Agler et al., 2005). Fig. 6 A illustrates the modulation of N-type currents in cells expressing this α1 construct with β2a upon activation of either M2R or M1R receptors. The chimera shows a smaller response to either receptor, as is summarized in Fig. 6 C. The M4R, a Gαq/11PCR, is anticipated to signal through the direct binding of

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**Figure 4.** Simultaneous measurement of current inhibition and PIP2 hydrolysis in single cells. All cells coexpressed Cav2.2(β2a) channel subunits, PH domain probes, and M1 receptors. (A) Cav2.2(β2a) current and PIP2 (FRET ratio, FRET) were measured simultaneously in single cells in the absence (top) or presence (bottom) of βARK-ct. 10 µM Oxo-M was applied during solid bars. (right) Scaled responses from the dashed boxes shown in the left panels. The initiation of the muscarinic response is indicated by arrows. (B) Normalized mean time courses of current suppression and PIP2 hydrolysis (FRET) from single cells without or with βARK-ct expression. (C) Summary of maximum current inhibition by Oxo-M in control and cells expressing βARK-ct. **, P < 0.01, compared with control. (D) Effect of βARK-ct on lag time between the initiation of current inhibition and the FRET change. ***, P < 0.001. (B–D) Data are mean ± SEM. (E) Analysis of the onset time (τ) for current inhibition and PIP2 hydrolysis (FRET) in control and βARK-ct-expressing cells. Data are mean ± SEM (n = 6–7). **, P < 0.01.
M₁R-induced, voltage-dependent modulation of CaV2.2 channels is dependent on CaV2.2 β subtypes

Our results reveal that M₁Rs use two pathways to suppress CaV2.2 currents. We now examine further whether the choice between inhibitory pathways might depend on the CaV channel β subunit. Fig. 7A shows that different β subunits localize differently. Expressed by themselves, β2a subunits are membrane localized and β2b and β3 subunits are soluble in the cytosol (Fig. 7A). Palmitoylation on two consecutive N-terminal cysteines makes β2a subunits membrane resident (Chien et al., 1995; Hurley et al., 2000), and when the cysteines are substituted by serines, the mutant β2a(C3,4S) moves to the cytosol. Appending a membrane-targeting Lyn sequence to β3 makes the chimeric Lyn-β3 subunit localize at the plasma membrane (Suh et al., 2012).

Using the Gβγ-resistant chimera CaV2.2(α1C-α1B), we saw that coexpression with β2a makes channels that are more sensitive to the Gβγ-dependent pathway and less sensitive to the PIP₂-dependent pathway, whereas coexpression with β3 makes channels more sensitive to the PIP₂-dependent pathway relative to the Gβγ-dependent pathway. The same switch applies to wild-type CaV2.2 subunits (Fig. 6E). However, interestingly, with the CaV2.2 subunit, the inhibition upon activation of M₁ muscarinic receptors or upon PIP₂ depletion by VSP was much higher than with β2a-containing chimeric channels. Indeed, it was more like the inhibition of wild-type CaV2.2 channels. This fits well with the concept that M₁ muscarinic inhibition of CaV2.2 channels with β3 is voltage independent and does not need Gβγ subunits.

Gβγ, so the chimera should lack modulation, exactly as seen. Inhibition dropped from 60 to 2%. However, now we find that signaling from the M₁R, a GαPCR, is also decreased by the chimera, giving only ~10% inhibition of current instead of the >40% seen in control cells (Fig. 1B), consistent with the concept that M₁Rs also can signal by the Gβγ pathway. Continuing on, as expected, CaV2.2(α1C-1B) (β2a) current inhibition by activation of Dr-VSP was not changed compared with control conditions (Fig. 6F and C).

We now consider whether switching from the CaV β2a subunit to the CaV β3 subunit alters the modulation of the CaV2.2(α1C-1B) chimera. Qualitatively, the modulation had similar features with either β subunit (Fig. 6B). As expected, activation of M₂Rs, which acts primarily through the Gβγ pathway, produced very little inhibition (Fig. 6E). However, interestingly, with the CaV β3 subunit, the inhibition upon activation of M₁ muscarinic receptors or upon PIP₂ depletion by VSP was much higher than with β2a-containing chimeric channels. Indeed, it was more like the inhibition of wild-type CaV2.2 channels. This fits well with the concept that M₁ muscarinic inhibition of CaV2.2 channels with β3 is voltage independent and does not need Gβγ subunits.

Prepulse, the inhibition percentage upon Oxo-M perfusion was much reduced in β2a expressing cells, ~40 to ~10%, and only slightly reduced in β2b, β3, and β2a(C3,4S)-expressing cells (Fig. 7B). Thus, the voltage-dependent inhibition of CaV2.2 depends on the subcellular location of the β subunits and is stronger in channels with membrane-binding β subunits. The voltage-independent inhibition is stronger in channels with cytosolic β subunits.

The PIP₂-dependent portion of inhibition was tested in cells with different β subunits (Fig. 8A) using the potent PIP₂ 5-phosphatase Dr-VSP. Cells transfected with CaV2.2, various β subunits, and Dr-VSP were depolarized to 120 mV for 1 s. The PIP₂ depletion-dependent inhibition of current was low at 10% with β2a compared with 40–60% with cytosolic β2b, β3, and β2a(C3,4S). When membrane-targeted Lyn-β3 was expressed, the PIP₂

Figure 5. Kinetic assays reveal participation of Gβγ in M₁ receptor–induced CaV2.2(β2a) current inhibition. (A) Summary of Fig. 4B. The estimated effect of M₁R-mediated release of Gβγ on CaV2.2 current (ΔCaV2.2, green) was calculated by subtracting the mean current of control and βARK-ct (orange) from that of control ARK-ct (red). Blue trace indicates M₁R-induced PIP₂ hydrolysis observed by FRET change. (B) Interpretation of the CaV2.2 current inhibition as a series of exponential curves in control and βARK-ct-expressing cells. Icontrol = exp(−t/4.05) (t > 0; red), IβARK-ct = 0.52*exp(−(t-4)/8.78) (t > 4; orange), FRET = exp(−(t-4)/8.78) (t > 4; blue). Predicted Gβγ-induced CaV current inhibition (dashed green) was calculated by subtracting the mean current of βARK-ct from that of control. The amplitude of IβARK-ct is determined by obtaining the relative current amplitude between control and βARK-ct-expressing cells.
sensitivity decreased to \(~20\%\) (Fig. 8 B). Fig. 8 B contrasts the inhibition percentages of PIP$_2$-dependent, voltage-independent and G$\beta$$\gamma$-mediated, voltage-dependent pathways. Though M$_1$ receptor stimulation suppresses all combinations of CaV$_{2.2}$ and $\beta$ subunits, depending on the types of CaV$\beta$ subunit, the modulatory mechanism by M$_1$ receptor is clearly different.

**DISCUSSION**

We have shown that with an appropriate choice of CaV$\beta$ subunit, a G$_q$PCR can signal by G$\beta$$\gamma$ subunits to suppress N-type Ca$^{2+}$ currents. This contrasts with the simpler view that PTX-insensitive, G$_q$PCRs modulate Ca$^{2+}$ channels exclusively by actions downstream of PLC and that only PTX-sensitive G$_i$/oPCRs can modulate through G$\beta$$\gamma$, and it extends earlier clear suggestions of G$\beta$$\gamma$ roles in M$_1$R signaling (Kammermeier et al., 2000; Melliti et al., 2001; Gamper et al., 2004; Suh et al., 2010; Vivas et al., 2013). Our adjusted working hypothesis is summarized as a flow chart in Fig. 9 A. M$_1$Rs inhibit CaV$_{2.2}$ not only through G$_q$ and PLC but also through the G$\beta$$\gamma$ pathway, whereas M$_2$Rs suppress principally through the G$\beta$$\gamma$ pathway. Furthermore, for M$_1$Rs, the choice between PLC and G$\beta$$\gamma$ pathways is biased by the subtype of CaV$\beta$ subunit expressed. Channels with the membrane–lipid-interacting $\beta$ subunit $\beta$2a were more sensitive to the G$\beta$$\gamma$-dependent pathway and less to the PIP$_2$ depletion, whereas channels with cytosolic $\beta$ subunits, including $\beta$2b, $\beta$3, and $\beta$2a(C3,4S), were more sensitive to PIP$_2$ depletion (Fig. 9 B). Our data also showed that even though the maximum inhibition of N-type CaV current by M$_1$ receptors ranged from 40 to 65\% for different cytosolic CaV$\beta$ subunits, the relative proportion of the total inhibition mediated by PIP$_2$ and

**Figure 6.** Voltage-dependent muscarinic modulation disappears in G$\beta$$\gamma$-insensitive chimeric CaV$_{2.2}$(a1C-1B) channels. (A) Effects on CaV$_{2.2}$(a1C-1B) ($\beta$2a) currents of M$_1$ and M$_2$ muscarinic receptor stimulation. The current amplitude was measured at 10 mV every 4 s. Insets show currents a and b superimposed. (B) Inhibition by Dr-VSP activation of CaV$_{2.2}$(a1C-1B) ($\beta$2a) currents. Cells received a test pulse (a) and then were depolarized to 120 mV for 1 s, followed by a second test pulse (b). Current traces before and after the Dr-VSP activation in cells expressing the a1C-1B and $\beta$3 subunits are shown. (C) Summary of current suppression by muscarinic stimulation or Dr-VSP activation. (D) Current traces before (a) and during (b) the Oxo-M application (left and middle) or Dr-VSP activation (right) in cells expressing the a1C-1B and $\beta$3 subunits. Effects on CaV$_{2.2}$(a1C-1B) ($\beta$3) currents of M$_1$ and M$_2$ muscarinic receptor stimulation and Dr-VSP activation were traced as above. (E) Summary of current suppression by muscarinic stimulation or Dr-VSP activation. (C and E) Data are mean ± SEM ($n=5$ for each bar).
Gβγ was almost the same for each of the cytosolic β subtypes. For M1 muscarinic inhibition with cytosolic β subunits, the fractional distribution between the Gβγ-dependent pathway and the PIP₂-dependent pathway was ~20 and ~80% of the total (Fig. 9 B). In contrast, in cells expressing the membrane-localized β2a subunits, the fractional distribution was reversed, ~20% and ~80%, and in cells expressing the membrane-targeted form of β3, Lyn-β3, the distribution was equal, ~50 and 50%. This intermediate effect of Lyn-β3 is consistent with its weaker effects on current inactivation and on PIP₂ depletion–mediated suppression compared with control β3 (Suh et al., 2012).

Several of our findings support Gβγ as one of the inhibitory signals in M1 muscarinic suppression of Ca₂⁺ channels. (a) M1 receptor activation shifts the voltage dependence of activation of channels rightward by ~5 mV and slows the activation kinetics, comparable with Gβγ-dependent regulation of N-type channels in sympathetic neurons (Elmslie et al., 1990; Beech et al., 1992; Boland and Bean, 1993). (b) Coexpression of the Gβγ chelator reduced inhibition of the Ca²⁺ currents by M₁R stimulation (Kammermeier et al., 2000; Melliti et al., 2001). (c) M₁ receptor activation induces a fast component of channel inhibition in addition to a slow one (Melliti et al., 2001). The fast, βARK-ct–sensitive component precedes the slow one by 3 s, about the time difference between Gq activation and PIP₂ depletion (Jensen et al., 2009). (d) A chimeric α1 calcium channel subunit unable to bind to Gβγ showed much less M₁ receptor–induced inhibition. Lastly (e), the suppression of Caᵥ2.2 current by M₁Rs could be reversed partially by applying a strong positive prepulse. Thus, for M₁R signaling, a Gβγ-mediated, voltage-dependent pathway coexists with the well-known slow PLC and PIP₂-sensitive voltage-independent pathway that is not affected by the expression of βARK-ct, chimeric α1 subunits, or depolarizing prepulses (Fig. 6 A; Melliti et al., 2001; Gamper et al., 2004; Suh et al., 2010). With M₁Rs, neither pathway is sensitive to PTX.

Our single-cell experiments combining FRET and patch clamp confirmed that M₁ receptors can suppress the N-type current through the fast Gβγ-mediated

![Figure 7](image-url)

**Figure 7.** Cytosolic β subunit decreases the Gβγ-mediated, voltage-dependent suppression of Caᵥ2.2 currents. (A) N-terminal amino acid sequences of β2a, β2a(C₃,₄S), β2b, and β3 subunit with GFP as a fluorescent label. In the palmitoylation-resistant mutant β2a(C₃,₄S), both palmitoylated cysteine residues (*) blue are replaced with serine (red). Lyn-β3 is labeled with YFP. (bottom) Confocal images of the β subunits expressed in tsA-201 cells. (B) Inhibition of Caᵥ2.2 current by M₁ receptors is significantly relieved by a prepulse (+PP) in cells with membrane-localized β subunits but not in cells with cytosolic β subunits. Cells were given a test pulse (-PP) and then depolarized to 130 mV for 20 ms, followed by the second test pulse after 20 ms (+PP). The experiments were performed before (control) and during the Oxo-M application (+Oxo-M). (bottom) Summary of the prepulse experiments in control and Oxo-M–perfused cells with different Caᵥ2.2 β subunits. The current amplitude after Oxo-M application is given as percentage of the initial control. Data are mean ± SEM (n = 5–6). *, P < 0.01.
signaling pathways and that the fast current inhibition is independent from and unable to be triggered by the slow PIP₂ depletion. Many previous studies suggested that N-type channel suppression by GₛPCRs occurs through both fast and slow pathways (Hille, 1994; Melliti et al., 2001; Mitra-Ganguli et al., 2009). Here, we clearly show that the M₁ receptor–mediated channel inhibition and the PIP₂ depletion are temporally separated (a lag time) in a live single cell. Current inhibition begins earlier than PIP₂ depletion, and the lag time was almost completely abolished by the Gₛβγ scavenger βARK-ct, resulting in almost the same time constants for the PIP₂ depletion and the current inhibition. This temporal separation can be interpreted as a Gₛγ-dependent Caᵥ current inhibition that occurs immediately after the receptor stimulation in synapse, followed by a PLC- and lipid-dependent slow current inhibition, if the receptor activation lasts longer than the lag time. Hence the lag time determines a threshold for diversity of signaling in synaptic transmission. For example, short (<2 s) M₁ receptor stimulation may suppress the Caᵥ currents only through the fast inhibitory pathway, whereas longer receptor stimulation may regulate slower signaling by PIP₂ depletion, PKC activation, Ca²⁺ release from the ER, and gene expression by activating the downstream PLC signaling. Thus, our new finding would provide clues to elucidate the role of M, R and Caᵥ channels in synaptic plasticity such as Gₛα₄-mediated long-term depression (Kamsler et al., 2010; Collingridge et al., 2010).

Caᵥ β subunit isoforms have profound effects on calcium channel trafficking, inactivation kinetics, and susceptibility to modulation. A key distinction governing the actions of isoforms is whether they are palmitoylated and membrane directed (β₂a, Lyn-β₃) or not (β₂a(C₃,4S), β₂b, β₃; Fig. 9; Chien et al., 1995; Hurley et al., 2000; Feng et al., 2001). Thus, Feng et al. (2001) showed that raising free Gₛβγ by transient expression of Gₛ subunits induced kinetic slowing of activation in Caᵥ₂.₂ channels expressed with lipidated β₂a subunit, whereas it had little effect in channels with other types of Caᵥ β subunit. Similarly, in our experiments, expression of β₂a gave a stronger Gₛβγ-mediated, voltage-dependent inhibition, whereas expression of β₃ gave a stronger PIP₂-mediated, voltage-independent regulation. The dependence on subcellular localization was confirmed by reversing the targeting of the β₂a and β₃ subunits. A cytosolic β subunit conferred reduced voltage dependency and increased voltage independency of the M₁ muscarinic inhibition of N-type calcium channels. So far, the mechanism of how the membrane-targeted Caᵥ β subunit regulates the Gₛβγ signaling to Caᵥ₂.₂ channel is not clear. However, it is well known that the intracellular I-II loop (as well as N and C termini) of the α₁ subunit is the major target site for both Gₛβγ and Caᵥ subunits, and thus binding of Caᵥ β₂a to the I-II loop through the BID domain and the plasma membrane through N-terminal palmitoyl groups at the same time may affect the mobility of this region in an unfavorable way, making the Caᵥ channel retain high Gₛβγ binding affinity and be more susceptible to βγ subunit–mediated inhibition (Zamponi and Currie, 2013).

Our findings are important to understand regulation of Caᵥ₂.₂ channels by neurotransmitter receptors...
that couple to Gq in excitable cells. Previous studies reported that the lipitated β2a subunit is highly expressed in chromaffin cells, form noninactivating N-type channel currents, and contribute to hormone release (Cahill et al., 2000). The β2a subunit is also expressed broadly in brain, heart, and aorta (Hullin et al., 1992; Pichler et al., 1997), though only a small proportion of endogenous CaV2.2 interacts with the β2a subunit (Scott et al., 1996). SCG neurons express mostly β2a subunits but also β3 and β4 (Heneghan et al., 2009), accounting for relatively slow N-type current inactivation and less sensitivity to Dr-VSP–mediated PIP2 depletion compared with expression systems with the β3 subunit alone (Suh et al., 2012). However, Gamper et al. (2004) and Vivas et al. (2013) clearly showed that the N-type Ca2+ current was still strongly suppressed by membrane PIP2 depletion in neurons, suggesting that a disproportionate fraction of β3 subunits become bound to N-type α1B subunits. This is supported by previous studies showing that the modulation of N-type currents by M1 receptors in SCG neurons appears as a mixture of voltage-dependent and -independent pathways (Kammermeier et al., 2000; Suh et al., 2010) and that β3 subunits are the predominant form associated with brain N-type Ca2+ channels (Vance et al., 1998). Furthermore, the temporal expression pattern of CaV β subunits varies across brain tissue and within a single cell type during the development (Vance et al., 1998; Wittemann et al., 2000). This implies that regulation of N-type channels in nerve might change with developmental stage.

In conclusion, our study provides some insight into the possible mechanism of how GqPCRs modulate the Ca2+ channel activity and thus regulate intracellular Ca2+ concentrations in excitable nerve terminals and tissues (Kubista et al., 2009). Our results showed that Gq/11PCRs can inhibit CaV2.2 channels through the Gβγ-mediated, voltage-dependent pathway and the PIP2-sensitive, voltage-independent pathway and that this dual mode of inhibition after GqPCR activation is tightly controlled by the type of CaV β subunit present. Considering the previous observations that CaV channels can be regulated by diverse intracellular signals, such as protein kinase C and SNARE proteins (Swartz et al., 1993; Zamponi et al., 1997; Hamid et al., 1999; Magga et al., 2000), our data provide further intricacy in the G protein modulatory mechanism of Ca2+ influx and therefore neurotransmitter release in the synaptic terminal.

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