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Modulation of Ca\textsubscript{v}1.3b L-type calcium channels by M\textsubscript{1} muscarinic receptors varies with Ca\textsubscript{v}β subunit expression

Mandy L. Roberts-Crowley\textsuperscript{1} and Ann R. Rittenhouse\textsuperscript{1,2*}

**Abstract**

**Objectives:** We examined whether two G protein-coupled receptors (GPCRs), muscarinic M\textsubscript{1} receptors (M\textsubscript{1}Rs) and dopaminergic D\textsubscript{2} receptors (D\textsubscript{2}Rs), utilize endogenously released fatty acid to inhibit L-type Ca\textsuperscript{2+} channels, Ca\textsubscript{v}1.3. HEK-293 cells, stably transfected with M\textsubscript{1}Rs, were used to transiently transfect D\textsubscript{2}Rs and Ca\textsubscript{v}1.3b with different Ca\textsubscript{v}β-subunits, allowing for whole-cell current measurement from a pure channel population.

**Results:** M\textsubscript{1}R activation with Oxotremorine-M inhibited currents from Ca\textsubscript{v}1.3b coexpressed with α\textsubscript{2}δ-1 and a β\textsubscript{1b}, β\textsubscript{2a}, β\textsubscript{3}, or β\textsubscript{4}-subunit. Surprisingly, the magnitude of inhibition was less with β\textsubscript{2a} than with other Ca\textsubscript{v}β-subunits. Normalizing currents revealed kinetic changes after modulation with β\textsubscript{1b}, β\textsubscript{3}, or β\textsubscript{4}, but not β\textsubscript{2a}-containing channels. We then examined if D\textsubscript{2}Rs modulate Ca\textsubscript{v}1.3b when expressed with different Ca\textsubscript{v}β-subunits. Stimulation with quinpirole produced little inhibition or kinetic changes for Ca\textsubscript{v}1.3b coexpressed with β\textsubscript{2a} or β\textsubscript{3}. However, quinpirole inhibited N-type Ca\textsuperscript{2+} currents in a concentration-dependent manner, indicating functional expression of D\textsubscript{2}Rs. N-current inhibition by quinpirole was voltage-dependent and independent of phospholipase A\textsubscript{2} (PLA\textsubscript{2}), whereas a PLA\textsubscript{2} antagonist abolished M\textsubscript{1}R-mediated N-current inhibition. These findings highlight the specific regulation of Ca\textsuperscript{2+} channels by different GPCRs. Moreover, tissue-specific and/or cellular localization of Ca\textsubscript{v}1.3b with different Ca\textsubscript{v}β-subunits could fine tune the response of Ca\textsuperscript{2+} influx following GPCR activation.

**Keywords:** Acetylcholine, Ca\textsubscript{v}β subunit, Dopamine, L-type calcium current

**Introduction**

Voltage-gated Ca\textsuperscript{2+} channels (VGCCs) control membrane excitability, gene expression, and neurotransmitter release \cite{1}. Alterations in these cellular functions occur when GPCR-activated signal transduction cascades modulate VGCCs. In medium spiny neurons (MSNs) of the striatum, GPCRs, including M\textsubscript{1}Rs and D\textsubscript{2}Rs, inhibit VGCC activity \cite{2,3}. These GPCRs specifically inhibit Ca\textsubscript{v}1.3 L-current, decreasing the output of MSNs \cite{3,4} and may have functional consequences for motor control \cite{5,6}.

Although present in MSNs, M\textsubscript{1}R signaling has been characterized most thoroughly in superior cervical ganglion (SCG) neurons. M\textsubscript{1}Rs couple to Go\textsubscript{q} and phospholipase C (PLC) to inhibit native L- and N-VGCC currents \cite{7–9}. This signal transduction cascade, referred to as the slow or diffusible second messenger pathway, is characterized as pertussis toxin (PTX)-insensitive, voltage-independent, and requiring intracellular Ca\textsuperscript{2+} to function \cite{10}. Our laboratory has identified arachidonic acid (AA) as a critical effector in the slow pathway \cite{9}. Exogenously applied AA inhibits L-current \cite{11–13}, which in SCG neurons most likely arises from Ca\textsubscript{v}1.3 \cite{14}. Moreover, Ca\textsuperscript{2+}-dependent cytosolic phospholipase A\textsubscript{2} (cPLA\textsubscript{2}) appears critical for release of AA from phospholipids following M\textsubscript{1}R activation; loss of cPLA\textsubscript{2} activity by pharmacological antagonists or gene knockout ablates L-current inhibition \cite{15,16}.
Additionally, D2Rs inhibit L-current via a diffusible second messenger pathway involving phospholipase C (PLC), InsP3, and calcineurin in MSNs [3]. While both GPCRs signal through PLC, they share another commonality: their activation releases AA from striatal neurons [17, 18] and transfected cell lines [19, 20]. Therefore, D2Rs may also inhibit L-(CaV1.3) and N-(CaV2.2) currents via a pathway utilizing cPLA2 to release AA. In the present study, we tested whether the M1R and D2R pathways converge to modulate recombinant L-VGCC activity.

**Materials and methods**

**Cell culture**

Human embryonic kidney cells, stably transfected with the M1 muscarinic receptor (HEK-M1) [a generous gift from Emily Liman, University of Southern California, originally transfected by [21]] were propagated at 37 °C with 5% CO2 in Dulbecco’s MEM (DMEM)/F12 supplemented with 10% FBS, 1% G418, 0.1% gentamicin, and 1% HT supplement (Gibco Life Technologies). Cells were passaged when 80% confluent.

**Transfection**

HEK-M1 cells, grown in 12-well plates (~60–80% confluent), were transfected with a 1:1:1 molar ratio of CaV1.3b or CaV2.2, αδ-1 and different CaVβs [22], using Lipofectamine PLUS (Invitrogen) according to the manufacturer’s instructions. Cells were co-transfected with green fluorescent protein (GFP) to identify transfected cells. Constructs for CaV1.3b (+exon11, Δexon32, +exon42α; GenBank accession AF370009), CaV2.2 (‘10, Δexon18a, Δexon24α, +exon31α, +exon37β, +exon46; AF055477), CaVβ3 (#M88751) and αδ-1 (#AF286488) were provided by Diane Lipscombe (Brown University). CaVβ1b (#X61394), CaVβ2α (#M80545), and CaVβ4 (#L02315) constructs were provided by Edward Perez-Reyes (University of Virginia). The D1aR (#AF199329) construct was provided by Hubert H. M. Van Tol (University of Toronto). D2R cDNA (#NM_000795) was obtained from the UMR cDNA Resource Center (https://www.cdna.org). Per well, a total of 0.5 μg of DNA (of which GFP cDNA was less than 10%) was used following the methods of Roberts-Crowley and Rittenhouse (2009) [13].

**Electrophysiology**

Whole-cell currents were recorded following the methods of Liu et al. [11]. High resistance seals were established in Mg2+ Tyrode’s (in mM): 5 MgCl2, 145 NaCl, 5.4 KCl, and 10 HEPES, brought to pH 7.50 with NaOH. Once a seal was established and the membrane ruptured, the Tyrode’s solution was exchanged for external bath solution (in mM): 125 NMG-aspartate, 20 Ba-acetate, 10 HEPES, brought to pH 7.50 with CsOH. Only cells with ≥0.2 nA of current were used. Data were acquired using Signal 2.14 software (CED) and stored for later analysis on a personal computer. Linear leak and capacitive currents were subtracted from all traces.

**Drugs**

All chemicals were purchased from Sigma unless otherwise noted. FPL 64176 (FPL), nimodipine (NIM), and oleoyloxyethyl phosphorylcholine (OPC, Calbiochem) were prepared as stock solutions in 100% ethanol. Quinpirole (quin) and Oxotremorine-M (Oxo-M, Tocris) were dissolved in DW and stored as 10 mM stock solutions at −70 °C. Stocks were diluted daily to the final concentration by at least 1000-fold with external solution. For ethanol-prepared stocks, the final ethanol concentration was less than 0.1%.

**Statistical analysis**

Data are presented as the mean ± s.e.m. Data were analyzed for significance using a Student’s paired t-test for two means, or a one-way ANOVA followed by a Tukey multiple-comparison post hoc test. Statistical significance was set at p < 0.05 or < 0.001. Analysis programs included Signal (CED), Excel (Microsoft), and Origin (OriginLab).

**Results**

**Characterization of recombinant CaV1.3 current as L-type in HEK-M1 cells**

Whole-cell L-currents, from β3-containing L-channels, elicited from a holding potential of −60 mV to a test potential of −10 mV, averaged −4699 ± 279 pA (n = 3) compared to −9 ± 1 pA for HEK-M1 cells transfected with only accessory subunits (n = 10, P < 0.001). Lack of current from cells transfected without CaV1.3b, confirmed that HEK-M1 cells exhibit little endogenous Ca2+ current and transfection of accessory subunits does not upregulate endogenous Ca2+ channels. Recombinant current was confirmed as L-type by showing sensitivity to the L-VGCC antagonist NIM. NIM inhibited β3-containing currents (Additional file 1A) in a concentration-dependent manner (Additional file 1B). Currents were also sensitive to FPL, which enhanced current from β2, and β3-containing channels and produced long-lasting tail currents upon repolarization (Additional file 1C, D). Additionally, FPL produced a slight hyperpolarizing voltage shift in the peak inward current and enhanced current amplitude at all voltages (Additional file 1E). Additional file 1F demonstrates that FPL enhanced the long-lasting tail current in a concentration-dependent manner. These pharmacological and biophysical
properties show that transfection of HEK-M1 cells with CaV1.3b and accessory subunits produce currents with L-type characteristics.

**The CaVβ-subunit varies the magnitude of CaV1.3 current inhibition by M1Rs**

In MSNs, M1R stimulation inhibits L-current in CaV1.2 knockout animals [4]. Only CaV1.2 and CaV1.3 constitute the L-type CaVα1 subunits expressed in brain [23], implying that M1Rs specifically inhibit CaV1.3 current. Using a cell line transfected with only CaV1.3 channels provides molecular proof for the identity of the inhibited channel. Therefore, to determine if activation of M1Rs inhibits CaV1.3 activity, peak current amplitudes were measured prior to and following application of the M1R agonist Oxo-M. Figure 1a compares representative current traces for CaV1.3b coexpressed with β1b, β2a, β3, or β4-subunits in the absence or presence of Oxo-M.

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**Fig. 1 CaV1.3b current inhibition and kinetic changes produced by M1R stimulation are CaVβ-subunit dependent.**

- **a** Representative current traces from CaV1.3b coexpressed with β1b, β2a, β3, or β4 before (black) or 1 min after applying 10 μM Oxo-M (red).
- **b** Current traces from a were normalized to the end of the test pulse.
- **c** Summary of Oxo-M inhibition of CaV1.3b with different CaVβ-subunits. Maximal inward current amplitudes were measured after the onset of the test pulse using a trough seeking function (peak current). Percent of current inhibition was calculated as:

\[
\% \text{I}_{\text{inhib}} = \frac{100 \times (I_{\text{CTL}} - I_{\text{DRUG}})}{I_{\text{CTL}}},
\]

where I_{CTL} and I_{DRUG} are the average maximum current amplitude of 5 traces prior to and after 1 min of application of test material (unless otherwise noted).

- **d** Schematic of quantification of kinetic changes.
- **e**, **f** Summary of kinetic changes (n = 4–6, **P < 0.001, *P < 0.05) open bars, control; hatched bars, Oxo-M.

- **e** Time to peak (TTP) was measured using a minimum seeking function in Signal within the test pulse duration.

- **f** Current remaining (r40) was measured from an average of five normalized current traces per condition using the equation:

\[
\text{r}40 = 100 \times \frac{I_{\text{end}}}{I_{\text{peak}}},
\]

where r40 is the percent of the maximum inward current remaining at the end of a 40 ms test pulse; I_{end} is the current amplitude at the end of the test pulse; I_{peak} is the maximum inward current measured during the test pulse.
After 1 min, Oxo-M significantly inhibited L-current by $58 \pm 8\%$ with $\beta_{1b}$; $36 \pm 12\%$ with $\beta_{2a}$; $66 \pm 6\%$ with $\beta_3$; and $72 \pm 10\%$ with $\beta_4$ (Fig. 1c). Oxo-M elicited kinetic changes that were visualized by normalizing individual traces to the end of the 40 ms test pulse (Fig. 1b), which were quantified by measuring TTP and r40 (Fig. 1d). TTP (Fig. 1e) and r40 (Fig. 1f) decreased following Oxo-M with $\beta_{1b}$, $\beta_3$, or $\beta_4$; however, no changes were detected with $\beta_{2a}$ ($P \geq 0.11$ for TTP; $P \geq 0.40$ for r40). These differences in the magnitude of current inhibition and kinetics suggest that the Ca$\alpha$-subunit affects M1R modulation of Ca$\alpha_{1.3}$b.

**Dopamine D$_2$ receptors inhibit Ca$\alpha_{2.2}$ but not Ca$\alpha_{1.3}$ currents**

Both M1Rs and D$_2$Rs activate pathways involving G proteins, PLC, and AA release (Fig. 2a). However, whether L-current inhibition by D$_2$Rs shows varied inhibition depending on Ca$\alpha$-subunit expression has not been examined. Therefore, we coexpressed D$_2$Rs with Ca$\alpha_{1.3}$b, Ca$\alpha_{2.2}$, and Ca$\alpha_1$-b subunits. While Oxo-M inhibited Ca$\alpha_{1.3}$b-$\beta_{2a}$ currents over time (Fig. 2b), quin, a D$_2$R agonist, had no effect on current amplitude (Fig. 2c) or kinetics (Fig. 2c inset, g). Since Ca$\alpha_{1.3}$b-$\beta_{2a}$ current shows less inhibition and no kinetic changes with Oxo-M, we tested whether Ca$\alpha_{1.3}$b-$\beta_3$ current was sensitive to modulation by quin. Figure 2d shows a time course of Ca$\alpha_{1.3}$b-$\beta_3$ current inhibition by Oxo-M whereas the time course with quin (Fig. 2e) shows no inhibition or kinetic change (Fig. 2e inset, g). Several concentrations of quin were tested but did not inhibit L-current to the same extent as Oxo-M (Fig. 2f). D$_2$Rs appeared to desensitize with 10 $\mu$M quin. Application of quin for 1 min to cells co-transfected with the D$_2$R-like family member, D$_4$R, inhibited L-current by $8.5 \pm 2.5\%$ and did not produce changes in TTP or r40 (Additional file 2).

To confirm that lack of L-current inhibition was not due to poor expression of D$_2$Rs, we repeated the experiment but substituted Ca$\alpha_{2.2}$ for Ca$\alpha_{1.3}$b to serve as a positive control since activated D$_2$Rs also inhibit Ca$\alpha_{2.2}$ [24–26]. Quin inhibited Ca$\alpha_{2.2}$ by $45 \pm 7\%$ after 30 s and $48 \pm 4\%$ after 1 min (Fig. 3a). Inhibition occurred specifically by activating transfected D$_2$Rs because cells transfected without D$_2$Rs showed no response to quin (Fig. 3a, n = 3). Moreover, N-current inhibition by quin occurred in a concentration-dependent manner (Fig. 3b, n = 3–5). Compared to lower concentrations, 10 $\mu$M quin resulted in less inhibition; inhibited current did not recover upon wash, suggesting this concentration causes receptor desensitization (data not shown). Thus, our findings indicate that transfected D$_2$Rs functionally express in HEK-M1 cells to modulate Ca$\alpha_{2.2}$, but not Ca$\alpha_{1.3}$b VGCC activity.

**M$_1$R and D$_2$R pathways use different signaling mechanisms to inhibit N-current**

To compare D$_2$Rs and M$_1$Rs signaling pathways on Ca$\alpha_{2.2}$ current, we first confirmed that activation of the stably transfected M$_1$Rs could suppress N-current. Indeed, Oxo-M inhibited currents from beta-3-containing channels by $70 \pm 5\%$ after 30 s (Fig. 3c). When incubated with the PLA$_2$ antagonist OPC, cells showed less N-current inhibition by Oxo-M, $14 \pm 8\%$ inhibition after 30 s (Fig. 3c). In contrast, low concentrations of quin still suppressed N-current in the presence of OPC (Fig. 3d). Inhibition was relieved by pre-pulse facilitation (Fig. 3e, g, h) and occurred in the presence of BSA, which acts as a scavenger of free AA (Fig. 3f–h), suggesting that quin mediates membrane-delimited inhibition of N-current. These findings suggest that M$_1$Rs and D$_2$Rs do not share a common pathway leading to N-current inhibition.

**Discussion**

Previously, the Ca$\alpha_{1.3}$b splice variant of L-VGCCs, found in MSNs, had not been specifically tested for modulation by GPCRs. Here, using HEK-M1 cells, we present the novel finding that M1R stimulation inhibits Ca$\alpha_{1.3}$b L-current with the accessory Ca$\alpha$-subunit determining the magnitude of inhibition. In contrast, stimulation of transfected D$_2$Rs with quin does not recapitulate L-current inhibition observed in MSNs [3]. Pharmacological sensitivity to both FPL and NIM confirmed that Ca$\alpha_{1.3}$b expressed in HEK-M1 cells behaves similarly to other recombinant Ca$\alpha_{1.3}$ VGCCs [22, 27].

We also report that N-current modulation by the D$_2$R short splice variant appears similar to membrane-delimited inhibition by the D$_2$R long form [24]. In this form of modulation, when G proteins are activated, G$\beta$G directly binds to and inhibits Ca$\alpha_{2.2}$ which can be reversed by strong prepulses [10, 28]. Indeed, D$_2$R-mediated inhibition of Ca$\alpha_{2.2}$ was independent of PLA$_2$, whereas blockers of PLA$_2$ abolished inhibition by M$_1$Rs. Thus, the membrane-delimited pathway may be at least partially responsible for the inhibition of Ca$\alpha_{2.2}$ by D$_2$Rs in MSNs [25].

In our experiments, the short splice variant of Ca$\alpha_{1.3}$ (Ca$\alpha_{1.3}$b) was unaffected by activation of D$_2$Rs, expressed in HEK-293 cells, similar to a previous report on Ca$\alpha_{1.3}$a, which has a longer C-terminus [24]. Since neither D$_2$R-long inhibited Ca$\alpha_{1.3}$a [24], nor D$_2$R-short inhibited Ca$\alpha_{1.3}$b (Fig. 2f), one possibility is that another channel/receptor combination occurs in vivo; however, D$_2$R-long and short equally couple to G$_i$ proteins [29]. On the other hand, Ca$\alpha_{1.3}$a binds a scaffolding protein found in the postsynaptic density of synapses known as Shank [30]. In MSNs, Ca$\alpha_{1.3}$a requires an association with Shank for current inhibition by D$_2$Rs [4]. Although lack of the longer
Fig. 2  M₁Rs but not D₂Rs inhibit recombinant L-current.  

*a* Comparison of M₁R and D₂R signaling pathways that inhibit L-VGCC activity. 

**b** Time course of Oxo-M applied at time 0 for Caᵥ₁.3b-β₂a current. 

**c** Time course of 10 nM quin applied at time 0 for Caᵥ₁.3b-β₂a current. Inset: (left) Individual current traces before (black) and after 1 min of quin, scale bar = 0.5 nA. (right) Normalized traces. 

**d** Time course of Oxo-M applied at time 0 for Caᵥ₁.3b-β₃ current. 

**e** Time course of 0.5 μM quin applied at time 0 for Caᵥ₁.3b-β₃ current. Inset: same as **c**, scale bar = 1 nA. 

**f** Concentration–response curve of quin on Caᵥ₁.3b-β₂a (filled circles) and Caᵥ₁.3b-β₃ (open circles) currents (n = 2–5). 

**g** Summary of kinetic analysis.
CaV1.3 C-terminus may explain the absence of channel modulation by D2Rs in our studies, we found that Oxo-M inhibits CaV1.3b currents, showing that this short splice variant of CaV1.3 can be modulated by a GqPCR. Therefore, a missing intermediary protein vital for D2R modulation of CaV1.3b may underlie the lack of inhibition reported here, or D2Rs may not modulate CaV1.3b.

**Conclusions**
These findings highlight the specific regulation of Ca2+ channels in a CaVβ-subunit dependent manner by different neurotransmitters. While M1R and D2R pathways contain similar signaling molecules and share a common functional output of inhibiting Ca2+ channels, differences between the two cascades exist. Expression and localization of CaV1.3b associated with different CaVβ-subunits in a tissue or cell may dictate how Ca2+ influx is modulated by nearby GPCRs, ultimately affecting Ca2+-dependent processes.

**Limitations**
Further experiments are needed to determine the differences in signaling between successful CaV1.3b inhibition by M1Rs versus none with D2Rs.

**Additional files**

Additional file 1. Pharmacological characterization of CaV1.3b L-current. HEK-M1 cells were washed with DMEM and the DNA mixture of CaV1.3b, α-δ-1, a β-subunit and GFP was added and incubated for 1 h at 37°C in a 5% CO2 incubator. Supplemented media, without antibiotics, was then returned to the cells to bring the volume up to 1 ml (normal medium volume). After 2 h, cells were washed with supplemented media and washed a final time 2 h later. 10 mM MgSO4 was added to the medium to block basal activity of CaV1.3b, which helped minimize excitotoxicity of transfected cells. Cells were transferred 24–72 h post-transfection using 2 mM EDTA in 1X PBS to poly-L-lysine-coated coverslips. Recording began 1 h after transfer to coverslips. A Individual traces of CaV1.3b-β3 current before (CTL) and after exposure to 0.3 μM NIM. B Concentration–response curve of L-current inhibition to NIM (n = 4–8). C CaV1.3b-β3 currents before and after exposure to FPL (1 μM). Cells were stepped to a test potential of −10 mV from a holding potential of −90 mV followed by repolarization to −90 or −50 mV. Control (CTL) currents from β3−containing L-VGCCs show little to no inactivation as observed previously [31]. D CaV1.3b-β1 currents before and after FPL. Cells were stepped to a test potential of −10 mV from a holding potential of −60 mV followed by repolarization to −60 mV. Following FPL, both β2a- and β3-containing channels exhibited slower activation and deactivation kinetics, hallmarks of agonist action on L-current [32]. E FPL enhancement of the CaV1.3b-β2a current–voltage plot from a holding potential of −90 mV (CTL, filled circles; FPL, open circles, n = 3, *P < 0.05). F Concentration–response curve of CaV1.3b-β2 tail current enhancement to FPL (n = 4–8). Currents inhibited by NIM and enhanced by FPL fully recovered by washing with bath solution (data not shown).

Additional file 2. D2Rs do not inhibit recombinant L-current: A Summary bar graph of CaV1.3b-β3 current inhibition by 0.5 μM quin (n = 5). B & C Summary bar graphs of TTP and 40 kinetic analysis.

**Abbreviations**
AA: arachidonic acid; cPLA2: Ca2+-dependent, cytosolic phospholipase A2; D2Rs: dopaminergic D2 receptors; FPL: FPL 64176; GFP: green fluorescent protein; GPCRs: G protein-coupled receptors; HEK-293 cells: human embryonic kidney cells; M1Rs: muscarinic M1 receptors; MSN: medium spiny neurons; NIM: nimodipine; NMG: N-methyl-D-glucamine; OPC: oleoyloxyethyl phosphorylcholine; Oxo-M: Oxtremorine-M; PLA2: phospholipase A2; PLC: phospholipase C; PTX: pertussis toxin; Quin: quinpirole; SCG: superior cervical ganglion; TTP: time to peak; VGCCs: voltage-gated Ca2+ channels.

**Authors’ contributions**
MLR conceived of the project, experimental design, collected and analyzed data, and wrote the manuscript. ARR contributed to the experimental design, analysis and editing of the manuscript. Both authors read and approved the final manuscript.

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**Competing interests**
The authors declare that they have no competing interests.

**Availability of data and materials**
The accession numbers for the constructs used in this study are as follows: CaV1.3b (+ exon11, Δ exon32, + exon42a), GenBank accession #AF370009; CaV2.2 (Δ exon10, Δ exon18a, Δ exon24a, + exon31a, + exon37b, + exon46), GenBank
accession #M80545; CaVβ1b, GenBank accession #M88751; CaVβ2a, GenBank accession #L02315, and α2δ-1, GenBank accession #AF266488. All data generated or analyzed during this study are included in this published article (and its additional files).

Consent for publication
Not relevant.

Ethics approval and consent to participate
Not applicable.

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