Selective Inhibition of Cav3.3 T-type Calcium Channels by Gαq/11-coupled Muscarinic Acetylcholine Receptors*

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T-type calcium channels play critical roles in controlling neuronal excitability, including the generation of complex spiking patterns and the modulation of synaptic plasticity, although the mechanisms and extent to which T-type Ca\(^{2+}\) channels are modulated by G-protein-coupled receptors (GPCRs) remain largely unexplored. To examine specific interactions between T-type Ca\(^{2+}\) channel subtypes and muscarinic acetylcholine receptors (mACHRs), the Cav3.1 (α1G), Cav3.2 (α1H), and Cav3.3 (α1I) T-type Ca\(^{2+}\) channels were co-expressed with the M1 Gαq/11-coupled mACHR. Perforated patch recordings demonstrate that activation of M1 receptors has a strong inhibitory effect on Cav3.3 T-type Ca\(^{2+}\) currents but either no effect or a moderate stimulating effect on Cav3.1 and Cav3.2 peak current amplitudes. This differential modulation was observed for both rat and human T-type Ca\(^{2+}\) channel variants. The inhibition of Cav3.3 channels by M1 receptors is reversible, use-independent, and associated with a concomitant increase in inactivation kinetics. Loss-of-function experiments with genetically encoded antagonists of Gα and Gβγ proteins and gain-of-function experiments with genetically encoded Gα subtypes indicate that M1 receptor-mediated inhibition of Cav3.3 occurs through Gαq/11. This is supported by experiments showing that activation of the M3 and M5 Gαq/11-coupled mACHRs also causes inhibition of Cav3.3 currents, although Gαi-coupled mACHRs (M2 and M4) have no effect. Examining Cav3.1-Cav3.3 chimeric channels demonstrates that two distinct regions of the Cav3.3 channel are necessary and sufficient for complete M1 receptor-mediated channel inhibition and represent novel sites not previously implicated in T-type channel modulation.

T-type calcium channels play critical roles in shaping the electrical, chemical, and plastic properties of neurons throughout the central and peripheral nervous systems. In thalamic reticular and relay neurons, T-type channels are involved in rhythmic rebound burst firing and spindle waves associated with slow-wave sleep (1–5). Studies on knock-out mice and a rat model of absence epilepsy indicate that altering T-type activity within thalamic cells can contribute to pathological conditions such as sleep disorders and epilepsy (1–5). Certain human epilepsies appear to be associated with T-type Ca\(^{2+}\) channel point mutations conferring channel gain-of-function phenotype (6–9). T-type channels also play crucial roles in dendritic integration and Ca\(^{2+}\) spiking in hippocampal pyramidal cells (10, 11). Within the olfactory bulb, T-type channels are implicated in modulating Ca\(^{2+}\) transients and synaptic release at dendrodendritic synapses (12, 13). In the periphery, antinociceptive oligonucleotides and pharmacological approaches have implicated T-type channels in contributing to both acute and chronic nociceptive behaviors (14, 15).

Previous studies have identified three main subtypes of T-type Ca\(^{2+}\) channel α1 subunits (Cav3.1/α1G, Cav3.2/α1H and Cav3.3/α1I) and characterized their voltage-dependent, kinetic, and pharmacological properties (16–21). Cav3.1 and Cav3.2 channels display “typical” T-type properties, including relatively small conductance, fast activation and inactivation kinetics, and slow deactivation kinetics, whereas Cav3.3 channels uniquely display a larger conductance, much slower activation and inactivation kinetics, as well as faster deactivation kinetics (17, 19). Some of the distinct biophysical properties associated with Cav3.3 T-type currents have been observed in certain populations of native T-type currents (4, 17, 19, 22). The biophysical differences between the T-type channels likely enable them to differentially shape and modulate firing patterns, with the more slowly inactivating Cav3.3 currents able to produce longer bursts of spikes and tonic firing patterns (17, 23, 24).

Although the basic properties of both cloned and native T-type channels have now been largely characterized, there remains relatively little information concerning their modulation by GPCR-linked pathways. Neurotransmitters such as acetylcholine have been shown to either attenuate or stimulate low threshold Ca\(^{2+}\) currents depending on the type of native cells examined, and sometimes multiple forms of modulation

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*This work was supported in part by operating grants from the Canadian Institutes for Health Research and Canada Research Tier 1 Chairs (to T. P. S. and G. W. Z.), a fellowship from the Heart and Stroke Foundation of Canada, and trainee fellowships from the Natural Sciences and Engineering Research Council of Canada and Michael Smith Foundation for Health Research (to M. E. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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2The abbreviations used are: GPCR, G-protein coupled receptor; mACHR, muscarinic acetylcholine receptor; pEGFP, enhanced green fluorescent protein; WT, wild type; P1(4,5)P2, phosphatidylinositol 4,5-biphosphate; CCh, carbachol; PLC, phospholipase C; PKC, protein kinase C; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid-ace-toxymethyl ester.
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can be observed within the same cell type (25–29). Multiple T-type Ca\(^{2+}\) channel subtypes are expressed in most native cells (30, 31), although pharmacological tools with the specificity needed to separate these currents have not been generated. In this regard, the description of the modulation of specific T-type Ca\(^{2+}\) channels in heterologous systems will provide insights crucial toward further investigations within native systems. This approach is also well suited for GPCR studies as most neurotransmitters activate multiple receptor subtypes in neurons.

Within thalamic reticular, hippocampal pyramidal, and olfactory granule cells, there is evidence for the expression of both T-type Ca\(^{2+}\) channels and Go\(_{q/11}\)-coupled muscarinic acetylcholine receptors (mAChRs) (25, 30–36). As both T-type Ca\(^{2+}\) currents and mAChRs have been independently shown to play important physiological roles within these cell types, their functional coupling could be relevant to a number of neuronal processes. Here we studied the modulatory effects of mAChRs on the three main subtypes of low threshold T-type Ca\(^{2+}\) channels expressed in the mammalian nervous system. We found the selective modulation of Cav3.3 Ca\(^{2+}\) channels by Go\(_{q/11}\)-coupled mAChRs and combined pharmacological, genetic, and chimeric channel approaches to examine the G-protein-mediated pathway and structural regions responsible for the distinct Cav3.3 signaling characteristics.

**EXPERIMENTAL PROCEDURES**

**Molecular Biology**—Human Cav3.1–Cav3.3 T-type Ca\(^{2+}\) channel \(\alpha_1\) subunit chimeras were constructed as described in detail by Hamid et al. (37).

**Cell Culture and Transfection**—Human embryonic kidney cells (HEK 293H, Invitrogen) were grown in standard Dulbecco’s modified Eagle’s medium (10% fetal bovine serum and 50 units/ml penicillin/streptomycin) to \(\sim\)80% confluence and maintained at 37 °C in a humidified incubator with 95% atmosphere and 5% CO\(_2\). The generation of stable T-type cell lines (in HEK 293, tsa-201) expressing rat brain Cav3.1, Cav3.2, or Cav3.3 \(\alpha_1\) subunits has been described previously (16). Stable cell lines were transiently transfected with human muscarinic M1, M2, M3, M4, or M5 cDNAs (all in pcDNA3.1) using Lipofectamine (Invitrogen). As a reporter for transfection, all transient transfections included co-transfection with either CD8 or pEGFP marker plasmids at a 1:0.25 molar ratio compared with receptor and/or channel plasmid DNA, unless otherwise indicated. Lipofectamine-mediated transfections used 1–1.25 \(\mu\)g of DNA/35-mm dish and 5 \(\mu\)l of Lipofectamine/dish. In G-protein experiments, stable Cav3.3 cells were co-transfected with M1 receptors and equal amounts of either MAS-GRK3ct (in pcDNA3.1), Go\(_q\) (in pcDNA3.1), or RGS2 (in pEGFP) using Lipofectamine. Only MAS-GRK3ct and Go\(_q\) transfections required co-transfection with marker plasmids as RGS2 expression could be directly detected with fluorescence. In Go\(_q\) transfection experiments, stable Cav3.3 cells were transfected with constitutively active mutants of Go\(_q\), Go\(_{11}\), or Go\(_{13}\) (Go\(_q\)-Q209L, Go\(_{11}\)-Q209L, and Go\(_{13}\)-Q226L, respectively, all in pcDNA3.1) using Lipofectamine. Twelve to 18 h after transfection, the medium was changed from Opti-MEM I to regular Dulbecco’s modified Eagle’s medium, and cells were transferred to a 28 °C incubator. The M1 to M5, Go\(_{q}\)-Q209L, Go\(_{11}\)-Q209L, and Go\(_{13}\)-Q226L cDNAs were all obtained from the UMR cDNA Resource Center (Rolla, MO), and the RGS2 and MAS-GRK3ct constructs were a generous gift from Dr. Brett Adams.

In separate experiments, HEK 293H cells were co-transfected with M1 and wild type (WT) or chimeric human Cav3.1 or Cav3.3 channels using standard Ca\(^{2+}\) phosphate transfection with 2 \(\mu\)g of total cDNA/dish, 0.15 to 0.4 \(\mu\)g of channel cDNA/dish, and 0.2 \(\mu\)g of M1 cDNA/dish. In a subset of these experiments involving co-transfection of WT Cav3.3 and M1, either 200 \(\mu\)M di-C8 PI(4,5)P\(_2\) (Echelon Biosciences Inc., Salt Lake City, UT) or 50 \(\mu\)g/ml PI(4,5)P\(_2\) IgG\(_{2b}\), antibody (\(\sim\)1:30 dilution) (Assay Designs, Ann Arbor, MI) was included in the internal solution to explore the role of PI(4,5)P\(_2\) signaling. As the PI(4,5)P\(_2\) antibody was supplied in a phosphate-buffered saline solution containing 10% calf serum and 0.05% sodium azide, the control Cav3.3 + M1 cells were recorded in an internal solution containing a 1:30 dilution of phosphate-buffered saline with 10% fetal bovine serum and 0.05% sodium azide. Electrophysiological recordings for all experiments were performed 24–48 h after transfection. Transiently transfected cells were selected for CD8 or pEGFP expression using either adherence of Dynabeads (Dynal, Great Neck, NY) or fluorescence of EGFP under UV light.

**Electrophysiological Recordings and Analysis**—Macroscopic currents were recorded using the perforated patch clamp technique to reduce current rundown and to preserve cytoplasmic signaling pathways. The external recording solution contained (in mM) 2 CaCl\(_2\), 1 MgCl\(_2\), 10 HEPES, 40 tetraethylammonium chloride, 92 CsCl, 10 glucose, pH 7.4, and the internal pipette solution contained (in mM) 120 Cs\(^+\) methanesulfonate, 11 EGTA, 10 HEPES, 2 MgCl\(_2\), 75–100 \(\mu\)M \(\beta\)-escin, pH 7.2. For these perforated patch recordings, experimental recording did not begin until the series resistance was below 20 megohms and constant, as measured by amplifier compensation. Whole-cell recordings were used for the transiently transfected WT or chimeric human Cav3.1 and Cav3.3 channel experiments as well as the Go\(_q\) transfection experiments. The internal solution for these recordings contained (in mM) 120 Cs\(^+\) methanesulfonate, 11 EGTA, 10 HEPES, 2 MgCl\(_2\), 4 Mg-ATP, 0.3 sodium GTP. Macroscopic currents were recorded using Axopatch 200A and 200B amplifiers (Axon Instruments, Foster City, CA), controlled and monitored with Pentium 4 personal computers running pClamp software version 9 (Axon Instruments). Patch pipettes (borosilicate glass BF150-86-10; Sutter Instruments, Novato, CA) were pulled using a Sutter P-87 puller and polished with a Narishige (Tokyo, Japan) microforge, with typical resistances of 3–6 megohms when filled with internal solution. The bath was connected to the ground via a 3 M KCl agar bridge.

Data were low-pass filtered at 2 kHz using the built-in Bessel filter of the amplifier, with sampling at 10 kHz. The amplifier was also used for capacitance and series resistance compensation between 70 and 85% on every cell. Leak subtraction of capacitative and leakage current was performed on-line using a P/5 protocol or else performed with Clampfit (Axon Instruments) during off-line analysis. Figures and fittings utilized the software program Microcal Origin (version 7.5).
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7.5, Northampton, MA). All recordings were performed at room temperature (20–22 °C).

The voltage dependence of activation for Cav3.1, Cav3.2, and Cav3.3 currents was measured by a series of 100–220-ms depolarizing pulses applied from a holding potential of −110 mV to membrane potentials from −80 to +10 mV, increasing at 5-mV increments, with 2 s between pulses. The potential that elicited peak currents (“peak potential” ranging from −45 to −25 mV) was obtained from this protocol and used in subsequent protocols. Series resistance was also monitored with a 5-ms depolarizing pulse to −105 mV immediately before the test pulse to ensure that this variable was relatively constant, and any changes in peak current levels were not because of significant changes in series resistance. Effects of saturating concentrations of mAChR agonist (1 mM CCh) on stable T-type currents were then investigated using steps to peak potential every 5 s (0.2 Hz) from a holding potential of −110 mV. These depolarizing steps were 80 ms in duration for Cav3.1 and Cav3.2 and 200 ms in duration for Cav3.3. The −140-mV prepulse protocol for Cav3.3 included a 1-s prepulse to −140 mV to remove any accumulated channel inactivation. To quantify the percent of channel inhibition, stimulation, or washout during CCh or control solution perfusion, the peak current magnitude at equilibrium was averaged (2–5 values). When distinct effects were observed (i.e. stimulation versus no effect of M1 on Cav3.1 currents), all cells displaying a >10% modulating effect with a clear exponential time course were grouped into one group, while the rest of the cells were grouped into the “no effect” group.

Current-voltage relationships were fitted with the modified Boltzmann equation, $I = \left( G_{\text{max}} \times (V_m - E_{\text{rev}}) \right) / \left( 1 + \exp \left( \frac{V_m - V_{0.5a}}{k_a} \right) \right)$, where $V_m$ is the test potential; $V_{0.5a}$ is the half-activation potential; $E_{\text{rev}}$ is the extrapolated reversal potential; $G_{\text{max}}$ is the maximum slope conductance, and $k_a$ reflects the slope of the activation curve. Data from CCh concentration-response studies were fitted with the equation, $y = (A_1 - A_2) / \left[ 1 + \left( x / x_{0.5} \right) ^ G \right] + A_2$, where $A_1$ is initial amplitude (=0) and $A_2$ is final block value; $x_{0.5}$ is IC\textsubscript{50} (concentration causing 50% inhibition of currents), and $G$ gives a measure of the steepness of the curve. The activation and inactivation rates during steps to peak potential were well described by single exponential curves to give $\tau_{\text{act}}$ and $\tau_{\text{inact}}$ values, respectively. Statistical significance was tested with Student’s $t$ tests with significance being determined at a confidence interval of $p < 0.02$.

Solutions, Drugs, and Perfusion—A 25 mM stock of β-escin (in distilled H\textsubscript{2}O) was prepared fresh, with dilution to working stocks in intracellular solution. Carbachol (CCh) was added directly to the extracellular recording solution. Wortmannin, okadaic acid, genistein, and H9 were all obtained from Tocris.

FIGURE 1. T-type Ca\textsuperscript{2+} channels are differentially modulated by M1 receptors. A and B, representative perforated patch current traces during depolarizing pulses from −110 to −30 mV demonstrating no effect on Cav3.1 currents (A) and Cav3.2 currents (B) when M1 is activated with 1 mM CCh. D and E, normalized peak current levels during perfusion of control recording solution (2 mM Ca\textsuperscript{2+}) followed by 1 mM CCh for Cav3.1 (+M1) currents (D) and Cav3.2 (+M1) currents (E). Perfusion of CCh usually had no effect on Cav3.1 peak current amplitudes (−2.1 ± 2.0%, $n = 18$) and Cav3.2 peak current amplitudes (−0.1 ± 2.3%, $n = 17$). C, representative perforated patch current traces during depolarizing pulses from −110 to −40 mV showing inhibition of Cav3.3 currents by M1. F, normalized peak current levels during perfusion of control recording solution (2 mM Ca\textsuperscript{2+}) followed by 1 mM CCh for Cav3.3 (+M1) currents. Perfusion of CCh caused a 45% (±2%, $n = 34$) decrease in Cav3.3 currents. All data points correspond to mean ± S.E.
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### RESULTS

**Muscarinic M1 Receptors Selectively Inhibit Cav3.3 T-type Ca\(^{2+}\) Channels**—To investigate the potential for T-type Ca\(^{2+}\) channel modulation by mACHRs, we transiently transfected HEK cell lines stably expressing individual subtypes of recombinant rat brain T-type channels with the human muscarinic M1 receptor. Perforated patch recordings with β-escin demonstrated that activation of M1 with 1 mM CCh caused a rapid (<30 s) and robust inhibition of exogenously expressed rat brain Cav3.3 T-type channel peak currents (−45 ± 2%, \(n = 34\)) (Fig. 1, C and F). Only a small subpopulation of stable Cav3.3 cells (<10%) was not affected by CCh application (likely representing cells untransfected with the M1 receptor).

Activation of M1 with 1 mM CCh had no significant effect (\(p > 0.05\)) on the voltage dependence of Cav3.3 currents but significantly increased both the rates of activation and inactivation (\(p < 0.001\); Table 1). In contrast to the clear inhibition of Cav3.3 T-type currents, activation of M1 receptors with 1 mM CCh largely had no effect on the peak current amplitude of either rat brain Cav3.1 (−2.1 ± 2.0%, \(n = 18\)) or Cav3.2 channels (−0.1 ± 2.3%, \(n = 17\)) (Fig. 1, A, B, D, and E). In a small subset of both Cav3.1 and Cav3.2 currents we noted a stimulation induced by M1 activation (Cav3.1 = 35 ± 12%, \(n = 4\); Cav3.2 = 36 ± 12%, \(n = 5\)), with a slower time course to equilibrium of greater than 1 min (\(n = 3\) and \(n = 4\), respectively). For the prevalent null effect on Cav3.1 and Cav3.2 currents, 1 mM CCh application had no significant effect on channel activation and inactivation kinetics or the voltage dependence of activation (\(p > 0.05\); Table 1).

Different Cav3.3 T-type channel isoforms with distinct carboxyl termini have been identified from both the rat and human brain (17, 19, 21, 38). To test whether inhibition of the Cav3.3 channel by M1 receptors was restricted to the rat brain short carboxyl-terminal isoform (17), we also examined the longer human Cav3.3 isoform (21) transiently co-transfected into HEK cells with the M1 receptor. Similar to that for the shorter rat brain isoform, application of 1 mM CCh resulted in significant inhibition of the human Cav3.3 peak current amplitude (−28 ± 2%, \(n = 15\)) and also significantly increased activation and inactivation kinetics (\(p < 0.001\); Fig. S4A; Table 1). Additionally, similar to that for the rat Cav3.1 T-type channel, application of 1 mM CCh to HEK cells co-transfected with the human Cav3.1 channel and M1 receptor had no significant effect on peak current amplitude (−0.3 ± 0.0%, \(n = 9\)) or channel kinetics (for 100% cells tested; \(p > 0.05\); Fig. S7B; Table 1). Overall, the differential modulation of T-type Ca\(^{2+}\) channel subtypes mediated by M1 receptors was consistent across both rat and human recombinant T-type channels.

### Table 1

| Effect | Control | Carbachol |
|--------|---------|-----------|
| \(\tau_{\text{act}}\) | \(\tau_{\text{inact}}\) | \(V_{\text{act}}\) | \(\tau_{\text{act}}\) | \(\tau_{\text{inact}}\) | \(V_{\text{act}}\) |
| 2 mM Ca\(^{2+}\) | 1 mM Ca\(^{2+}\) |
| Cav3.3 + M1 (inhibition) | 6.0 ± 0.4, \(n = 27\) | 86 ± 6, \(n = 27\) | −51 ± 2, \(n = 13\) | 4.1 ± 0.3, \(n = 27\) | 31 ± 2, \(n = 27\) | −49 ± 1, \(n = 13\) |
| Human Cav3.3 + M1 (whole-cell; inhibition) | 9.7 ± 0.8, \(n = 10\) | 117 ± 6, \(n = 10\) | −44 ± 1, \(n = 4\) | 5.4 ± 0.6, \(n = 10\) | 41 ± 5, \(n = 10\) | −47 ± 1, \(n = 4\) |
| Cav3.3 + M2 (no effect) | 6.1 ± 0.7, \(n = 10\) | 81 ± 9, \(n = 10\) | −53 ± 3, \(n = 4\) | 5.5 ± 0.7, \(n = 10\) | 76 ± 9, \(n = 10\) | −52 ± 3, \(n = 4\) |
| Cav3.3 + M3 (inhibition) | 9.5 ± 0.9, \(n = 9\) | 104 ± 13, \(n = 9\) | −53 ± 4, \(n = 4\) | 6.2 ± 0.6, \(n = 9\) | 56 ± 6, \(n = 8\) | −50 ± 4, \(n = 4\) |
| Cav3.3 + M4 (no effect) | 6.7 ± 0.9, \(n = 6\) | 110 ± 18, \(n = 6\) | −50 ± 1, \(n = 5\) | 6.0 ± 0.9, \(n = 6\) | 101 ± 16, \(n = 6\) | −51 ± 2, \(n = 5\) |
| Cav3.3 + M5 (inhibition) | 8.6 ± 0.9, \(n = 6\) | 127 ± 23, \(n = 7\) | −48 ± 1, \(n = 5\) | 5.6 ± 0.6, \(n = 6\) | 49 ± 6, \(n = 9\) | −45 ± 1, \(n = 5\) |
| Cav3.3 + Control Plasmid | 6.0 ± 0.4, \(n = 5\) | 78 ± 13, \(n = 5\) | −42 ± 2, \(n = 6\) | 2.1 ± 0.2, \(n = 5\) | 71 ± 12, \(n = 5\) | −39 ± 3, \(n = 11\) |
| Cav3.1 + M1 (no effect) | 2.3 ± 0.2, \(n = 17\) | 19 ± 2, \(n = 17\) | −36 ± 2, \(n = 17\) | 1.2 ± 0.1, \(n = 9\) | 13 ± 1, \(n = 9\) | −51 ± 2, \(n = 4\) |
| Human Cav3.1 + M1 (whole-cell; no effect) | 2.0 ± 0.4, \(n = 9\) | 14 ± 1, \(n = 9\) | −42 ± 2, \(n = 6\) | 4.3 ± 0.2, \(n = 16\) | 34 ± 2, \(n = 15\) | −40 ± 3, \(n = 7\) |
| Cav3.2 + M1 (no effect) | 4.7 ± 0.2, \(n = 16\) | 36 ± 2, \(n = 15\) | −42 ± 1, \(n = 7\) | 17 ± 1, \(n = 17\) | 17 ± 1, \(n = 17\) | −39 ± 3, \(n = 11\) |

\(n = 3\) and \(n = 2\), respectively. \(p < 0.001\). \(p < 0.02\).
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FIGURE 2. Mechanistic properties of inhibition of Cav3.3 currents by M1 receptors. A, inhibition of Cav3.3 channels by M1 is reversible. Representative Cav3.3 perforated patch current traces during depolarizing pulses from −110 to −40 mV before (trace 1), during (trace 2), and after (trace 3) perfusion of 1 mM CCh. Note the increase in inactivation kinetics when CCh is applied (Table 1). B, plot of peak current amplitude (for same cell as in A) showing the rate of inhibition by 1 mM CCh perfusion and the rate of washout, with the selected traces from A (traces 1–3) labeled. C, application of 1 mM CCh increases Cav3.3 inactivation kinetics in a reversible manner. The inactivating component of every trace from B was fit with an exponential equation to give $\tau_{\text{inact}}$. D, application of 1 mM CCh dramatically reduces the amount of Ca\textsuperscript{2+} influx through Cav3.3 channels. The effects of M1 activation on normalized Ca\textsuperscript{2+} influx is shown for all Cav3.3 cells. Perfusion of 1 mM CCh caused a 77% (± 2%, n = 20) decrease in Ca\textsuperscript{2+} influx. E, inhibition of Cav3.3 currents by 1 mM CCh occurs through M1 receptors. Control experiments show elimination of the inhibition because of 1 mM CCh when a muscarinic antagonist (atropine) is co-applied or when a control vector (pBluescript) is transfected instead of M1. A lack of depolarizing test pulses during initial CCh perfusion, increase in test pulse frequency to 0.5 Hz, or a hyperpolarizing prepulse to −140 mV for 1 s had no significant (p > 0.05) effect on the magnitude of Cav3.3 inhibition by M1. * indicates significance at p < 0.001 compared with control (0.2 Hz). F, CCh inhibited Cav3.3 currents in a dose-dependent manner. CCh concentration versus percentage block data were fit with a Hill equation, and the IC\textsubscript{50} for CCh inhibition of Cav3.3 currents was 27 μM. All data points correspond to mean ± S.E.

In addition, experiments, indicating that M1 effects on Cav3.3 are use-independent. Increasing the test pulse frequency to 0.5 Hz also caused no significant (p > 0.05) change in the level of Cav3.3 inhibition, indicating that the M1 effects on Cav3.3 are also frequency-independent (Fig. 2E). Another possibility is that M1 receptor activation inhibits Cav3.3 currents by shifting steady-state inactivation to more hyperpolarized potentials, reducing the proportion of channels available (in the closed state) to open at the holding potential of −110 mV. A protocol with a 1-s prepulse to −140 mV to remove accumulated channel inactivation demonstrated no significant (p > 0.05) difference in inhibition compared with the control protocol, suggesting that the inhibitory effect is not because of changes in the steady-state inactivation of Cav3.3 channels (Fig. 2E).

Control experiments with mock transfections of an empty control vector or with a preincubated mACHR antagonist (atropine) demonstrated that the CCh-induced inhibition of Cav3.3 currents is mediated specifically via the transfected M1 receptor (Fig. 2E). Testing the effects of varying concentrations of CCh on stable Cav3.3 cells with transfected M1 receptors revealed that the inhibitory effect is dose-dependent (Fig. 2F). The IC\textsubscript{50} for inhibition of Cav3.3 currents by CCh = 27 μM, consistent with that reported for phosphatidylinositol hydrolysis triggered by M1 receptor activation in both HEK 293 and Chinese hamster ovary cells (39, 40).

Inhibition of Cav3.3 Channels by M1 Receptors Requires G_Gβγ_or Gα−mediated processes. To test for the involvement of Gβγ, a membrane-targeted version of the carboxyl terminus of β-arrestin kinase, MAS-GRK3ct (41), was co-transfected with M1 receptors into the stable Cav3.3 HEK cell line. Control experiments showed that the MAS-GRK3ct construct was able to completely abolish the well described Gβγ-dependent inhibition of N-type Ca\textsuperscript{2+} channels (data not shown). MAS-GRK3ct only partially inhibited Cav3.3 currents, indicating that M1 effects on Cav3.3 are use-independent. Increasing the test pulse frequency to 0.5 Hz also caused no significant (p > 0.05) change in the level of Cav3.3 inhibition, indicating that the M1 effects on Cav3.3 are also frequency-independent (Fig. 2E). Another possibility is that M1 receptor activation inhibits Cav3.3 currents by shifting steady-state inactivation to more hyperpolarized potentials, reducing the proportion of channels available (in the closed state) to open at the holding potential of −110 mV. A protocol with a 1-s prepulse to −140 mV to remove accumulated channel inactivation demonstrated no significant (p > 0.05) difference in inhibition compared with the control protocol, suggesting that the inhibitory effect is not because of changes in the steady-state inactivation of Cav3.3 channels (Fig. 2E).

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![Graphs showing inhibition of Cav3.3 currents by M1 and RGS2](image)

FIGURE 3. Inhibition of Cav3.3 channels by M1 requires G\(\alpha_{q/11}\) signaling. A, In perforated patch recordings of Cav3.3 stable cells co-transfected with M1 and a membrane targeted form of the carboxyl terminus of \(\beta\)-arrestin kinase that sequesters active G\(\beta\)\(\gamma\) subunits (MAS-GRK3ct), application of 1 mM CCh caused inhibition of Cav3.3 currents (−24.8 ± 3.4%, \(n = 10\)). B, similarly, inhibition by perfusion of 1 mM CCh was observed for Cav3.3 cells co-transfected with the G\(\beta\)\(\gamma\) buffer Go\(_{transducin}\) (Go\(_{q}\)) and M1 (−25.1% ± 2.5%, \(n = 10\)). C, In perforated patch recordings of Cav3.3 cells co-transfected with M1 and RGS2 (antagonist of active G\(\alpha_{q/11}\) subunits), application of 1 mM CCh predominantly had no effect (1 ± 5%, \(n = 7\)) on Cav3.3 currents. D, Bar graph comparing various genetic and pharmacological manipulations to control conditions where stable Cav3.3 cells are transfected with M1 and inhibited by 1 mM CCh. Inhibitors of serine/threonine kinases (500 nM staurosporine, \(n = 7\); 50 \(\mu\)M H9, \(n = 6\)), PKC (10 \(\mu\)M chelerythrine, \(n = 5\); 500 nM Go 6976, \(n = 6\)), tyrosine kinases (10 \(\mu\)M genistein, \(n = 7\)), phosphatases (100 nM okadaic acid, \(n = 6\)), phosphoinositide 3-kinases (200 nM wortmannin, \(n = 7\)), PTX-sensitive G proteins (0.5 \(\mu\)g/ml PTX, \(n = 6\)), cAMP (10 \(\mu\)M (R\(_g\)-cAMP, \(n = 5\)), and internal Ca\(^{2+}\) (10 \(\mu\)M BAPTA-AM, \(n = 5\)) had no significant effect (\(p > 0.02\)) on the inhibition of Cav3.3 currents by M1. RGS2, MAS-GRK3ct, and Go\(_{q}\) (\(p < 0.001\)) caused a significant elimination or reduction in the inhibition of Cav3.3 currents by M1. All data points correspond to mean ± S.E. * indicates significance at \(p < 0.001\) compared with control.

Reduced M1-mediated Cav3.3 current inhibition (−24.8 ± 3.4%, \(n = 10\)) in most cells, suggesting that inhibition is distinct from the previously reported pure G\(\beta\)\(\gamma\)-mediated inhibition of N- and P/Q-type and Cav3.2 Ca\(^{2+}\) channels (42, 43) (Fig. 3, A and D). A smaller subset of MAS-GRK3ct co-transfected cells displayed no exponential inhibitory effect (−9.3 ± 3.0%, \(n = 7\)). Co-expression of transducin (Go\(_{q}\)), which also buffers G\(\beta\)\(\gamma\) signaling (41), caused the same reduction in M1-mediated inhibition of Cav3.3 currents (−25.1% ± 2.5%, \(n = 10\)), with a small number of cells not being inhibited at all (7.0 ± 10.1%, \(n = 3\)) (Fig. 3, B and D).

In contrast to the partial effect of G\(\beta\)\(\gamma\) signaling antagonists, co-expression of the regulator of G-protein signaling 2 (RGS2), an effector antagonist for Go\(_{q/11}\) (44), completely prevented the M1 receptor-induced inhibition of Cav3.3 currents for all cells examined. In perforated patch recordings of Cav3.3 cells co-transfected with M1 and RGS2, application of 1 mM CCh either downstream effectors (PLC) as a negative control. We also performed controls wherein empty vectors were transfected. By comparing traces 30 s after forming the whole-cell configuration with traces 2 min after whole-cell in Fig. 4, A–D, we found that dialysis of the cell with the GTP-containing pipette internal solution caused both a significant reduction in peak current levels and an increase in inactivation kinetics only for the Go\(_{q/11}\) mutants by dialysis of GTP would cause a reduction in current amplitude and an increase in inactivation kinetics. Similar to a study that analyzed inhibition of KCNQ2/KCNQ3 channels by Go\(_{q/11}\) (46), we used constitutively active Go\(_{q}\) (Go\(_{q}\)-Q209L) and Go\(_{q}\) (Go\(_{q}\)-Q209L) mutants to test for the hypothesized effect and a constitutively active Go protein (Go\(13\)-Q226L) that does not couple to the same had no effect (Fig. 3, C and D; 1% ± 5%, \(n = 7\)) or caused a stimulation of Cav3.3 currents (30% ± 9%, \(n = 5\)). RGS2 has been thoroughly characterized and shown to be a selective GTPase-activating protein for Go\(_q\)/Go\(_{11}\), but not for other Go proteins (44), and is an effector antagonist that does not block the G\(\beta\)\(\gamma\)-mediated inhibition of R-type Ca\(^{2+}\) channels (45). Constitutively Active Go\(_{q/11}\) Proteins Modulate Cav3.3 T-type Ca\(^{2+}\) Channels—To further test whether active Go\(_{q/11}\) G-proteins are sufficient to produce inhibition of Cav3.3 currents, stable Cav3.3 cells were transiently transfected with various constitutively active Go subunit constructs. These constructs contain missense mutations that confer constitutive activity by reducing GTPase activity. If Go\(_{q/11}\) is the downstream signal of M1 receptor activation mediating the effects on Cav3.3 currents, then it is hypothesized that activation of the co-expressed Go\(_q\) or Go\(_{11}\) mutants by dialysis of GTP would cause a reduction in current amplitude and an increase in inactivation kinetics.
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FIGURE 4. The \(\text{Ga}_{q/11}\) subtypes of Ga proteins specifically cause inhibition of Cav3.3 currents. A–D, representative whole-cell current traces of stable Cav3.3 cells transfected with various control or Ga plasmids during depolarizing steps from \(-110\) to \(-30\) mV. Traces were obtained 30 s (black) and 2 min (gray) after the whole-cell conformation was formed, using an internal solution that contained 4 mM ATP and 0.3 mM GTP. The stable Cav3.3 cells were mock-transfected with empty plasmid (A) or transfected with the constitutively active forms (lack of GTPase activity) of Ga proteins as follows: \(\text{Go}_{13,0226L}(B), \text{Go}_{q/13,0226L}(C), \text{Go}_{11,0226L}(D)\). \(\text{Go}_{q/13,0226L}\) and \(\text{Go}_{11,0226L}\) cause a time-dependent reduction in Cav3.3 current magnitude. The peak current levels at 2 min were divided by the peak current levels at 30 s to determine the level of inhibition because of internal solution dialysis for the various types of transfected Cav3.3 cells, as described above. The Cav3.3 currents co-transfected with \(\text{Ga}_{q/13,0226L}\) and \(\text{Ga}_{11,0226L}\) had a significant \((p < 0.001)\) reduction in current ratio compared with the control transfection, whereas the \(\text{Ga}_{13,0226L}\) transfected caused no significant change \((p > 0.05)\). F, rate of inactivation \(\tau_{\text{inact}}\) was determined during depolarizing steps from \(-110\) to \(-30\) mV for all transfection types. The \(\tau_{\text{inact}}\) was significantly \((p < 0.001)\) faster for \(\text{Ga}_{q/13,0226L}\) and \(\text{Ga}_{11,0226L}\) compared with control transfections, whereas the \(\tau_{\text{inact}}\) was not significantly \((p > 0.02)\) different for \(\text{Ga}_{13,0226L}\). All data points correspond to mean \(\pm\) S.E. * indicates significance at \(p < 0.001\) compared with transfected control.

compared with control transfections, whereas the \(\tau_{\text{inact}}\) was not significantly different for \(\text{Ga}_{13,0226L}\) \((p > 0.02); \text{Fig. 4F}\).

\(\text{Ga}_{q/11}\) Inhibits Cav3.3 Channels through an Unidentified Nonclassical Pathway—The active GTP-bound form of \(\text{Go}_{q/11}\) causes the activation of PLC, which then produces inositol 1,4,5-trisphosphate- and diacylglycerol/PKC-mediated signals. Various pharmacological antagonists were used to investigate the role of potential cellular signals downstream of \(\text{Go}_{q/11}\). Specific inhibitors of PKC, including 10 \(\mu\)M chelerythrine \((n = 5)\) and 500 \(\mu\)M Go 6976 \((n = 6)\), had no significant \((p > 0.05)\) effect on the M1-mediated inhibition of Cav3.3 currents \((\text{Fig. 3D})\). To ensure pharmacological activity of these antagonists, the PKC-mediated stimulation of Cav3.2 channels by 300 \(\mu\)M phorbol 12-myristate 13-acetate \((65 \pm 17\%, n = 7)\); see Ref 47) was shown to be significantly \((p < 0.02)\) abolished by both 10 \(\mu\)M chelerythrine \((-15 \pm 6\%, n = 5)\) and 500 \(\mu\)M Go 6976 \((3 \pm 5\%, n = 5)\) (data not shown). Inhibitors of serine/threonine kinases \((500 \mu\text{M} \text{staurosporine}, n = 7; 50 \mu\text{M} \text{H9}, n = 6)\), tyrosine kinases \((10 \mu\text{M} \text{genistein}, n = 7)\), phosphatases \((100 \mu\text{M} \text{okadaic acid}, n = 6)\), phosphoinositide 3-kinases \((200 \mu\text{M} \text{wortmannin}, n = 7)\), pertussis toxin-sensitive \(\text{Ga}\) proteins \((0.5 \mu\text{g/\text{ml}} \text{pertussis toxin}, n = 6)\), cAMP \((10 \mu\text{M} \text{Rq}-\text{cAMP}, n = 5)\), and internal \(\text{Ca}^{2+}\) \((10 \mu\text{M} \text{BAPTA-AM}, n = 5)\) also had no significant effect \((p > 0.02)\) on the inhibition of Cav3.3 currents by M1 receptors \((\text{Fig. 3D})\). In this regard, classical \(\text{Ga}_{q/11}\) downstream effectors such as PKC, cAMP-dependent protein kinase, and increased cytosolic \(\text{Ca}^{2+}\) concentration appear not to be directly involved in the M1 receptor-mediated inhibition of Cav3.3 T-type \(\text{Ca}^{2+}\) currents. Phospholipase C activity has been shown recently to directly inhibit voltage-gated ion channels through the depletion of membrane PI(4,5)P\(_2\) levels, which are thought to stabilize active channels in the membrane \((48)\). Dialyzing cells with a PI(4,5)P\(_2\) antibody \((50 \mu\text{g/\text{ml}})\) to reduce available PI(4,5)P\(_2\) levels had no significant effect \((p > 0.05)\) on the M1 receptor-mediated inhibition of Cav3.3 currents \((\text{Fig. 5, B and D})\). Similarly, dialyzing cells with synthetic PI(4,5)P\(_2\) \((200 \mu\text{M} \text{di-C8 PI(4,5)P}_2)\) to saturate membrane PI(4,5)P\(_2\) levels also had no significant effect \((p > 0.05)\) on M1 receptor-mediated inhibition of Cav3.3 currents \((\text{Fig. 5, C and D})\). As a positive control for di-C8 PI(4,5)P\(_2\) activity and as previously shown \((49)\), dialysis of \(200 \mu\text{M} \text{di-C8 PI(4,5)P}_2\) into HEK 293 cells stably expressing HERG K\(^+\) channels caused a significant \((p < 0.02)\) stimulation of K\(^+\) channel currents \((n = 8)\) compared with control recordings \((n = 5)\); data not shown). Taken together, these results indicate that inhibition of Cav3.3 by M1 receptors occurs either directly through \(\text{Go}_{q/11}\) or a downstream pathway that is independent of PI(4,5)P\(_2\) metabolism and other classical effectors.

\(\text{Ga}_{q/11}\)-coupled Muscarinic Receptors Selectively Inhibit Cav3.3 Channels—If inhibition of Cav3.3 T-type \(\text{Ca}^{2+}\) channels by M1 receptors is primarily dependent on \(\text{Go}_{q/11}\) signaling, then all \(\text{Ga}_q/\text{Ga}_{11}\)-coupled mAChRs should similarly inhibit Cav3.3 currents, whereas \(\text{Ga}_q\)-coupled mAChRs should have no effect. Indeed, activation of co-expressed \(\text{Ga}_q\)-coupled M2 and M4 receptors with 1 \(\mu\text{M} \text{CCH}\) had no effect on Cav3.3 current amplitude \((M2 = -4 \pm 2\%, n = 11; M4 = -4 \pm 3\%, n = 8)\) or kinetics \((\text{Fig. 6, A, C, E, and G}; \text{Table 1})\). In contrast, upon transfection of either the \(\text{Ga}_q/\text{Ga}_{11}\)-coupled M3 or M5 receptor subtypes into stable Cav3.3 cells, perforated patch recordings revealed a significant CCh-mediated inhibition \((M3 = -25 \pm 3\%, n = 10; M5 = -31 \pm 3\%, n = 10)\) as well as a concomitant increase in both activation and inactivation kinetics \((\text{Fig. 6, B, D, F, and H}; \text{Table 1})\). Overall, experiments with genetically encoded antagonists of \(\text{Go}_{q/11}\) (RG522) and GB\(_5\) (MAS-GRK3ct) and genetically encoded \(\text{Ga}\) subtypes, as well as inhibition experiments with various mAChRs, all support the assertion that inhibition of Cav3.3 channels by mAChRs specifically occurs through \(\text{Go}_{q/11}\).
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Two Distinct Cav3.3 Channel Regions Are Involved in M1-mediated Inhibition—Most modulation of Ca\textsuperscript{2+} channels by intracellular signaling pathways involves physical interactions between various effectors and cytoplasmic channel domains (42, 50). Chimeric T-type Ca\textsuperscript{2+} channels between human Cav3.1 and human Cav3.3 were generated to determine the molecular regions of the Cav3.3 channel involved in the M1 receptor-mediated inhibition (Fig. 7). The Cav3.1 and Cav3.3 full-length channels were initially divided into four approximately equal portions, and chimeric channels were constructed using restriction enzyme digestion and religation (see Ref. 37). The four channel portions were named as follows: region 1 = amino terminus + domain I; region 2 = domain I–II linker, domain II + the first 39–63 amino acids of the domain II–III linker; region 3 = remainder of the domain II–III linker + domain III; and region 4 = the domain III–IV linker, domain IV + the carboxyl terminus. Chimeric channel names were assigned based on whether the chimera contained Cav3.1 (G) or Cav3.3 (I) sequence in each of the four regions described (e.g. the chimeric Cav3.3 channel that contained region 2 from Cav3.1 is called IGII).

Co-expression of M1 receptors with chimeric GIII and IIGI T-type channels both showed a similar degree of M1 receptor-mediated peak current inhibition compared with that of the inhibition of the WT Cav3.3 channel (IIII) (Fig. 7, A and E). In contrast, when the IGII chimera was co-transfected with M1 receptors, application of 1 mM CCh resulted in a significantly attenuated degree of inhibition (−5.6 ± 2.1%, n = 11, (p < 0.001)) compared with the wild type IIII channel (−26.9 ± 2.3%, n = 9; Fig. 7E). Interestingly, although the chimeric IGII channels exhibited lowered M1 receptor-mediated inhibition, they still possessed significantly increased inactivation kinetics (p < 0.001; Table 2). Finally, although the IIIG chimeric channels showed similar degree of M1 receptor-mediated peak current inhibition compared with the wild type IIII, the rate of inhibition was notably slower (not shown). The changes in the rate of inhibition for IIIG and the significant decrease in the amount of inhibition for IGII suggested that both regions 2 and 4 might be involved in the M1-induced inhibition of Cav3.3 channels. To explore this, a double chimer (IGIG) was co-transfected into HEK cells with M1 receptors. Fig. 7, C and E, shows that the inhibiting effect of 1 mM CCh application on peak current amplitude was completely abolished for the IGIG chimera (0.9 ± 2.5%, n = 8).

Activation of M1 with 1 mM CCh still caused a significant increase (p < 0.001) in the inactivation kinetics of IGIG, but the \( \tau_{\text{inact}} \) decreased by less than 25% for IGIG, and it decreased by 40–65% for all the single chimeric and wild type Cav3.3 channels (Table 2).

The chimeric channel loss-of-function experiments indicate that both regions 2 and 4 are involved in the M1-mediated inhibition of current amplitude and increase in inactivation kinetics of Cav3.3 currents. In gain-of-function experiments, substitution of either region 2 (GIGG) or region 4 (GGGG) into the Cav3.1 channel resulted in 1 mM CCh-induced inhibition (GIGG = −14.3 ± 0.8%, n = 7; GGGG = −91.1 ± 2.6%, n = 9) that was significantly different (p < 0.001 and p < 0.02, respectively) when compared with GGGG (−0.3 ± 2.2%, n = 9; Fig. 7, B and E; Table 2). In contrast, inclusion of either region 1 or region 3 of Cav3.3 into Cav3.1 resulted in no significant change (p > 0.05) in M1-mediated inhibition when compared with GGGG (Fig. 7E). Although both GIGG and GGGG were inhibited by M1, the level of inhibition was significantly lower (p < 0.001) than the inhibition of IIII by M1 (Fig. 7E). When the effect of 1 mM CCh application on GIGG current amplitude was tested, M1 activation was found to produce a significant level of...
GIGI inhibition (−25.1 ± 2.4%, n = 11; p < 0.001) compared with GGGG that was not significantly (p > 0.05) different from the inhibition of IIII (Fig. 7, A, B, D, and E). Application of 1 mM CCh also significantly increased (p < 0.001) the rate of inactivation for the GIGI Cav3.1 chimera but not for GIGG, GGII, or the other Cav3.1 single chimeras (Table 2). Overall, the combined substitution of regions 2 and 4 from the Cav3.3 channel into the Cav3.1 channel completely restores M1-induced inhibition together with the associated increase in channel inactivation kinetics.

**DISCUSSION**

In this study we systematically explored the effects of activated muscarinic GPCRs on the three main T-type Ca\(^{2+}\) channel isoforms expressed in the mammalian nervous system, and we report for the first time the differential modulation between a G-protein signaling pathway and Cav3.3 T-type Ca\(^{2+}\) channels. Most studies on T-type Ca\(^{2+}\) channel modulation have involved the Cav3.2 (\(\alpha_{1H}\)) subtype, revealing specific modulatory responses to G\(_{\beta\gamma}\)2, CAMKII, and redox modulation that are not observed for the Cav3.1 and Cav3.3 T-type Ca\(^{2+}\) channel isoforms (42, 51, 52). The exclusive inhibition of Cav3.3 channels by G\(_{\beta\gamma}\)q/11-coupled mAChRs is the first example of specific GPCR-mediated modulation of a T-type Ca\(^{2+}\) channel subtype other than for Cav3.2.

**Differential Effects of mAChRs on T-type Ca\(^{2+}\) Channel Isoforms—**Examination of the literature shows that activation of mAChRs can result in multiple effects on native T-type Ca\(^{2+}\) currents, including causing stimulation (26, 27, 53), inhibition (28), or having no effect (54). Given the heterogeneous nature of native low threshold Ca\(^{2+}\) currents, without investigating interactions between specific mAChR gene products and specific T-type Ca\(^{2+}\) channel isoforms, the published differences in modulation...
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**TABLE 2**

Effects of M1 receptor activation on chimeric T-type channel inactivation kinetics

|       | III  | IGII | IIIG | IGIG | GGGG | GIGG | GGGI | GIGI |
|-------|------|------|------|------|------|------|------|------|
| Control \(\tau_{\text{inact}}\) (ms) | \(117 \pm 6, n = 10\) | \(86 \pm 7, n = 11\) | \(103 \pm 11, n = 10\) | \(62 \pm 2, n = 8\) | \(14 \pm 1, n = 9\) | \(20 \pm 1, n = 9\) | \(38 \pm 3, n = 8\) | \(58 \pm 2, n = 10\) |
| 1 mM CCh \(\tau_{\text{inact}}\) (ms) | \(41 \pm 5, n = 10\*\) | \(43 \pm 3, n = 11\*\) | \(58 \pm 4, n = 10\*\) | \(47 \pm 3, n = 8\*\) | \(13 \pm 1, n = 9\) | \(17 \pm 2, n = 7\) | \(32 \pm 2, n = 8\) | \(38 \pm 1, n = 10\*\) |

\*\(p < 0.001\)

\*\(p < 0.02\)

are nearly impossible to interpret. Our results using exogenous expression of cloned T-type Ca\(^{2+}\) channels indicates that M1 receptor activation has a robust inhibitory effect on Cav3.3 currents and has either no effect or a small stimulation on both Cav3.1 and Cav3.2 currents. Similarly, experiments examining native Cav3.2 Ca\(^{2+}\) channels in NIH3T3 cells transiently transfected with mAChRs demonstrated that M1 receptor activation had either no effect or a stimulatory effect if a PKC inhibitor was applied (27). Active G\(_{\beta\gamma}\) subunits have been shown to specifically inhibit Cav3.2 currents (42, 55), and the lack of inhibition of Cav3.2 channels by M1 receptors in our study is likely because of the absence of any functional coupling between M1 receptors and G\(_{\beta\gamma}\) proteins (56). We also found that all G\(_{\alpha_{i}q_{11}}\)-coupled mAChR subtypes (M1, M3, and M5) cause attenuation of Cav3.3 currents, whereas G\(_{\alpha_{i}}\)-coupled M2 and M4 receptors had no effect on Cav3.3 currents. Thus it is likely that any stimulation of T-type Ca\(^{2+}\) currents by mAChRs in native systems does not involve Cav3.3 channels. Experiments testing the effects of recombinant M2–M5 receptors on the Cav3.2 and Cav3.1 Ca\(^{2+}\) channel isoforms in a heterologous system are required to further facilitate the possibility of interactions between these T-type channels and mAChRs.

**Functional Effects of M1 Receptor Activation on Cav3.3 Currents**—Activation of M1 receptors dramatically altered Cav3.3 currents by both reversibly attenuating peak current levels and increasing the rate of inactivation, resulting in a significant reduction in the influx of Ca\(^{2+}\). The relationship between these effects was explored using both structural channel chimera and classical gating property studies. In chimeric studies (see below), the activation of M1 receptors primarily caused an increase in inactivation kinetics of the IGII chimera and, conversely, primarily a decrease in peak current levels for the GIGG chimera. Both this isolation of the two specific M1 receptor-mediated effects and the gating results discussed below suggest that the effects of M1 on current amplitude and inactivation kinetics are complementary but distinct phenomena. For gating studies, reduction of Cav3.3 current magnitude by M1 receptor activation was equally robust when the Cav3.3 channels were held in various states including: 1) during a prolonged hyperpolarization with no test depolarizations (channels mostly in closed state); 2) after a stronger hyperpolarizing prepulse to \(-140\) mV; and 3) during 200-ms test depolarizations to peak potential at 0.2 and 0.5 Hz. Combining this lack of use dependence with the observed reduction in peak current amplitude and the increase in activation and inactivation kinetics indicates that all states of the Cav3.3 channel are subject to modulation by M1 receptor activation. The acceleration of Cav3.3 channel kinetics by M1 receptor activation also supports the hypothesis that modulation affects channel biophysical properties and not channel density via internalization, which has recently been shown to occur for the voltage-independent, GPCR-mediated inhibition of N-type Ca\(^{2+}\) channels on a relatively fast time scale (57). Physiologically, the combined decrease in Cav3.3 peak currents and the increased activation and inactivation kinetics would be predicted to alter neuronal firing patterns and perhaps eliminate rhythmic oscillations (23, 58). In support of this notion, the concomitant reduction in peak current and increase in inactivation kinetics of Cav3.3 currents triggered by anandamide have been shown to completely eliminate the sustained, rhythmic Cav3.3 current during an action potential voltage clamp experiment with an oscillating thalamic waveform (59).

**Signal Transduction Pathway of M1 Receptor-mediated Cav3.3 Inhibition**—Use of genetically encoded antagonists of G\(_{\beta\gamma}\) (MAS-GRK3ct and G\(_{\alpha_{i}}\)) and G\(_{\alpha_{i}q_{11}}\) (RG52) demonstrated that G\(_{\beta\gamma}\) may partially contribute to the M1-mediated inhibition of Cav3.3 currents, whereas G\(_{\alpha_{i}q_{11}}\) is absolutely required for complete inhibition. The potential involvement of both G\(_{\alpha_{i}q_{11}}\) and G\(_{\beta\gamma}\) in a nonclassical, voltage-independent mechanism of Ca\(^{2+}\) channel inhibition by mAChRs has been described previously for HVA Ca\(^{2+}\) channels. In rat superior cervical ganglion sympathetic neurons, application of a muscarinic agonist causes the voltage-independent inhibition of endogenous N-type Ca\(^{2+}\) channels that is abolished by co-expression of RG52, G\(_{\alpha_{i}}\), or MAS-GRK3ct and exhibits a time course similar to the Cav3.3 inhibition reported here (41). As G\(_{\beta\gamma}\) is a co-factor for PLC\(_{\beta}\) activity, a possible explanation is that sequestering G\(_{\beta\gamma}\) reduces PLC\(_{\beta}\) activity (60). Although G\(_{\beta\gamma}\) may potentiate the inhibitory effect of M1 receptor activation, transfection of constitutively active G\(_{\alpha_{i}q_{11}}\) mutants into...
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stable Cav3.3 cells demonstrated that active Go\textsubscript{q/11} alone is sufficient to induce the inhibition of Cav3.3 currents. In support of this notion, only Go\textsubscript{q/11}-coupled mACHRs (M1, M3, and M5) inhibited Cav3.3 currents, whereas Go\textsubscript{i/o} coupled M2 and M4 receptors that activate G\beta\gamma signaling have no effect on Cav3.3 currents. Unlike that reported for the attenuation of Cav3.2 channels by G\beta\gamma\gamma, this novel form of T-type Ca\textsuperscript{2+} channel inhibition involves the Go\textsubscript{q/11} subunit and also affects channel kinetics. This inhibitory mechanism for the Cav3.3 T-type isoform may be applicable to all Go\textsubscript{q/11}-coupled receptors as we have also found a similar inhibition of Cav3.3 channels by mGlur\textsubscript{R3} receptors (61).

Pharmacological antagonists eliminated the potential involvement of various intracellular signals downstream of Go\textsubscript{q/11} activation that may be involved in the inhibition of Cav3.3 by M1 receptor activation. Abolishing the activity of PKC, serine/threonine kinases (including cAMP-dependent protein kinase), tyrosine kinases, phosphatases, phosphoinositide 3-kinases, and intracellular Ca\textsuperscript{2+} signaling all had no effect on inhibition. This profile of M1/Go\textsubscript{q/11}-mediated Ca\textsuperscript{2+} channel inhibition resistant to common antagonists of cytoplasmic signaling is not unique and has been reported for the inhibition of L-type channels by Go\textsubscript{q/11}-coupled M1/3/5 receptors in HEK cells (62). Like the inhibition of Cav3.3 via M1 receptors, this inhibition is voltage-independent, relatively slow kinetically (\t\tau \text{on} \approx 13 \text{s}), and insensitive to antagonists of protein kinases and protein phosphatases (62).

A more recent explanation for the Go\textsubscript{q/11}-mediated inhibition of ion channels, including voltage-gated K\textsuperscript{+} channels and HVA Ca\textsuperscript{2+} channels, has emerged wherein channel activity is suppressed through the depletion of membrane PI(4,5)P\textsubscript{2} levels via PLC activity (46, 63, 64). In these studies, Go\textsubscript{q/11}-mediated inhibition was shown to be inhibited via dialysis of synthetic PI(4,5)P\textsubscript{2} or a PI(4,5)P\textsubscript{2}-specific antibody into the cytoplasm. In our experiments, adding di-C8 PI(4,5)P\textsubscript{2} or the PI(4,5)P\textsubscript{2} antibody into the internal pipette solution and dialyzing cells for up to 25 min had no significant effect on M1-mediated inhibition of Cav3.3 channels, suggestive of another to-be-defined mechanism whereby Go\textsubscript{q/11} signaling causes the inhibition of voltage-gated ion channels. Further biochemical and biological experiments are required to clarify the nature of the intracellular messengers and/or scaffolding proteins that can modulate Cav3.3 T-type Ca\textsuperscript{2+} channels and also whether Go\textsubscript{q/11} can interact directly with the channel through a novel mechanism.

Go\textsubscript{q/11}-mediated Inhibition of Cav3.3 Involves Two Discrete Channel Regions—Replacing both regions 2 and 4 in the Cav3.3 channel with the corresponding Cav3.1 T-type Ca\textsuperscript{2+} channel sequences abrogated both the M1 receptor-mediated peak current inhibition and concomitant increase in inactivation kinetics. Conversely, substituting regions 2 and 4 from Cav3.3 into Cav3.1 conferred M1 receptor-mediated inhibition and increased inactivation kinetics. These data suggest that regions 2 and 4 of the Cav3.3 channel are both necessary and sufficient to recapitulate M1 receptor-mediated channel modulation. Region 2 of the Cav3.1 and Cav3.3 sequence contains the highly divergent domain I–II linker, the highly conserved domain II, and 39–63 amino acids of the domain II–III linker, and region 4 contains most of the III–IV linker, the highly conserved domain IV, and the highly divergent carboxyl terminus. Based on their putatively intracellular regions and their high divergence between the two T-type isoforms, the I–II linker, proximal region of the II–III linker, the III–IV linker, and the carboxyl terminus are all candidates for modulation sites within regions 2 and 4. Interestingly, the only identified sites of alternative splicing within the rat and human Cav3.3 channel occur both in the I–II linker and the carboxyl-terminal regions (38, 65). The effects of these splicing variations on the biophysical properties (activation kinetics) of the human Cav3.3 channel are interdependent rather than additive, suggesting a possible direct interaction between the I–II linker and carboxyl terminus that affects channel kinetics (24). Both the human and rat Cav3.3 channels inhibited by M1 receptor activation in our study lack exon 9 located in the I–II linker, whereas both the rat and human Cav3.1 channels have a 10-amino acid insertion in this region in a manner similar to that for the +exon 9 Cav3.3 splice variant. Thus, several observations suggest that the I–II linker may be a target region in the inhibition of Cav3.3 by M1, and some evidence points to a possible role for the carboxyl terminus. However, as multiple structural determinants contribute to the slow inactivation kinetics of Cav3.3 compared with Cav3.1 in a nearly additive manner (37), and M1 activation dramatically speeds up Cav3.3 inactivation kinetics, it is also possible that multiple intracellular loci within regions 2 and 4 of the Cav3.3 channel may be involved in the M1-mediated effect.

In summary, we find that activation of known Go\textsubscript{q/11}-coupled mACHRs results in the selective inhibition of Cav3.3 T-type Ca\textsuperscript{2+} currents with a concomitant increase in inactivation kinetics. The Go\textsubscript{q/11}-mediated signaling pathway appears to be mediated via two disparate regions of the Cav3.3 channel. Functional interactions between mACHRs and Cav3.3 Ca\textsuperscript{2+} channels could potentially impact firing patterns of various cell types, including thalamic nRT cells. Biophysical and pharmacological evidence suggests that primarily Cav3.3 channels compose dendritic T-type currents in nRT cells (22), whereas immunostaining suggests the presence of M3 receptors in these cells (33). This raises the possibility that the inhibition of Cav3.3 T-type Ca\textsuperscript{2+} channels by M3 receptors in the dendrites of nRT cells could be involved in cholinergic modulation of thalamic firing patterns.

Acknowledgments—We thank Dr. Brett Adams for the kind gifts of MAS-GRK3ct and EGFP-RGS2 and Drs. Aaron Beedle, Arnaud Monteil, and Philippe Lory for the human Cav3.1 and Cav3.3 channels used in the chimeric channel experiments. We also thank Dr. Colin Thacker, Tracy Evans, Paul Adams, and Dr. John Tyson for providing stable cell lines, and to Dr. Philippe Isope for comments on the manuscript.

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