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

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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 \( \alpha_{2\delta}β-1 \) and a \( \beta_{1b}, β_{2a}, β_{3}, \) or \( β_{4} \)-subunit. Surprisingly, the magnitude of inhibition was less with \( β_{2a} \) than with other Ca\textsubscript{V}β-subunits. Normalizing currents revealed kinetic changes after modulation with \( β_{1b}, β_{3}, \) or \( β_{4} \), but not \( β_{2a} \)-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 \( β_{2a} \) or \( β_{3} \). 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 [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 [2, 3]. These GPCRs specifically inhibit Ca\textsubscript{V}1.3 L-current, decreasing the output of MSNs [3, 4] and may have functional consequences for motor control [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 [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 [10]. Our laboratory has identified arachidonic acid (AA) as a critical effector in the slow pathway [9]. Exogenously applied AA inhibits L-current [11–13], which in SCG neurons most likely arises from Ca\textsubscript{V}1.3 [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 [15, 16].

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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]. 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.

Main text
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, +exon42a; GenBank accession #AF370009), CaV2.2 (‘10, Δexon18a, Δexon24a, +exon31a, +exon37b, +exon46; #AF055477), CaVβ3 (#M88751) and αδ-1 (#AF286488) were provided by Diane Lipscombe (Brown University). CaVβ1b (#X61394), CaVβ2a (#M80545), and CaVβ4 (#L03215) constructs were provided by Edward Perez-Reyes (University of Virginia). The D4R (#AF1199329) 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 DDW 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 β2a- 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.

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: 

\[
\%I_{\text{inhib}} = 100 \times \frac{I_{\text{CTL}} - I_{\text{DRUG}}}{I_{\text{CTL}}}
\]

where \(I_{\text{CTL}}\) and \(I_{\text{DRUG}}\) 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 < 0.01, ***P < 0.001) 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:

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

where \(r_{40}\) is the percent of the maximum inward current remaining at the end of a 40 ms test pulse; \(I_{\text{end}}\) is the current amplitude at the end of the test pulse; \(I_{\text{peak}}\) is the maximum inward current measured during the test pulse.
After 1 min, Oxo-M significantly inhibited L-current by 58 ± 8% with β1b, 36 ± 12% with β2a; 66 ± 6% with β3; and 72 ± 10% with β4 (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 β1b, β3, or β4; however, no changes were detected with β2a (P ≥ 0.11 for TTP; P ≥ 0.40 for r40). These differences in the magnitude of current inhibition and kinetics suggest that the Caβ-subunit affects M1R modulation of Ca2.3b.

**Dopamine D2 receptors inhibit Ca2.2 but not Ca2.1 currents**

Both M1Rs and D2Rs activate pathways involving G proteins, PLC, and AA release (Fig. 2a). However, whether L-current inhibition by D2Rs shows varied inhibition depending on Caβ-subunit expression has not been examined. Therefore, we coexpressed D2Rs with Ca1.3b, αδ-1 and different Caβ-subunits. While Oxo-M inhibited Ca1.3b-β2a currents over time (Fig. 2b), quin, a D2R agonist, had no effect on current amplitude (Fig. 2c) or kinetics (Fig. 2c inset, g). Since Ca1.3b-β2a current shows less inhibition and no kinetic changes with Oxo-M, we tested whether Ca1.3b-β3 current was sensitive to modulation by quin. Figure 2d shows a time course of Ca1.3b-β3 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). D2Rs appeared to desensitize with 10 μM quin. Application of quin for 1 min to cells co-transfected with the D2R-like family member, D4.4R, inhibited L-current by 8.5 ± 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 D2Rs, we repeated the experiment but substituted Ca2.2 for Ca2.1b to serve as a positive control since activated D2Rs also inhibit Ca2.2 [24–26]. Quin inhibited Ca2.2 by 45 ± 7% after 30 s and 48 ± 4% after 1 min (Fig. 3a). Inhibition occurred specifically by activating transfected D2Rs because cells transfected without D2Rs 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 μM 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 D2Rs functionally express in HEK-M1 cells to modulate Ca2.2, but not Ca2.1b VGCC activity.

**M1R and D2R pathways use different signaling mechanisms to inhibit N-current**

To compare D2Rs and M1Rs signaling pathways on Ca2.2 current, we first confirmed that activation of the stably transfected M1Rs could suppress N-current. Indeed, Oxo-M inhibited currents from β2a-containing channels by 70 ± 5% after 30 s (Fig. 3c). When incubated with the PLA2 antagonist OPC, cells showed less N-current inhibition by Oxo-M, 14 ± 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 M1Rs and D2Rs do not share a common pathway leading to N-current inhibition.

**Discussion**

Previously, the Ca2.1b 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 Ca2.1b L-current with the accessory Caβ-subunit determining the magnitude of inhibition. In contrast, stimulation of transfected D2Rs with quin does not recapitulate L-current inhibition observed in MSNs [3]. Pharmacological sensitivity to both FPL and NIM confirmed that Ca2.1b expressed in HEK-M1 cells behaves similarly to other recombinant Ca2.1b VGCCs [22, 27].

We also report that N-current modulation by the D2R short splice variant appears similar to membrane-delimited inhibition by the D2R long form [24]. In this form of modulation, when G proteins are activated, Gβγ directly binds to and inhibits Ca2.2 which can be reversed by strong prepulses [10, 28]. Indeed, D2R-mediated inhibition of Ca2.2 was independent of PLA2, whereas blockers of PLA2 abolished inhibition by M1Rs. Thus, the membrane-delimited pathway may be at least partially responsible for the inhibition of Ca2.2 by D2Rs in MSNs [25].

In our experiments, the short splice variant of Ca1.3 (Ca2.1b) was unaffected by activation of D2Rs, expressed in HEK-293 cells, similar to a previous report on Ca1.3a, which has a longer C-terminus [24]. Since neither D2R-long inhibited Ca1.3a [24], nor D2R-short inhibited Ca1.3b (Fig. 2f), one possibility is that another channel/receptor combination occurs in vivo; however, D2R-long and short equally couple to G proteins [29]. On the other hand, Ca1.3a binds a scaffolding protein found in the postsynaptic density of synapses known as Shank [30]. In MSNs, Ca1.3a requires an association with Shank for current inhibition by D2Rs [4]. Although lack of the longer
Fig. 2  M_1Rs but not D_2Rs inhibit recombinant L-current. a Comparison of M_1R and D_2R signaling pathways that inhibit L-VGCC activity. b Time course of Oxo-M applied at time 0 for CaV1.3b-β_2a current. c Time course of 10 nM quin applied at time 0 for CaV1.3b-β_2a 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-β_3 current. e Time course of 0.5 μM quin applied at time 0 for CaV1.3b-β_3 current. Inset: same as c, scale bar = 1 nA. f Concentration–response curve of quin on CaV1.3b-β_2a (filled circles) and CaV1.3b-β_3 (open circles) currents (n = 2–5). g Summary of kinetic analysis
Ca\textsubscript{v}.1.3 C-termini may explain the absence of channel modulation by D\textsubscript{2}Rs in our studies, we found that Oxo-M inhibits Ca\textsubscript{v}.1.3b currents, showing that this short splice variant of Ca\textsubscript{v}.1.3 can be modulated by a G\textsubscript{q}PCR. Therefore, a missing intermediary protein vital for D2R modulation of Ca\textsubscript{v}.1.3b may underlie the lack of inhibition reported here, or D2Rs may not modulate Ca\textsubscript{v}.1.3b.

Conclusions

These findings highlight the specific regulation of Ca\textsuperscript{2+} channels in a Ca\textsubscript{v}.\beta-subunit dependent manner by different neurotransmitters. While M\textsubscript{1}R and D\textsubscript{2}R pathways contain similar signaling molecules and share a common functional output of inhibiting Ca\textsuperscript{2+} channels, differences between the two cascades exist. Expression and localization of Ca\textsubscript{v}.1.3b associated with different Ca\textsubscript{v}.\beta-subunits in a tissue or cell may dictate how Ca\textsuperscript{2+} influx is modulated by nearby GPCRs, ultimately affecting Ca\textsuperscript{2+}-dependent processes.

Limitations

Further experiments are needed to determine the differences in signaling between successful Ca\textsubscript{v}.1.3b inhibition by M\textsubscript{1}Rs versus none with D\textsubscript{2}Rs.

Additional files

**Additional file 1.** Pharmacological characterization of Ca\textsubscript{v}.1.3b L-current. HEK-M1 cells were washed with DMEM and the DNA mixture of Ca\textsubscript{v}.1.3b, α\textsubscript{2}δ-1, a β3-subunit and GFP was added and incubated for 1 h at 37 °C in a 5% CO\textsubscript{2} 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 MgSO\textsubscript{4} was added to the medium to block basal activity of Ca\textsubscript{v}.1.3b, which helped minimize excitotoxicity of transfected cells. Cells were transfected 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 Ca\textsubscript{v}.1.3b-β\textsubscript{3} 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 β2a-containing L-VGCCs show little to no inactivation as observed previously [31]. D Ca\textsubscript{v}.1.3b-β\textsubscript{3} 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 Ca\textsubscript{v}.1.3b-β\textsubscript{3} 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 Ca\textsubscript{v}.1.3b-β\textsubscript{3} 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.** D\textsubscript{2}Rs do not inhibit recombinant L-current: A Summary bar graph of Ca\textsubscript{v}.1.3b-β3 current inhibition by 0.5 μM quin (n = 9). B & C Summary bar graphs of TTP and 40 kinetic analysis.

**Abbreviations**

AA: arachidonic acid; cPLA\textsubscript{2}: Ca\textsuperscript{2+} dependent, cytosolic phospholipase A\textsubscript{2}; D\textsubscript{2}Rs: dopaminergic D\textsubscript{2} receptor; FPL: FPL 64176; GFP: green fluorescent protein; GPCRs: G protein-coupled receptors; HEK-293 cells: human embryonic kidney cells; M\textsubscript{1}Rs: muscarinic M\textsubscript{1} receptors; MSN: medium spiny neurons; NIM: nimodipine; NMG: N-methyl-D-glucamine; OPC: oleoyloxyethyl phosphorylcholine; Oxo-M: Oxo-tetramitamine-M; PL\textsubscript{A}2: phospholipase A\textsubscript{2}; PLC: phosphatidylinositol C; PTX: pertussis toxin; Quin: quinpirole; SCG: superior cervical ganglion; TTP: time to peak; VGCCs: voltage-gated Ca\textsuperscript{2+} 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: Ca\textsubscript{v}.1.3b (+-exon11, Δexon32, +-exon42a), GenBank accession #F4370009; Ca\textsubscript{v}.2.2 (+-exon10, Δexon18a, Δexon24a, +exon31a, +exon37b, +exon46), GenBank
accession #M80545; CaVβ4 GenBank accession #M88751; CaVβ5 GenBank accession #L02315, and α6-1, GenBank accession #AF266488. All data generated or analyzed during this study are included in this published article (and its additional files).

Consent for publication
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Ethics approval and consent to participate
Not applicable.

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