

RESEARCH PAPER

Roscovotine, a cyclin-dependent kinase inhibitor, affects several gating mechanisms to inhibit cardiac L-type ($\text{Ca}_{\text{v}}1.2$) calcium channels

V Yarotsky¹ and KS Elmslie^{1,2}¹Department of Anesthesiology, Penn State College of Medicine, Penn State University, Hershey, PA, USA and ²Department of Pharmacology, Penn State College of Medicine, Penn State University, Hershey, PA, USA

Background and purpose: L-type calcium channels ($\text{Ca}_{\text{v}}1.2$) play an important role in cardiac contraction. Roscovotine, a cyclin-dependent kinase inhibitor and promising anticancer drug, has been shown to affect $\text{Ca}_{\text{v}}1.2$ by inhibiting current amplitude and slowing activation. This research investigates the mechanism by which roscovotine inhibits $\text{Ca}_{\text{v}}1.2$ channels.

Experimental approach: $\text{Ca}_{\text{v}}1.2$ channels were transfected into HEK 293 cells, using the calcium phosphate precipitation method, and currents were measured using the whole-cell patch clamp technique.

Key results: Roscovotine slows activation at all voltages, which precludes one previously proposed mechanism. In addition, roscovotine enhances voltage-dependent, but not calcium-dependent inactivation. This enhancement resulted from both an acceleration of inactivation and a slowing of the recovery from inactivation. Internally applied roscovotine failed to affect $\text{Ca}_{\text{v}}1.2$ currents, which supports a kinase-independent mechanism and extracellular binding site. Unlike the dihydropyridines, closed state inactivation was not affected by roscovotine. Inactivation was enhanced in a dose-dependent manner with an $\text{IC}_{50} = 29.5 \pm 12 \mu\text{M}$, which is close to that for slow activation and inhibition.

Conclusions and implications: We conclude that roscovotine binds to an extracellular site on $\text{Ca}_{\text{v}}1.2$ channels to inhibit current by both slowing activation and enhancing inactivation. Purine-based drugs could become a new option for treatment of diseases that benefit from L-channel inhibition such as cardiac arrhythmias and hypertension.

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Abbreviations: BNZ, benzothiazepine; $\text{Ca}_{\text{v}}1.2$, cardiac L-type voltage-dependent calcium channel; $\text{Ca}_{\text{v}}2.2$, N-type voltage-dependent calcium channel; CDI, calcium-dependent inactivation; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethylsulphoxide; FBS, fetal bovine serum; HeBS, HEPES-buffered saline; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethane sulphonic acid; HERG, human ether-a-go-go-related gene; K_{v} , voltage-dependent potassium channel; NMG, N-methyl-D-glucamine; PAA, phenylalkylamine; VDI, voltage-dependent inactivation

Introduction

L-type calcium channels ($\text{Ca}_{\text{v}}1$) play a vital role in regulating a wide range of cellular processes such as cardiac and smooth muscle cell contraction (Bodi *et al.*, 2005), hormone secretion (Bourinet *et al.*, 2004) and gene transcription (Gomez-Ospina *et al.*, 2006). The cardiac L-channel ($\text{Ca}_{\text{v}}1.2$) generates the Ca^{2+} influx that shapes the cardiac action potential and triggers Ca^{2+} release from the sarcoplasmic reticulum (Roden *et al.*, 2002) to drive contraction.

Drugs that target $\text{Ca}_{\text{v}}1.2$ channels are important for the treatment of certain types of arrhythmias as well as hypertension (Roden *et al.*, 2002; Elmslie, 2004).

It has been recently shown that roscovotine, a membrane permanent inhibitor of cyclin-dependent kinases (Meijer *et al.*, 1997), modulates Ca_{v} channels in a kinase-independent manner. Roscovotine has been shown to increase neurotransmitter release in central and peripheral neurons (Yan *et al.*, 2002; Cho and Meriney, 2006) through slowed deactivation of $\text{Ca}_{\text{v}}2$ channels (Yan *et al.*, 2002; Buraei *et al.*, 2005, 2007), which resulted in enhanced calcium influx into nerve terminals. Therefore, roscovotine can be considered an agonist drug for $\text{Ca}_{\text{v}}2$ channels (Buraei *et al.*, 2007). It has also been demonstrated that roscovotine inhibited

Correspondence: Dr KS Elmslie, Department of Anesthesiology H187, Penn State College of Medicine, 500 University Drive, Hershey, PA 17033, USA.
E-mail: kse10@psu.edu

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voltage-dependent potassium (K_v) channels by blocking the open channels, and inhibited Ca_v1.2 channels while slowing activation (Buraei *et al.*, 2007).

Roscovitine is currently undergoing phase II clinical trials as an anticancer treatment (Fischer and Gianella-Borradori, 2003). Thus, it is important to explore the inhibitory effects of roscovitine on Ca_v1.2 channels so that we can predict the potential impact of this drug on cardiovascular function. We show that therapeutic concentrations of roscovitine inhibit these channels by slowing their activation and enhancing their inactivation. Further, roscovitine slows down the recovery from inactivation of the Ca_v1.2 current. We conclude that the combination of slowed activation and enhanced inactivation is responsible for the inhibition of Ca_v1.2 channel activity.

Materials and methods

HEK293 cell transfection

We utilized the calcium phosphate precipitation method to transfect HEK293 cells with cardiac Ca_v1.2 channels, which provided highly reproducible expression over 24–72 h after transfection. HEK293 cells were maintained in standard Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS) and 1% antibiotic–antimycotic solution at 37°C in 5% CO₂ incubator. For transfection, the medium was changed to DMEM/F-12 containing 10% FBS. HEK293 cells were transfected by adding 1 ml of precipitated transfecting solution containing 4-(2-hydroxyethyl)-1-piperazine ethane sulphonic acid (HEPES)-buffered saline (HeBS), 50 mM CaCl₂ and cDNA plasmids pcDNA3 (Invitrogen, Carlsbad, CA, USA) as follow: 11 μg α_{1C} (rabbit heart, GenBank accession no. X15539), 8.5 μg α_{2δ}, 5.5 μg β_{1b}, 2.15 μg TAG (to increase expression efficiency) and 1 μg green fluorescent protein (to visualize transfected cells) (provided by Dr Blaise Peterson, Penn State College of Medicine, Hershey, PA, USA). Cells were incubated for 8 h after which the medium was replaced by the standard DMEM. The 35-mm dishes served as the recording chamber.

Measurement of ionic currents

Cells were voltage-clamped using the whole-cell configuration of the patch clamp technique. Pipettes were pulled from Schott 8250 glass (Garner Glass, Claremont, CA, USA) on a Sutter P-97 puller (Sutter Instruments Co., Novato, CA, USA). Series resistance ranged from 2 to 15 MΩ, and was compensated at 80% to yield a mean voltage error of 1.2 ± 0.8 mV (± s.d., *n* = 40). Currents were recorded using an Axopatch 200A amplifier (Molecular Devices, Sunnyvale, CA, USA) and digitized with ITC-18 data acquisition interface (InstruTECH Corporation, Port Washington, NY, USA). Experiments were controlled by a Power Macintosh G3 computer (Apple Computer, Cupertino, CA, USA) running S5 data acquisition software written by Dr Stephen Ikeda (NIH, NIAAA, Bethesda, MD, USA). Leak current was subtracted online using a P/4 protocol. All recordings were carried out at room temperature and unless otherwise noted the holding potential was –120 mV. Whole-cell currents were digitized depending on

step duration at 50 (25 ms), 10 (200 ms) and 4 kHz (300 and 1000 ms) after analog filtering at 1–10 kHz.

Electrophysiological data analysis

These data were analyzed using IgorPro (WaveMetrics, Lake Oswego, OR, USA) running on a Macintosh computer. Step currents were measured as the average of 1 ms of current at the end of the voltage step. Tail currents were measured as the average of 0.3 ms of current beginning 0.3 ms following hyperpolarization to –60 mV. Activation τ (τ_{Act}) was determined by fitting a single exponential function to the step current after a 0.3 ms delay (Buraei *et al.*, 2007). Inactivation τ (τ_{Inact}) was determined by fitting a single exponential function from peak step current to the end of the step.

Solutions

For the transfection, HeBS contained (in mM) 140 NaCl, 25 HEPES, 1.4 Na₂HPO₄, with pH 7.10 adjusted using 5 N NaOH. The internal pipette solution contained (in mM) 104 N-methyl-D-glucamine (NMG)-Cl, 14 creatine-PO₄, 6 MgCl₂, 10 NMG-HEPES, 5 Tris-ATP, 0.3 Tris-GTP and 10 NMG-glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid with osmolarity of 280 mosmol and pH 7.3. The external recording solution contained (in mM) 100 NMG-Cl, 10 NMG-HEPES and 30 BaCl₂. For some experiments, 30 mM BaCl₂ was replaced with 1 MgCl₂ and either 10 BaCl₂ or 10 CaCl₂. The NMG-Cl concentration was adjusted to maintain osmolarity of 300 mosmol and pH was set to 7.3 using NMG base. Roscovitine was prepared as a 50 mM stock solution in DMSO and stored at –30°C. All external solutions contained the same DMSO concentration so that the roscovitine concentration was the sole variable when changing solutions. Test solutions were applied from a gravity-fed perfusion system with an exchange time of 1–2 s.

Statistical analysis

Group data were calculated and are presented as mean ± s.d. throughout the paper. The paired *t*-test was used for within-cell comparisons.

Chemicals

All experiments utilized R-roscovitine (referred to as roscovitine) from LC Labs (Woburn, MA, USA). DMEM/F12, DMEM, FBS, 100 × antibiotic–antimycotic were from Invitrogen. Other chemicals were obtained from Sigma (St Louis, MO, USA).

Results

Effects of roscovitine on activation of Ca_v1.2 channels

It was shown previously that roscovitine inhibited step current and slowed activation of Ca_v1.2 channels (Buraei *et al.*, 2007). We have extended this observation to show the effect of 100 μM roscovitine over a range of voltages (Figures 1a and b). The inhibition was similar across the

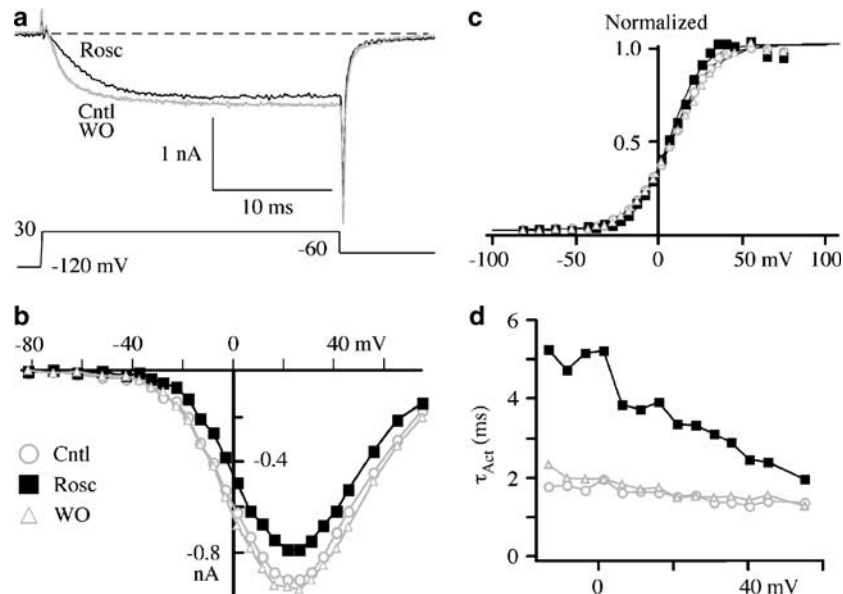


Figure 1 Roscovitine slowed activation of $Ca_{(V)}1.2$ channels. (a) $Ca_{(V)}1.2$ currents were activated during 25-ms voltage steps to 30 mV. Roscovitine (Rosc; 100 μ M) induced a slowing of activation and inhibition compared to currents before (Cntl) and upon recovery from (WO) roscovitine application. (b) The current–voltage (I – V) relationship shows roscovitine-induced inhibition across all current-generating voltages compared to control and washout. (c) The activation–voltage relationship was measured from tail currents and is shown normalized to maximum current to highlight the small changes induced by roscovitine (symbols are same as in b). The smooth lines are fits using a single Boltzmann function with $V_{1/2}$ = 7.9, 6.6 and 9.1 mV, and slope (k) = 12.1, 9.9 and 12.9 for control, roscovitine and washout, respectively. (d) Activation τ (τ_{Act}) was generated from single exponential fits to current activation (after a 0.3-ms delay) in control, 100 μ M roscovitine and washout (symbols are same as in b).

current–voltage relationship (I – V ; Figure 1b), so that there was no obvious voltage dependence to the roscovitine-induced inhibition. On average, peak current (+30 mV) was inhibited $26.3 \pm 8.2\%$, while current at +60 mV was inhibited $33.1 \pm 14.3\%$ ($n = 6$) by 100 μ M roscovitine. The activation–voltage relationship was measured from tail currents (–60 mV) and was well fitted by a single Boltzmann equation (Figure 1c). Roscovitine slightly, but significantly, left-shifted $V_{1/2}$, which was 12.0 ± 0.5 , 9.0 ± 0.5 and 13.6 ± 0.8 mV ($P < 0.01$, $n = 4$) for control, 100 μ M roscovitine and recovery, respectively. The voltage dependence of activation became slightly more steep in roscovitine with activation changing e-fold for 17.1 ± 0.4 , 15.1 ± 0.4 and 18.2 ± 0.5 mV ($P < 0.01$, $n = 4$) for control, 100 μ M roscovitine and recovery, respectively. The inhibitory effect of roscovitine was accompanied by slowed activation at all voltages generating measurable current (Figure 1d). The activation τ (τ_{Act}) was more than doubled at hyperpolarized voltages by 100 μ M roscovitine, but this increase declined with depolarization (Figure 1d). However, τ_{Act} was significantly higher even at the most depolarizing voltage examined (+50 mV, $P < 0.001$, $n = 9$).

We speculated previously that the roscovitine-induced slowed activation could result from voltage-dependent dissociation of roscovitine from $Ca_{(V)}1.2$ channel (Buraei *et al.*, 2007), which is analogous to G-protein-mediated inhibition of $Ca_{(V)}2.2$ channels (Elmslie *et al.*, 1990). The absence of obvious voltage dependence to inhibition measured from the I – V relationship failed to support this idea (Figure 1b). However, a more direct test comes from examining the effect of a strong depolarizing conditioning pulse on inhibition (Elmslie *et al.*, 1990). If roscovitine

binding is voltage-dependent, the conditioning pulse will induce dissociation so that current activates normally during a postpulse delivered 10 ms later. On the contrary, as shown in Figure 2, activation was similarly affected by 100 μ M roscovitine before (prepulse) and after (postpulse) a depolarizing pulse to 0 mV. Prepulse activation was slowed from 3.0 ± 0.6 to 5.6 ± 0.4 ms ($P < 0.001$, $n = 5$) and postpulse activation was slowed from 2.6 ± 0.5 to 4.5 ± 0.5 ms ($P < 0.01$, $n = 5$). The percent change of τ_{Act} was slightly, but significantly, reduced for the postpulse relative to the prepulse (Figure 2c), but the persistence of significant slowing of postpulse activation suggested that roscovitine did not dissociate from $Ca_{(V)}1.2$ channels in a voltage-dependent manner.

Roscovitine enhances inactivation of $Ca_{(V)}1.2$

Close inspection of the roscovitine effect on 25 ms voltage steps revealed that inhibition was largest at both the beginning and end of the step (Figure 1a). The late increase of inhibition could be explained by roscovitine-induced enhancement of voltage-dependent inactivation (VDI), since these currents were recorded in solutions containing Ba^{2+} to eliminate Ca^{2+} -dependent inactivation (CDI). Currents induced by 1-s voltage steps showed both increased magnitude and speed of apparent inactivation in the presence of 100 μ M roscovitine (Figure 3). The effect was quantified by calculating the I_{End}/I_{Peak} ratio, where I_{End} was measured at the end of the 1-s step and I_{Peak} was measured at peak step current. This ratio, which indicates the fraction of current remaining at the end of the step, was reduced from

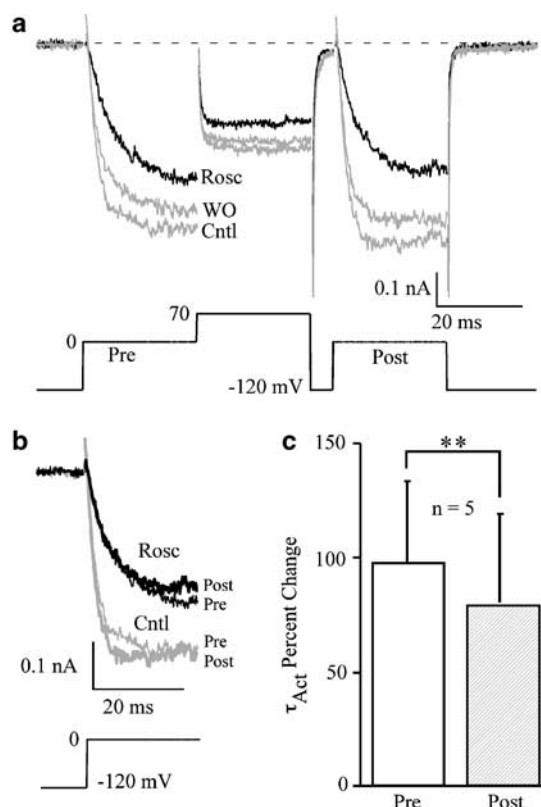


Figure 2 Roscovitine did not dissociate from $Ca_{(V)}1.2$ channels upon channel opening. (a) $Ca_{(V)}1.2$ currents were evoked by two 25-ms steps to 0 mV (prepulse (Pre) and postpulse (Post)) bracketing a +70-mV conditioning step in control, 100 μM roscovitine and washout. The interval between the conditioning step and postpulse was 10 ms. (b) Superimposed prepulse- and postpulse-evoked currents show slowed activation in the presence of 100 μM roscovitine compared to control. (c) Roscovitine (100 μM) increased activation τ (τ_{Act}) for both prepulse and postpulse stimulation, although these results were significantly different (mean \pm s.d. $**P < 0.01$, $n = 5$).

0.53 ± 0.20 to 0.16 ± 0.09 in control vs roscovitine, respectively ($n = 7$, $P < 0.001$). Single exponential fitting of inactivation showed that the inactivation τ (τ_{Inact}) was decreased by 100 μM roscovitine (Figure 3b). The roscovitine-induced enhancement of apparent inactivation was fully developed within 10 s of application and complete recovery was observed in ≤ 30 s of washout (data not shown). The speed of this effect is similar to that observed for slowed activation and inhibition (Buraei *et al.*, 2007), and suggests the possibility that roscovitine interacts with an extracellular site to enhance $Ca_{(V)}1.2$ channel inactivation.

Roscovitine blocks potassium channels by binding to open channels, which gives the appearance of enhanced inactivation (Buraei *et al.*, 2007). Thus, it is possible that enhanced inactivation of $Ca_{(V)}1.2$ channels resulted from open-state block. This was tested by measuring the effect of 10–300 μM roscovitine on $Ca_{(V)}1.2$ channel inactivation (Figure 4). The speed of an open-channel effect is directly related to the concentration by the relationship $\tau^{-1} = k_{Off} + k_{On}[Rosc]$, where k_{Off} and k_{On} are rate constants, and [Rosc] is roscovitine concentration (Buraei *et al.*, 2005). Thus, both the magnitude and speed of apparent inactivation should

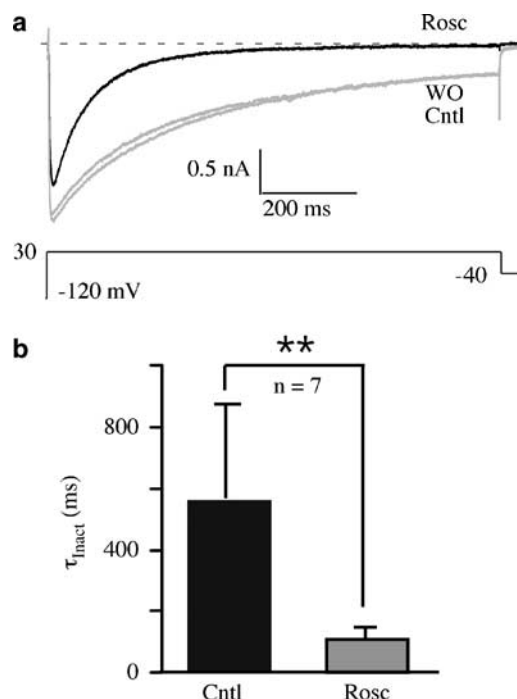


Figure 3 Roscovitine-enhanced inactivation of $Ca_{(V)}1.2$ channels. (a) $Ca_{(V)}1.2$ currents evoked by 1-s steps to 30 mV show the enhancement of inactivation by 100 μM roscovitine relative to control and washout. (b) Mean and s.d. of inactivation τ (τ_{Inact}) obtained from fitting a single exponential function to inactivating currents in control and 100 μM roscovitine. Data are significantly different ($**P < 0.01$, $n = 7$).

increase with concentration if roscovitine blocks open $Ca_{(V)}1.2$ channels. On the other hand, roscovitine could be binding to a site on $Ca_{(V)}1.2$ channels to increase VDI, which predicts saturation of the effect at high roscovitine concentrations. Inactivation, measured as the I_{End}/I_{Peak} ratio, increased with roscovitine concentration, but saturated at concentration ≥ 100 μM. I_{End}/I_{Peak} ratio was well fitted by the Hill equation yielding an $EC_{50} = 29.5 \pm 12.0$ μM and a Hill coefficient = 2.3 ± 2.8 (Figure 4b; $n = 6$), which supports a roscovitine enhancement of VDI. For comparison, we also measured the dose–response relationship of the other roscovitine-induced effects and found for slow activation (+10 mV), an EC_{50} of 20.9 ± 1.2 μM and Hill coefficient of 1.2 ± 0.1 . For inhibition (+30 mV) measured at 25 ms, the IC_{50} was 23.4 ± 4.8 μM, with Hill coefficient of 1.6 ± 0.5 . Thus, it is possible that all three effects result from roscovitine binding to a single site with an apparent affinity of 20–30 μM. The differences in Hill coefficient (1.2–2.3) could result from separate binding sites, but statistical analysis showed no significant differences between these values (analysis of variance with Tukey HSD).

Roscovitine enhances inactivation of $Ca_{(V)}1.2$ channels by interacting with externally located binding site(s)

Roscovitine is known to inhibit intracellularly located cyclin-dependent kinases (Meijer *et al.*, 1997), and altered calcium channel phosphorylation can affect inactivation (Werz *et al.*, 1993). However, the speed of the roscovitine

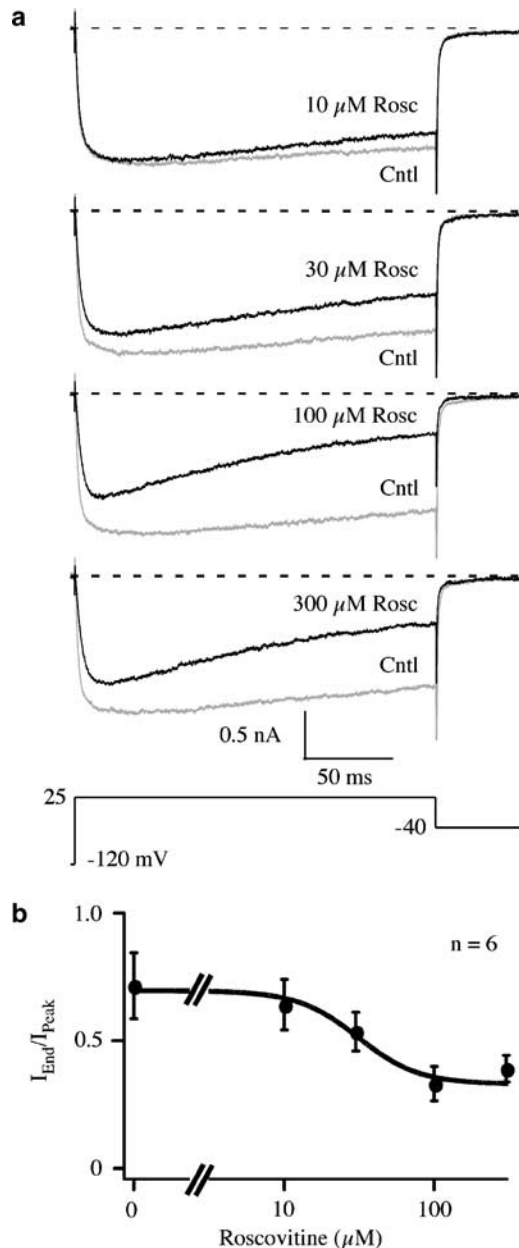


Figure 4 The roscovitine-induced enhancement of inactivation saturated at high drug concentrations. (a) Representative traces from a single cell show the enhancement of inactivation induced by application of 10, 30, 100 and 300 μM roscovitine compared to control. Currents were evoked by 200-ms steps to 25 mV. (b) Inactivation was quantified as the $I_{\text{End}}/I_{\text{Peak}}$ ratio, where I_{End} was measured at the end of the 200-ms step and I_{Peak} was measured at the peak current during the step. The smooth line is a fit using the Hill equation with an $\text{EC}_{50} = 29.5 \mu\text{M}$ and a Hill coefficient of 2.3. Data are presented as mean \pm s.d. of six cells.

effect is consistent with an externally facing binding site (Buraei *et al.*, 2005). To test this hypothesis further, 300 μM roscovitine was added to the internal solution and currents were elicited by 200 ms voltage steps (+10 mV) to examine both activation and inactivation. Control cells were dialyzed with internal solutions containing 0.6% DMSO to control for vehicle. We found no significant difference in the $I_{\text{End}}/I_{\text{Peak}}$ ratio between control (0.75 ± 0.14 , $n = 8$) and internally

applied roscovitine (0.65 ± 0.11 , $n = 8$, not significant). In addition, internal roscovitine failed to alter the effect of externally applied roscovitine (100 μM). The fraction of current remaining in external roscovitine was 0.40 ± 0.10 for internal roscovitine vs 0.36 ± 0.07 for DMSO control ($n = 8$; not significant). Activation was also not altered by internally applied roscovitine ($\tau_{\text{Act}} = 2.42 \pm 0.69$ vs control $\tau_{\text{Act}} = 3.0 \pm 1.1$ ms; $n = 8$, not significant) and external roscovitine significantly slowed activation with either internal roscovitine ($\tau_{\text{Act}} = 4.6 \pm 0.9$ ms) or DMSO ($\tau_{\text{Act}} = 4.2 \pm 0.9$ ms; $n = 8$). This evidence supports an extracellular binding site for the roscovitine-induced effects on $\text{Ca}_{\text{v}}1.2$ channels.

Roscovitine slows recovery from inactivation

We observed that roscovitine speeds inactivation, but recovery from inactivation could also be affected. Recovery from inactivation was examined using a three-pulse protocol, where the first (pre) and third (post) pulses were set to +30 mV (peak current) and the second (inactivating) pulse was +50 mV (duration = 300 ms). The interval between the inactivating pulse and postpulse was varied and the $I_{\text{Post}}/I_{\text{Pre}}$ ratio was monitored to follow the recovery from inactivation, which was composed of fast and slow components (Figure 5). The fast component ranged from 80 to 100% of total recovery time course with mean relative amplitude = $93 \pm 7\%$ in control and $87 \pm 2\%$ in roscovitine ($n = 4$, not significant). Roscovitine significantly slowed the fast component of recovery, but had little or no effect on the slow component. The fast recovery τ (τ_{Recov}) was increased to more than double the control value in 100 μM roscovitine (Figure 5c). The roscovitine-induced increase of inactivation thus appears to result from both accelerated inactivation and slowed recovery from inactivation.

The slowed recovery from inactivation suggests that roscovitine-induced inhibition could be frequency dependent. However, increasing stimulation frequency from 0.1 to 2 Hz (25 ms steps) did not alter the percent inhibition ($\sim 22\%$ for each condition) (see Supplementary Figure 1). This was expected since the slowed recovery from inactivation ($\tau_{\text{Recov}} \sim 72$ ms) would not impact inhibition until the interval between stimuli was ≤ 100 ms. Thus, use-dependent inhibition is not observed over the frequency range used to observe use-dependent block of $\text{Ca}_{\text{v}}1.2$ current by phenylalkylamines and benzothiazepines (Hering *et al.*, 1996; Johnson *et al.*, 1996; Motoike *et al.*, 1999; Bodi *et al.*, 2002).

Roscovitine does not affect calcium-dependent inactivation

Our previous results used Ba^{2+} as the charge carrier to isolate VDI. To determine if CDI was also affected (Peterson *et al.*, 1999, 2000), we compared the effect of 100 μM roscovitine on inactivation in either 10 mM Ca^{2+} or Ba^{2+} . A three-pulse protocol, similar to that described above, was used to examine the voltage dependence of inactivation. The 200 ms inactivating pulse was varied from -120 to +80 mV and inactivation was measured from the $I_{\text{Post}}/I_{\text{Pre}}$ ratio. In control, inactivation in Ca^{2+} was minimal at hyperpolarized voltages, peaked at +20 mV and declined with further depolarization (Figure 6a), which mirrored

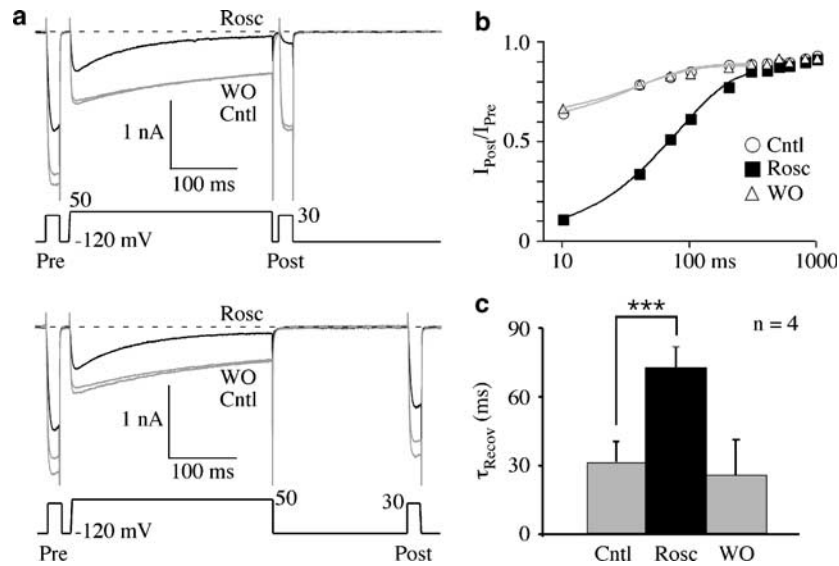


Figure 5 Roscovitine slowed recovery from inactivation. (a) This set of $\text{Ca}_v1.2$ currents shows the recovery from inactivation for 10-ms (top) and 200-ms (bottom) intervals between the inactivating pulse and postpulse. Roscovitine ($100 \mu\text{M}$) increased inactivation during the 300-ms step to 50 mV and slowed recovery from inactivation at -120 mV relative to control and washout. Pre = prepulse; Post = postpulse. (b) The $I_{\text{Post}}/I_{\text{Pre}}$ ratio is plotted vs recovery time for data from the same cell as in (a). Roscovitine ($100 \mu\text{M}$) significantly slowed the fast recovery component relative to control and washout. The smooth curves are fits using a single exponential function. (c) The mean and s.d. of recovery τ (τ_{Recov}) are shown for control, $100 \mu\text{M}$ roscovitine and washout. Data are significantly different (***) $P < 0.001$, $n = 4$.

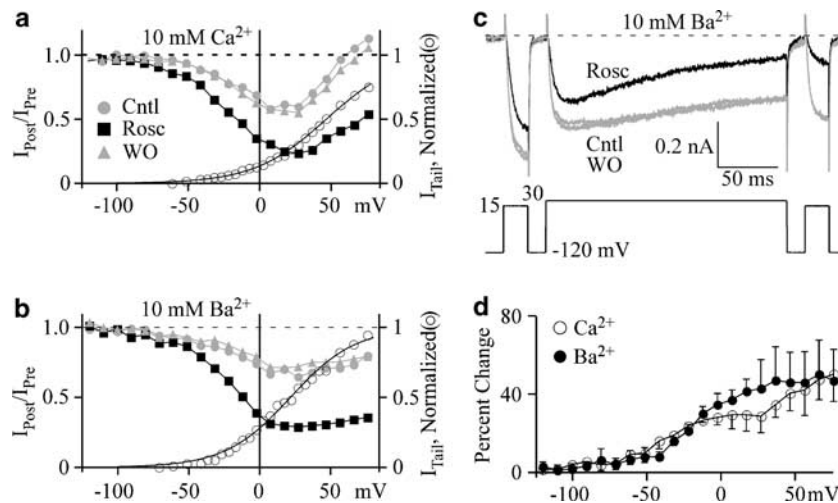


Figure 6 Roscovitine enhanced voltage-dependent (VDI) but not calcium-dependent inactivation (CDI). (a) The $I_{\text{Post}}/I_{\text{Pre}}$ ratio (left axis) was measured as in Figure 5 and is plotted vs inactivation voltage to show inactivation in 10 mM Ca^{2+} . Data are shown for control, $100 \mu\text{M}$ roscovitine and washout. The activation–voltage relationship in control (right axis, open circle) was measured as in Figure 1 and is superimposed here for comparison with the voltage dependence of inactivation. Data were collected in the presence of 10 mM Ca solution. (b) The voltage dependence of inactivation in 10 mM Ba^{2+} was measured as in (a). The same cell was first recorded in 10 mM Ca^{2+} (a), which was then replaced with 10 mM Ba^{2+} external solution. (c) $\text{Ca}_v1.2$ currents evoked by the triple-pulse inactivation protocol used to generate the data of (a) and (b). The 200-ms inactivation pulse to $+30$ mV is flanked by two 25-ms steps to 15 mV (prepulse and postpulse). Currents were recorded in 10 mM Ba^{2+} external solution in control, $100 \mu\text{M}$ roscovitine and washout. (d) $100 \mu\text{M}$ roscovitine induced a monotonic increase of inactivation with voltage in both 10 mM Ca^{2+} ($n = 7$) and Ba^{2+} ($n = 5$). The roscovitine-induced percent change in the $I_{\text{Post}}/I_{\text{Pre}}$ ratio was calculated by averaging control and washout values. There was no significant difference in the roscovitine-induced percent change of inactivation between Ca^{2+} and Ba^{2+} at any voltage.

Ca^{2+} influx as expected for CDI. Inactivation in Ba^{2+} increased monotonically with voltage as expected for an open-state inactivation mechanism typical for VDI (Figures 6b and c). Thus $100 \mu\text{M}$ roscovitine enhanced inactivation of $\text{Ca}_v1.2$ channels in the presence of both external Ca^{2+} and Ba^{2+} , but this could be explained by enhanced VDI that

functions in Ca^{2+} as well as Ba^{2+} (Giannattasio *et al.*, 1991). To determine if CDI was affected, we measured the percent effect of roscovitine with voltage (Figure 6d). If CDI was affected, we would expect to observe a peak in this relationship corresponding to peak CDI ($+20$ mV) in Ca^{2+} , but not Ba^{2+} . Contrary to this prediction, the percent

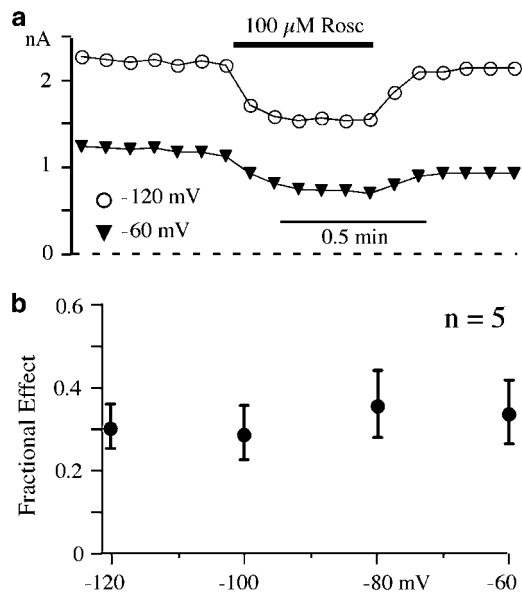


Figure 7 Closed-state inactivation was not affected by roscovitine. (a) Time course of inhibition of currents evoked by 25-ms steps to 30 mV from holding potential of -120 vs -60 mV. (b) Average fractional of inhibition by $100 \mu\text{M}$ roscovitine of currents generated by steps to 30 mV from holding potentials ranging from -120 to -60 mV (20 mV increments). Data are presented as mean \pm s.d. of five cells.

enhancement of inactivation was not significantly different between Ca^{2+} and Ba^{2+} at any voltage, which demonstrates that roscovitine does not affect CDI. While VDI was enhanced, roscovitine did not alter voltage dependence as quantified by a single Boltzmann equation fitted to the data from -120 to $+30$ mV ($30 \text{ mM } Ba^{2+}$ external solution), which yielded $V_{1/2} = 16.0 \pm 5.1$ and 16.0 ± 5.2 mV and slope $= -14.9 \pm 2.8$ and -17.1 ± 3.0 ($n = 6$, not significant) for control and $100 \mu\text{M}$ roscovitine, respectively.

Roscovitine does not affect closed state inactivation

The correlation between the voltage dependence of activation and inactivation (Figure 6b) supports a roscovitine-induced enhancement of open-state inactivation (VDI). We also investigated the effect of roscovitine on closed-state inactivation, which is involved in dihydropyridine-induced inhibition (Bean, 1984; Sanguinetti and Kass, 1984). However, $Ca_{(V)}1.2$ current inhibition induced by $100 \mu\text{M}$ roscovitine was not affected by altering the holding potential from -120 to -60 mV (Figures 7a and b). Holding potential was maintained at least 1 min before applying $100 \mu\text{M}$ roscovitine. The percent inhibition was $30.7 \pm 5.4\%$ at -120 mV and $34.2 \pm 7.8\%$ at holding potentials of -60 mV ($n = 5$, not significant), despite a 50% reduction in available channels at holding potential of -60 vs -120 mV.

Roscovitine inhibited physiologically activated $Ca_{(V)}1.2$ current

The slowing of activation and speeding of inactivation ($\tau = 100$ ms) suggest that roscovitine will inhibit $Ca_{(V)}1.2$ current activated by the cardiac action potential. A cardiac

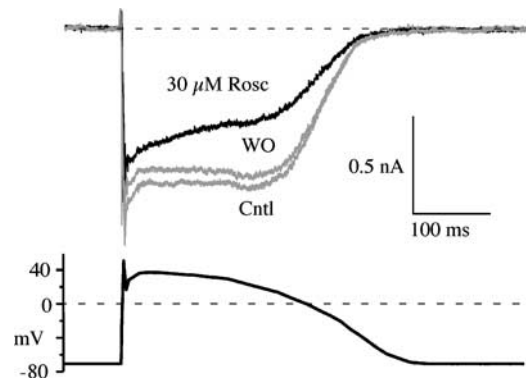


Figure 8 Roscovitine inhibited $Ca_{(V)}1.2$ current activated by a cardiac action potential waveform. Currents are shown for control, washout and in $30 \mu\text{M}$ roscovitine.

action potential waveform was generated to match that measured from human ventricular myocytes (Li *et al.*, 1999). Roscovitine inhibited current activated by the cardiac action potential in a time-dependent manner with inhibition increasing with duration as expected from enhanced inactivation (Figure 8). The charge influx carried by Ba^{2+} , calculated by integrating inward current during the cardiac action potential, was inhibited by roscovitine in a concentration-dependent manner with inhibitions of 16.1 ± 4.5 , 30.0 ± 6.8 , 53.2 ± 8.7 and $49.3 \pm 8.9\%$ for 10, 30, 100 and $300 \mu\text{M}$ roscovitine, respectively. Hence, clinically relevant doses of roscovitine (10 – $50 \mu\text{M}$; McClue *et al.*, 2002; Hahntow *et al.*, 2004; Raynaud *et al.*, 2005) will significantly inhibit $Ca_{(V)}1.2$ current.

Discussion

Roscovitine is a purine-based compound with a broad spectrum of effects. Initially used as a selective inhibitor of cyclin-dependent kinases (Meijer and Raymond, 2003), roscovitine has developed into a promising anticancer therapy (Hahntow *et al.*, 2004; Raynaud *et al.*, 2005; Benson *et al.*, 2007). However, roscovitine has more recently been recognized as a drug that can affect voltage-dependent ion channels (Yan *et al.*, 2002; Buraei *et al.*, 2005, 2007; Cho and Meriney, 2006). It has been demonstrated previously that roscovitine inhibits and slows activation of $Ca_{(V)}1.2$ current. We can now add the enhancement of VDI to roscovitine's effect on $Ca_{(V)}1.2$ channels. This enhancement resulted from both speeding of inactivation and slowing of recovery from inactivation. Internally applied roscovitine failed to reproduce enhanced inactivation or slowed activation, which suggests an extracellularly exposed binding site. In addition, the similar EC_{50} (20 – $30 \mu\text{M}$) for the roscovitine-induced effects was consistent with a single binding site mediating these effects, but verification awaits further investigation.

Roscovitine-induced slow activation of $Ca_{(V)}1.2$ channels

Our results support and extend findings that roscovitine slows activation by binding to the closed state (Buraei *et al.*,

2007). We had speculated previously that slowed activation could result from the voltage-dependent dissociation of roscovitine from Ca_v1.2 channels upon channel opening (Buraei *et al.*, 2007), which is analogous to G-protein-mediated inhibition of N-type (Ca_v2.2) calcium channels (Elmslie *et al.*, 1990). Our results demonstrate that this possibility is unlikely because activation was slowed at all voltages and current was similarly inhibited across voltages (Figure 1). In addition, activation was slowed in currents following a strong depolarizing pulse designed to activate fully Ca_v1.2 channels. If roscovitine binding was voltage-dependent, we would expect the strong depolarizing pulse to induce complete dissociation, which should have resulted in normal activation of postpulse-evoked current (Elmslie *et al.*, 1990). The absence of such an effect further supports the notion that roscovitine binding is voltage-independent. Thus, slowed activation is an effect induced by roscovitine occupying a site on Ca_v1.2 channels.

The change in τ_{Act} with voltage was dramatically increased by roscovitine. The τ_{Act} was weakly voltage-dependent in control conditions (e-fold change for 125 ± 11 mV, $n = 9$) as expected if the voltage-independent closed–open transition was rate limiting for channel activation (Marks and Jones, 1992). In $100 \mu\text{M}$ roscovitine, τ_{Act} changed e-fold for 54 ± 2 mV ($n = 9$). The increased voltage dependence is likely to result from roscovitine-induced slowing of voltage-dependent close–close transitions, which become rate-limiting to channel opening. It is unlikely that open–close transitions were greatly affected since we found no effect of roscovitine on deactivation of Ca_v1.2 channels, which suggests that mean open time was not affected. Slowed activation is responsible for the inhibition of Ca_v1.2 channels at the beginning of the voltage step, but did not appear to have a large impact at early times during the cardiac action potential. The minimal early inhibition of the cardiac action potential-induced Ca_v1.2 currents likely results from the very depolarized phase 0 and phase 1 portions of the cardiac action potential, which reaches voltages where τ_{Act} was less affected by roscovitine.

Roscovitine-enhanced inactivation

We investigated two possible mechanisms for the roscovitine-induced increase of apparent inactivation, open-channel block and enhanced VDI. K_v channels show open-channel block by roscovitine, which appears as enhanced inactivation. Assuming a first-order reaction process, the speed of block should increase with concentration without saturation (Buraei *et al.*, 2005). Contrary to this prediction, τ_{Inact} saturates at roscovitine concentrations $\geq 100 \mu\text{M}$, which suggests that roscovitine binds to Ca_v1.2 channels to enhance inactivation.

The majority of our studies on inactivation used Ba²⁺ to isolate VDI (Shi and Soldatov, 2002; Dafi *et al.*, 2004). The magnitude and speed of VDI measured in our study corresponded well with VDI measured from cardiac L-channels (Ferreira *et al.*, 2003). The correlation of roscovitine-enhanced inactivation with the voltage-dependent activation combined with the absence of an effect on holding potential-induced inactivation suggests that ros-

covitine enhances open-state inactivation (Faber *et al.*, 2007). This enhancement appears to result from an increase in the open to inactivated rate constant and from a decrease in the effective recovery from inactivation rate constant, which combine to increase the number of inactivated channels at steady state. On the other hand, CDI was not affected since the voltage dependence of enhanced inactivation did not differ between Ca²⁺ and Ba²⁺ (Figure 6d).

Comparison with other Ca_v1.2 antagonists

Phenylalkylamines (PAAs), such as gallopamil and verapamil, and benzothiazepines (BNZs), such as diltiazem, enhance VDI (Motoike *et al.*, 1999; Sokolov *et al.*, 2001), but the mechanism of roscovitine's action appears to be unique. For example, the block by PAA and BNZ is use-dependent as it builds with each pulse, but roscovitine block is rapid and nearly complete in the 10-s interval between sweeps. Use dependence results from these blockers preferentially binding to the inactivated and open states combined with slow dissociation so that the block develops with each depolarization that opens Ca_v1.2 channels (Hockerman *et al.*, 1997). However, roscovitine appears to bind to closed Ca_v1.2 channels since activation is slowed.

PAAs are thought to bind within the inner vestibule of the Ca_v1.2 channels, which helps explain use-dependent block and slow dissociation of these drugs from the channel (Hockerman *et al.*, 1997). In contrast, BNZs are thought to bind to an extracellular site on Ca_v1.2 channels (Hering *et al.*, 1993; Seydl *et al.*, 1993). In spite of this, the binding sites for PAA and BNZ appear to be either partially overlapping or allosterically linked, since mutation studies have identified amino acids that affect both (Hockerman *et al.*, 1997; Striessnig *et al.*, 1998). The absence of an effect of intracellularly applied roscovitine shows that the PAA site is not involved, while the apparent binding of roscovitine to closed channels (slowed activation) suggests that neither the PAA or BNZ sites are involved. On the basis of the similar EC₅₀ for roscovitine-induced slow activation and enhanced inactivation, we tentatively conclude that roscovitine binds to a unique externally facing site to affect Ca_v1.2 channel gating.

Potential cardiac effects of roscovitine

PAA and BNZ have proven useful as treatments for certain arrhythmias that result from Ca_v1.2 channel-induced early afterdepolarization (Marban *et al.*, 1986; Wu *et al.*, 1999; Roden *et al.*, 2002). However, the PAA verapamil has been shown to block K_v channels (Zhang *et al.*, 1999), which will increase cardiac action potential duration and can exacerbate, instead of ameliorate, the arrhythmias. The current thinking is that inhibition of Ca_v1.2 channels effectively counteracts the pro-arrhythmic effect of K_v channel block (Bril *et al.*, 1996; Sanguinetti and Tristani-Firouzi, 2006). Roscovitine has also been found to block K_v channels (Buraei *et al.*, 2007), including the human ether-a-go-go-related gene (HERG) potassium channel that is crucial for cardiac action potential repolarization (SB Ganapathi and KS Elmslie, submitted). However, cardiac arrhythmias have not

been reported as a side effect from either phase I or phase II drug trials (Fischer and Gianella-Borradori, 2003; Benson *et al.*, 2007), even though HERG channels were blocked by therapeutic roscovitine concentrations (10–50 μM) (McClue *et al.*, 2002; Hahntow *et al.*, 2004; Raynaud *et al.*, 2005). Like verapamil, it seems likely that roscovitine-induced inhibition of Ca_v1.2 prevents the negative cardiovascular effects of K_v channels block. In addition, the incomplete inhibition of Ca_v1.2 currents by roscovitine could provide a 'safety factor', as even saturating concentrations would allow considerable residual Ca_v1.2 channel activity.

In spite of its promiscuity, roscovitine appears to interact with previously uncharacterized sites to uniquely alter the gating of each of class of affected ion channel. Particularly notable is the agonist effect of roscovitine on Ca_v2 (Buraei *et al.*, 2005, 2007) and the slowed activation and faster inactivation Ca_v1.2 channels (detailed here). Further investigation of these sites is likely to define unique characteristics that can be exploited for the development of drugs specific for each site. As the intensive study of the ATP-binding site on protein kinases has led to the development of specific inhibitors, the study of roscovitine and its analogues could lead to isolation of a novel class of purine-based drugs that is specific for each class of affected ion channel.

Conflict of interest

The authors state no conflict of interest.

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