Roscovitine Inhibits CaV3.1 (T-Type) Channels by Preferentially Affecting Closed-State Inactivation

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Received August 18, 2011; accepted November 15, 2011

ABSTRACT

T-type calcium channels (CaV3) play an important role in many physiological and pathological processes, including cancerogenesis. CaV3 channel blockers have been proposed as potential cancer treatments. Roscovitine, a trisubstituted purine, is a cyclin-dependent kinase (CDK) inhibitor that is currently undergoing phase II clinical trials as an anticancer drug and has been shown to affect calcium and potassium channel activity. Here, we investigate the effect of roscovitine on CaV3.1 channels. CaV3.1 channels were transiently expressed in human embryonic kidney 293 cells, and currents were recorded by using the whole-cell patch-clamp technique. Roscovitine blocks CaV3.1 channels with higher affinity for depolarized cells (EC_{50} of 10 μM), which is associated with a negative shift in the voltage dependence of closed-state inactivation. Enhanced inactivation is mediated by roscovitine-induced acceleration of closed-state inactivation and slowed recovery from inactivation. Small effects of roscovitine were also observed on T-channel deactivation and open-state inactivation, but neither could explain the inhibitory effect. Roscovitine inhibits CaV3.1 channels within the therapeutic range (10–50 μM) in part by stabilizing the closed-inactivated state. The ability of roscovitine to block multiple mediators of proliferation, including CDKs and CaV3.1 channels, may facilitate its anticancer properties.

Introduction

T-type calcium channels (CaV3) are low voltage-activated channels with fast-inactivation and slow-deactivation kinetics that consist of three family members CaV3.1 (α1D), CaV3.2 (α1H), and CaV3.3 (α1L) (Perez-Reyes, 2003). CaV3 channels are widely distributed among different cell types including neurons, cardiomyocytes, and smooth and skeletal muscles (Perez-Reyes, 2003). In spite of establishing many aspects of function and cell specificity, nicely reviewed by Edward Perez-Reyes (Perez-Reyes, 2003), their role in many physiological and pathophysiological processes remains unclear. However, an emerging body of evidence suggests that CaV3 channels can participate in pathological processes such as chronic pain (Jagodic et al., 2008) and cancer cell proliferation (Gray and Macdonald, 2006; Lee et al., 2006; Heo et al., 2008; Lu et al., 2008; Taylor et al., 2008a,b). These findings, in particular regarding the channels’ role in cancer, have made CaV3 channels an attractive clinical target (Gray and Macdonald, 2006).

Roscovitine is a trisubstituted purine, which initially was proposed as an anticancer therapy because of its blocking effect on cyclin-dependent kinases (CDKs) (Meijer et al., 1997; Fischer and Gianella-Borradori, 2003; Wesierska-Gadek et al., 2007). This drug is also known as CYC202 and seliciclib and is currently undergoing phase II clinical trials as a treatment for non–small-cell lung cancer and nasopharyngeal cancer. Emerging evidence suggests that roscovitine may have additional targets involved with tumor development. For example, we have shown that roscovitine can also inhibit human ether-a-go-go related gene (HERG) potassium channel activity (Ganapathi et al., 2009), and HERG channel block can reduce the growth of certain cancer types (Pardo et al., 2005). CaV3 channels are potential targets for anticancer therapy (Gray and Macdonald, 2006). CaV3.1 channels are expressed in many human cancer cell types including liver, (Jagodic et al., 2008) and cancer cell proliferation (Gray and Macdonald, 2006; Lee et al., 2006; Heo et al., 2008; Lu et al., 2008; Taylor et al., 2008a,b). These findings, in particular regarding the channels’ role in cancer, have made CaV3 channels an attractive clinical target (Gray and Macdonald, 2006).

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ovarian, and breast cancers, and proliferation is reduced by inhibition of these channels by either down-regulation (short interfering RNA) or drug application (Lu et al., 2008; Taylor et al., 2008a; Li et al., 2009, 2011). Through its unique effects on ion channels, roscovitine has provided critical insights into gating mechanisms and the treatment of disease (Buraei et al., 2005, 2007; Cho and Meriney, 2006; Yarotskyy and Elmslie, 2007; Buraei and Elmslie, 2008; Ganapathi et al., 2009; Yarotskyy et al., 2009; Yazawa et al., 2011), which motivated us to test the effect of roscovitine on CaV3.1 channels. We find that CaV3.1 channels are inhibited by roscovitine. This inhibition is potentiated by depolarized voltages so that at a membrane potential of −70 mV the EC90 is 10 μM, which is at the low end of the therapeutic range (10–50 μM) for roscovitine block of cancer cell proliferation (Meijer et al., 1997; Fischer and Gianella-Borradori, 2003; Wiesierska-Gadek et al., 2007). We conclude that the inhibition of CaV3.1 channels could provide a third anticancer mechanism for roscovitine in addition to CKD and HERG block, which will probably enhance the therapeutic efficacy of roscovitine as an anticancer drug.

Materials and Methods

**HEK Cell Transfection.** We used either calcium phosphate precipitation (Yarotskyy and Elmslie, 2007) or Lipofectamine 2000 (Yarotskyy et al., 2010) to transfect HEK293 cells with CaV3.1 channels (cloned from rat pancreatic β cells, a generous gift from Dr. Ming Li, Tulane University Medical School, New Orleans, LA; Genbank no. AF125161) (Zhuang et al., 2000), which provided highly reproducible expression (cloned from rat pancreatic β cells, a generous gift from Dr. Ming Li, Tulane University Medical School, New Orleans, LA; Genbank no. AF125161) (Zhuang et al., 2000), which provided highly reproducible expression. CaV3.1 channels were transfected in HEK293 cells by adding cDNA plasmids as follows: 11.5 μg of α1D (CaV3.1), 2.15 μg of δ1 (CaV3.1), and 1 μg of green fluorescent protein (to visualize transfected cells). They were incubated at 5% CO2 for 8 h after which the transfecting medium was replaced by the standard DMEM. The transfected cells were split the next day into 35-mm dishes that served as the recording chamber. Recordings were performed 24 to 48 h after transfection.

**Measurement of Ionic Currents.** Cells were voltage-clamped by using the whole-cell configuration of the patch-clamp technique. Pipettes were pulled from Schott 8250 glass (Garner Glass, Claremont, CA) on a Sutter P-97 puller (Sutter Instrument Company, Novato, CA). Currents were recorded by using an Axopatch 200A amplifier (Molecular Devices, Sunnyvale, CA) and digitized with an ITC-18 data acquisition interface (Instrutech Corporation, Port Washington, NY). Experiments were controlled by a Power Macintosh G3 computer (Apple Computer, Cupertino, CA) running S8 data acquisition software written by Dr. Stephen Ikeda (National Institutes of Health, National Institute on Alcohol Abuse and Alcoholism, Bethesda, MD). Leak current was subtracted online by using a P/4 protocol. Recordings were carried out at room temperature, and the holding potential (HP) was −120 mV, unless otherwise stated. Some studies examined CaV3.1 current activated by an action potential wave form (AP) that was generated by using a series of voltage steps and ramps with the following values (HP = −120 mV): −80 mV for 1 ms, −80 to 37 mV in 0.5 ms, 37 to 40 mV in 0.1 ms, 40 to 37 mV in 0.1 ms, 37 to −80 mV in 1.5 ms, and −80 mV at 11.5 ms. A 20-Hz 10-AP train was generated by using the same voltage changes except that the 1-ms step to −80 mV was removed and voltage between APs within the train was −120 mV. Whole-cell currents were digitized depending on voltage step duration at 50 kHz (up to 100 ms), 10 kHz (200 ms), and 4 kHz (2000 ms) after analog filtering at 1 to 10 kHz.

**Data Analysis.** Data were analyzed by using IgorPro versions 5 and 6 (WaveMetrics, Lake Oswego, OR) run on a Macintosh computer. Percentage of inhibition was measured by comparing the steady-state drug effect to the average of current measured before [control (Cntl)] and after full recovery [washout (W)]. Activation time constant (τAct) was determined by fitting a single exponential function to the step current after a 0.3 ms delay (Buraei et al., 2007). The effect of roscovitine on T-channel inactivation was measured by using either 100- or 1000-ms inactivating voltage steps followed by a 20-ms test step to −20 mV. The Inact ratio for the current measured from the test step was plotted against inactivating voltage (either 100- or 1000-ms steps) and fitted by a single Boltzmann equation to yield half-maximal voltage (V1/2), slope factor (k), and the magnitude of inactivation. Inactivation time constant (τInact) was determined by fitting a single exponential function from peak step current to the end of the step. The development of closed-state inactivation was determined by measuring the effect of increasing duration of voltage steps to −70 mV on a 20-ms step to −20 mV (Serrano et al., 1999) (see Fig. 6A). The recovery from inactivation protocol used a 1000-ms inactivating step to either −20 or −70 mV followed by a 20-ms test step to −20 mV after an increasing recovery time at −120 mV. Group data were calculated as mean ± S.D. A paired t test was used for within-cell comparisons. One-way analysis of variance with Tukey honestly significant difference post hoc test was used to test for differences among three or more independent groups.

**Solutions.** The internal pipette solution contained 104 mM N-methyl-d-glucamine (NMG)-Cl, 14 mM creatine-Po4, 6 mM MgCl2, 10 mM NMG-HEPES, 5 mM Tris-ATP, 0.3 mM Tris-GTP, and 10 mM Mg-EGTA with osmolarity of 280 mOsm and pH 7.3. The external recording solution contained 30 mM BaCl2, 100 mM NMG-Cl, and 10 mM NMG-HEPES with osmolarity of 300 mOsm and pH 7.3. The extracellular solution contained dimethyl sulfoxide and stored at −30°C. All external solutions contained the same dimethyl sulfoxide concentration so that the roscovitine concentration was the sole variable when changing solutions. Test solutions were applied from a gravity-fed perfusion system that provided complete solution exchange within 1 to 2 s.

**Chemicals.** All experiments used Rosc (De Azevedo et al., 1997) from LC Labs (Woburn, MA). DMEM, fetal bovine serum, and 100× antibiotic/antimycotic (penicillin, streptomycin, and amphotericin B) were from Invitrogen (Carlsbad, CA). Other chemicals were obtained from Sigma (St. Louis, MO).

**Results.** Roscovitine Blocks T-Type Channels. Roscovitine has provided surprising insights into ion channel gating mechanisms and disease treatment (Buraei et al., 2005, 2007; Cho and Meriney, 2006; Yarotskyy and Elmslie, 2007; Buraei and Elmslie, 2008; Ganapathi et al., 2009; Yarotskyy et al., 2009; Yazawa et al., 2011). Thus, we were interested in determining whether T-channels would be affected. CaV3.1 channels expressed in HEK293 cells were activated by using 15-ms depolarizing steps ranging from −100 to +20 mV, followed by repolarization to −100 mV (Fig. 1). Roscovitine (45 μM) strongly blocked the current in a voltage-independent manner (Fig. 1, A–C). Figure 1C shows no significant difference in the activation versus voltage relationship. The activation versus voltage relationship was examined in more detail by plotting tail current amplitude (Iact) versus step voltage (Fig. 1D), which showed a very small right shift induced by 45 μM roscovitine. Single Boltzmann function fitting yielded V1/2 for control = −38.0 ± 4.5 mV, roscovitine = −36.7 ± 4.4 mV, and washout = −37.6 ± 4.6 mV (n = 9) (Fig. 1D) and slope factor (k)
Roscovitine Does Not Affect Activation but Slows Deactivation of CaV3.1 Channels. We have reported previously that roscovitine slowed CaV1.2 channel activation, which contributes to the inhibitory effect (Yarotskyy and Elmslie, 2007; Yarotsky et al., 2009). For that reason, we examined the effect of roscovitine on CaV3.1 channel activation speed by comparing \( \tau_{\text{Act}} \) determined by fitting step current onset with a single exponential function. We found no effect of roscovitine on \( \tau_{\text{Act}} \) at any CaV3.1 current-generating voltage. Indeed, normalized step currents were superimposed in control versus 45 \( \mu \)M roscovitine (Supplemental Fig. 1).

The absence of an effect on CaV3.1 current activation suggests that the small shift in activation \( V_{\text{0.5}} \) observed in Fig. 1D is induced by a different mechanism. Because there is precedent for roscovitine to slow calcium channel deactivation (Buraei et al., 2005, 2007), we measured the speed of CaV3.1 channel closing over voltages ranging from –50 to –160 mV. CaV3.1 current was activated by 10-ms steps to –10 mV followed by a 72-ms repolarizing (tail) step (Fig. 2). The tail current was fitted by a single exponential function to yield the deactivation time constant (\( \tau_{\text{Deact}} \)), which was plotted against tail voltage (Fig. 2C). Roscovitine significantly increased \( \tau_{\text{Deact}} \) at voltages ranging from –140 to –50 mV (\( p < 0.05 \)). The effect was small and can be better demonstrated by the roscovitine-induced change in \( \Delta \tau_{\text{Deact}} \) (Fig. 2D). Qualitatively, this effect is similar to that of roscovitine on CaV2.1 and CaV2.2 channels (~7-fold increase in \( \tau_{\text{Deact}} \)) (Buraei et al., 2005, 2007), but the increase of CaV3.1 current \( \tau_{\text{Deact}} \) was small and did not exceed 20%. This small effect on \( \Delta \tau_{\text{Deact}} \) is likely to mediate the minor roscovitine-induced right shift in activation \( V_{\text{0.5}} \) (Fig. 1D) and could possibly reduce the overall CaV3.1 current inhibition by a small amount.

**Roscovitine Blocks CaV3.1 Channels in a Dose-Dependent Manner.** To determine the dose-dependent effect of roscovitine, we tested the effect of 1, 10, 30, 45, and 100 \( \mu \)M roscovitine on CaV3.1 current (Fig. 3). Fractional block was plotted against roscovitine concentration and fitted by Hill’s equation yielding \( E_{50} \) of 40.5 ± 7.6 \( \mu \)M and Hill’s coefficient of 1.55 ± 0.11 (\( n = 5 \)). This \( E_{50} \) was in the range obtained for CaV1.2 channels (Yarotskyy and Elmslie, 2007). Some CaV3 channel blockers, such as mibebradil (McDonough and Bean, 1998), octanol (Eckle and Todorovic, 2010), and T-type antagonist A2 (Uebele et al., 2009), can preferentially affect inactivated channels. We determined whether roscovitine had apparent higher affinity at a more depolarized holding potential at which CaV3.1 channels inactivate. We found that a ~70-mV holding potential significantly decreased the roscovitine \( E_{50} \) of 10.0 ± 1.0 \( \mu \)M, and Hill’s coefficient of 1.22 ± 0.15 compared with the results from HP –120 mV (Fig. 3D). This result supports the idea that roscovitine preferentially affects inactivated channels.

**Roscovitine Slows Open-State Inactivation.** Based on the effect of holding potential on the dose-response relationship, we expected to observe a significant enhancement of inactivation by roscovitine. Using 100-ms voltage steps to inactivate CaV3.1 channels, we found that roscovitine had the opposite effect with a significant decrease of inactivation at voltages ≥ –40 mV (Fig. 4). There was a small roscovitine-induced enhancement of inactivation, but only at –80 and –70 mV. The more widespread effect was a ~20% decrease of...
inactivation (Fig. 4A) that corresponded with a significant slowing of inactivation at voltages >–40 mV (Fig. 4, B and C). Although decreased inactivation was unexpected, we realized that the reduced inactivation corresponded to current-generating voltages, whereas a small, but significant, enhancement of inactivation was observed at voltages hyperpolarized to –70 mV, and washout (gray triangles). D, the percentage of change in inactivation (%ΔT\text{Deact}) induced by roscovitine (from C) was calculated from the mean T\text{Deact} of control and washout data. The mean ± S.D. for control (gray circles), 45 μM roscovitine (black squares), and washout (gray triangles). D, the percentage of change in T\text{Deact} (%ΔT\text{Deact}) induced by roscovitine (from C) was calculated from the average of control and washout data. The mean ± S.D. are shown with the data significantly different from zero indicated by the line (p < 0.05).

Roscovitine Affects Closed-State Inactivation. The 4-fold reduction in roscovitine EC₅₀ with depolarized holding potential (–120 to –70 mV) supports the idea that the inactivated state is stabilized by roscovitine (Fig. 3). Because holding potential changes generally affect closed-state (also called steady-state) inactivation, we tested the effect of longer voltage steps (1000 ms) (Fig. 5), which hyperpolarized the inactivation V₀.5 by 20 mV from –52 mV for 100 ms to –73 mV for 1000 ms (Fig. 4A versus Fig. 5A) so that much of the inactivation was now observed from closed Caᵥ₃.1 channels. Using this paradigm, 45 μM roscovitine significantly left-shifted the inactivation versus voltage relationship ∼10 mV to enhance inactivation at voltages around the resting potential (e.g., –70 mV) (Jones, 1989). Boltzmann equation fits of the inactivation-voltage relationship yielded V₀.5 = –73.4 ± 2.8, –82.5 ± 2.5, and –72.6 ± 2.8 mV (n = 6) for control, roscovitine, and washout, respectively (Fig. 5B). The Boltzmann slope factor was also significantly increased, whereas maximum inactivation was significantly decreased for current generating voltages as observed for inactivation measured from 100-ms steps (Fig. 4A). The enhancement of closed-state inactivation is reminiscent of the inhibitory effect of roscovitine on N-type channels (Buraei and Elmslie, 2008). The decrease in open-state inactivation could have a potentiating effect on Caᵥ₃.1 current, but the enhancement of closed-state inactivation seems to dominate and increase roscovitine-induced inhibition of Caᵥ₃.1 channel activity.

Roscovitine Affects Inactivation Kinetics. The enhancement of closed-state inactivation by roscovitine could result from either an increased speed of inactivation, decreased recovery from inactivation, or both. We examined the time course for the development of closed-state inactivation.
The recovery from inactivation was investigated by using 1000-ms inactivating steps (−20 mV) to inactivate Ca₃.1 channels and short test steps to −20 mV of increasing time from the inactivating step to measure the recovery of current (Fig. 7A). The recovery voltage was −120 mV. We found that 45 μM roscovitine significantly slowed recovery from inactivation (Fig. 7, A and B). The recovery time course was best fit by using a double exponential function, which yielded t_inact and t_slow (Fig. 7B). Roscovitine (45 μM) doubled t_inact from 121 ± 36 and 131 ± 21 ms for control and washout, respectively, to 257 ± 89 ms (p < 0.05; n = 5), and t_slow was nearly doubled to 1181 ± 227 ms from 652 ± 111 and 695 ± 100 ms (n = 5; p < 0.05) in control and washout, respectively. The relative amplitude of the slow recovery component was increased from 56% (average control and washout) to 71% of the total (not significant; p = 0.08). Thus, the enhancement of closed-state inactivation could result from slowed recovery from inactivation as well as faster inactivation. This was further investigated by measuring the recovery from inactivation after 1000-ms steps to −70 mV (Fig. 7C). As observed after −20-mV steps, 45 μM roscovitine significantly slowed the recovery from inactivation generated by −70-mV steps (Fig. 7D). Thus, recovery from closed-state inactivation is slowed by roscovitine. One difference was that the recovery was well described by a single exponential equation with the recovery τ in between the t_inact and t_slow measured after the −20-mV step. The expectation was that the τ values would be similar because the recovery voltage was −120 mV for both data sets. However, it is clear that a slow component of recovery exists after the −70-mV step because the amplitude
of the single exponential function reaches only 93% \((p < 0.05)\) of the maximum recovery observed at 15 s (Fig. 7C). The relatively small magnitude of the slow component (7% for \(-70 \text{ mV}\) versus 56% for \(-20 \text{ mV}\)) that bracketed a \(-70 \text{ mV}\) step with duration varying from 10 to 1900 ms. B, the postpulse to prepulse current ratio \((I_{\text{post}}/I_{\text{pre}})\) was plotted against the inactivating step duration, and the data were fitted by single exponential function to yield the time constant of \(\tau_{\text{CS}}\) and residual \(I_{\text{SS}}\). Symbols have the same meaning as in Fig. 5 \((n = 5)\). C, roscovitine significantly decreased \(\tau_{\text{CS}}\) \((R; 45 \mu M)\), compared with control \((C)\) and WO. D, \(I_{\text{SS}}\) was significantly decreased in 45 \(\mu M\) roscovitine \((R)\) compared with control \((C)\) and WO \((n = 5); p < 0.05)\).

**Physiological Impact of Roscovitine.** Based on our voltage step data, we speculated that the dominant effect of roscovitine would be inhibition of physiologically activated current. However, deactivation kinetics critically shape \(\text{Ca}^{2+}\) influx through action potential-activated \(\text{Ca}_V\) channels (Llinás et al., 1981, 1982; Buraei et al., 2005), and roscovitine significantly slows \(\text{Ca}_V,3.1\) channel deactivation, which could potentially offset inhibition. Thus, we determined the effect of 45 \(\mu M\) roscovitine on \(\text{Ca}_V,3.1\) current activated by a 2-ms AP (Fig. 8). As predicted, roscovitine-induced inhibition dominated with a 53 \(\pm\) 4% inhibition of charge influx via \(\text{Ca}_V,3.1\) channels (Fig. 8C).

Compared with the 53 \(\pm\) 6% inhibition of step current by 45 \(\mu M\) roscovitine \((\text{HP} \, -120 \text{ mV}; \text{Fig. 3D})\), it seems that slowed deactivation has little or no effect on the roscovitine-induced inhibition of AP-activated \(\text{Ca}_V,3.1\) current.

A separate issue involves the effect of roscovitine on reducing open-state inactivation. We wanted to determine whether this reduction would have an impact on AP-activated current, which was accomplished by examining the effect of 45 \(\mu M\) roscovitine on \(\text{Ca}_V,3.1\) currents generated during a 10-AP, 20-Hz train (Fig. 9). Under control conditions, \(\text{Ca}_V,3.1\) current decreased with each AP within the train as expected for accumulated inactivation (termed accommodation; Fig. 9, A, B, and D), and roscovitine significantly enhanced accommodation over this 10-AP train (Fig. 9D). As a result, roscovitine-induced inhibition was significantly increased from the first to the 10th AP within the train (Fig. 9, C and E). Thus, the roscovitine-induced reduction of open-state inactivation has little or no effect and is dominated by slowed recovery from inactivation to increase inhibition during the AP train.

**Discussion**

We have found that roscovitine blocks \(\text{Ca}_V,3.1\) channels in a dose-dependent and holding potential-dependent manner. Depolarizing the holding potential from \(-120\) to \(-70 \text{ mV}\) decreased the \(\text{EC}_{50}\) by 4-fold. Given that more than 50% of the channels are inactivated at the \(-70 \text{ mV}\) holding potential, we tested the idea that roscovitine enhanced \(\text{Ca}_V,3.1\) channel inactivation. We were surprised to find that inactivation measured from current-generating voltages was slowed by roscovitine to yield a small, but significant, decrease of inactivation. Thus, open-state inactivation was not enhanced by roscovitine. However, longer voltage steps \((1000 \text{ ms})\) increased inactivation from closed states, which was significantly enhanced by roscovitine. The development of closed-state inactivation was accelerated by roscovitine, whereas recovery from inactivation was slowed. Thus, increased occupancy of closed-state inactivation is a major mechanism by which roscovitine inhibits T-channel activity.

**Roscovitine Inhibits \(\text{Ca}_V,3.1\) Current by Preferentially Affecting Inactivated Channels.** We have previously shown that roscovitine-induced inhibition of \(\text{Ca}_V,2.2\) and \(\text{Ca}_V,1.2\) calcium channels was associated with enhanced voltage-dependent inactivation (Yarotskyy and Elmslie, 2007; Buraei and Elmslie, 2008). For \(\text{Ca}_V,2.2\) channels, the roscovitine effect resulted from enhanced closed-state inactivation (Buraei and Elmslie, 2008), whereas \(\text{Ca}_V,1.2\) channel open-state inactivation was selectively affected by roscovitine (Yarotskyy and Elmslie, 2007). Like the \(\text{Ca}_V,2.2\) channel, roscovitine-induced \(\text{Ca}_V,3.1\) channel inhibition was enhanced at depolarized holding potentials that were associated with a 10-mV left shift in the \(\text{Ca}_V,3.1\) channel closed-state inactivation versus voltage relationship. This enhancement results from roscovitine speeding the development of closed-state inactivation and slowing the recovery from inactivation. The acceleration of closed-state inactivation is in stark contrast to the significant slowing of open-state inactivation, which mediated a small, but significant, decrease of open-state inactivation. Thus, roscovitine demonstrates that open- and closed-state inactivation can be differentially modulated in \(\text{Ca}_V,3.1\) channels, which may have clinical benefits. For \(\text{Ca}_V,1.2\) channels, the preferential enhancement of closed-state inactivation by dihydropyridine antagonists (e.g., nifedipine or amlopidine) makes them potent antihypertensive drugs without negative cardiac effects (e.g., negative ionotropy or bradycardia) (Elmslie, 2004).

Our data show that the recovery from inactivation after 1-s
steps to $-20$ mV was best fit by a double exponential equation in both control and roscovitine supporting multiple components. It is tempting to relate the two recovery components to open-state (fast recovery) and closed-state (slow recovery) inactivation. However, previous work using CaV3.2 channels demonstrated that recovery was well described by a single exponential process and the time constant was similar if inactivation occurred from either the open or closed state (Serrano et al., 1999), which supports a common recovery pathway for these inactivation processes. Serrano et al. (1999) used short (60 ms) steps to inactivate CaV3.2 channels whereas our step duration was 1 s for studying recovery kinetics. Our measurement of recovery from closed-state inactivation (1 s at $-70$ mV) clearly had multiple components with the majority of current (93%) recovering by 2.2 s at $-120$ mV, but full recovery was achieved only within the 15-s interval between sweeps at $-120$ mV. Unfortunately, this slow component (7%) was too small for an

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fractional amplitude of the recovery component was 0.59, 0.72, and 0.58 for control, roscovitine, and washout, respectively. The recovery at 2200 ms was 95, 92, and 92% of the maximal value (at 15 s) for control, roscovitine, and washout, respectively. The data are presented as mean ± S.D. ($*$, $p < 0.05$).
Fig. 9. Increased action potential frequency enhances roscovitine-induced inhibition of CaV3.1 current. A, currents were generated by a 20-Hz AP train (HP = 120 mV) in Cntl (gray trace) and 45 μM Rosc (black trace). For clarity, currents generated upon washout of roscovitine are not shown (but see B). The smooth lines are single exponential fits to the data in B. B, current induced by each AP was integrated over 15 ms and plotted versus the AP number within each train. The smooth lines are single exponential fits to the data. Data are shown in control (gray trace) and 45 μM Rosc (black trace). Although our data strongly support the dominance of inhibition for CaV3.1 current activated by voltage steps, deactivation kinetics play an important role in determining Ca\(^{2+}\) influx via action potential-activated Ca\(_V\) channels (Llinás et al., 1981, 1982; Buraei et al., 2005). Because roscovitine significantly slowed CaV3.1 channel deactivation, we wanted to determine whether this had a measureable impact. However, there was no difference between the roscovitine-induced inhibition of CaV3.1 step current versus AP-activated charge influx. Thus, slowed deactivation was probably too small to significantly affect inhibition.

The differential effect of roscovitine on open-state versus closed-state inactivation could also have a physiological impact. Although the decrease of open-state inactivation is small, it is possible that this effect would be measurable under conditions where open-state inactivation dominates. One such condition could be an AP train, where open-state inactivation is expected to accumulate with each pulse. In our test, we used −120 mV as the interpulse potential to limit the potential impact of closed-state inactivation. However, roscovitine still increased inhibition during the AP train so that current during the 10th pulse was significantly smaller than that of the first pulse. This increase is probably the result of the slowed recovery from inactivation induced by roscovitine. Even though open-state inactivation is reduced, the recovery from open-state inactivation at −120 mV is slowed by roscovitine, and it is this slowed recovery that enhances inhibition during the AP train.

**Does Closed-State Inactivation Fully Explain Roscovitine-Induced Inhibition?** Many T-channel antagonists show enhanced block at depolarized holding potentials, including mibebradil (McDonough and Bean, 1998; Martin et al., 2000), octanol (Eckle and Todorovic, 2010), and T-type antagonist A2 (Uebele et al., 2009). However, significant block of CaV3 current at holding potentials that maximally recover CaV3 channel inactivation suggests that closed channel block can also occur, but with lower affinity (McDonough and Bean, 1998). This seems to be the case with roscovitine as well. We observed 50% inhibition at −120 mV induced by 45 μM roscovitine. Although this concentration left-shifted the inactivation-voltage relationship 10 mV (1000-ms steps), maximal recovery from inactivation was still achieved at −120 mV (Fig. 5A). Thus, it seems that closed CaV3.1 channels are sensitive to block by roscovitine, accurate determination of the time course. It has long been recognized that Ca\(_V\)3 channels can recover from inactivation with multiple components and the slower recovery component can be increased with longer step durations (Bosso and Feltz, 1986; Herrington and Lingle, 1992). Thus, the slow recovery component may correspond to a slow inactivation state for which occupancy requires longer and stronger depolarization. It is noteworthy that roscovitine significantly slowed recovery to contribute to the enhancement of closed-state inactivation and, thus, inhibition.
but this state has at least a 4-fold lower affinity for the drug than the closed inactivation state.

One surprise was that the enhancement of closed-state inactivation by roscovitine is common between CaV2.2 and CaV3.1 channels, but open-state inactivation of CaV1.2 channels is selectively enhanced. Sequence comparisons between the three CaV gene families shows much closer homology between the CaV1 and CaV2 families (~52%) compared with the CaV2 and CaV3 families (~25%) (Catterall et al., 2005).

Based on this, it seems more likely that CaV2.2 channels would share a common mechanism with CaV1.2 channels than with CaV3.1 channels. Roscovitine reveals the potential for similar closed-state inactivation mechanisms between the distinctly related CaV2.2 and CaV3.1 channels. However, block of closed channels distinguishes CaV3.1 channel, because no inhibition was observed at holding potentials from which CaV2.2 channels were fully recovered from closed-state inactivation (Buraei and Elmslie, 2008).

**Roscovitine Is a Promising Anticancer Drug that Exhibits Beneficial Polypharmacy.** Roscovitine is undergoing phase II clinical trials as an anticancer drug based on its CDK blocking effect (Meijer et al., 1997; Hahntow et al., 2004; Benson et al., 2007; Wiesierska-Gadek et al., 2007). Until very recently the anticancer properties were solely linked to CDK block with a therapeutic window of 10 to 50 μM (Meijer et al., 1997; Fischer and Gianella-Borradori, 2003; Hahntow et al., 2004). We showed that roscovitine's anticancer effect may also involve potassium channel blockade (Ganapathi et al., 2009). HERG potassium channels can regulate cancer cell proliferation, and HERG blockers have been shown to reduce proliferation and invasiveness (Arcangeli, 2005; Masi et al., 2005; Pardo et al., 2005; Raschi et al., 2008). Thus, HERG blockers have been proposed as an adjuvant cancer therapy (Pillozzi et al., 2007; Raschi et al., 2008).

Roscovitine blocked HERG channels with an EC50 of 27 μM, which could complement the CDK inhibition to more potently suppress the cancer cell development. Here, we reveal a third potential anticancer activity of this drug, which is to inhibit calcium entry through CaV3.1 channels with an EC50 of 10 μM at the −70-mV holding potential (at the low end of the roscovitine therapeutic window). Although polypharmacy was once thought to be an undesirable property for a drug, more recent insights have revealed the important benefits of multiple drug actions in treating disease, and polypharmacy has gained new importance in the pharmaceutical industry (Hopkins, 2008; Howitz and Sinclair, 2008; Yang et al., 2008). CaV3.1 channels seem to support abnormal calcium entry to enhance the proliferation of cancer cells, and CaV3.1 channel blockers are promising anticancer drugs (Gray and Macdonald, 2006; Lee et al., 2006; Heo et al., 2008; Lu et al., 2008; Taylor et al., 2008a,b). Roscovitine more potently blocks CaV3.1 channels in the closed inactivated state, which increases its affinity at more depolarized holding potentials. Changes in membrane potential that are controlled by potassium channel activity (e.g., HERG) seem to be critical in the control of cell proliferation by enhancing calcium entry and controlling cell volume (Pardo, 2004). Roscovitine block of potassium channel activity would depolarize the cancer cell to increase CaV3.1 channel closed-state inactivation and enhance roscovitine inhibition of CaV3.1 channel activity. Thus, the polypharmacy action of roscovitine could have synergistic benefits.

**Authorship Contributions**

Participated in research design: Yarotsky and Elmslie. Conducted experiments: Yarotsky and Elmslie. Performed data analysis: Yarotsky and Elmslie. Wrote or contributed to the writing of the manuscript: Yarotsky and Elmslie.

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