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(R)-ROSCOVITINE PROLONGS THE MEAN OPEN TIME OF UNITARY N-TYPE CALCIUM CHANNEL CURRENTS

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Abstract—(R)-roscovitine (Ros) is a cyclin-dependent kinase inhibitor that also has been shown to have direct agonist and antagonist actions on $Ca_v2.1$ (P/Q-type) and Ca_v 2.2 (N-type) families of voltage-gated calcium channels. These kinase-independent effects represent a novel opportunity to advance our understanding of calcium channel function and calcium-triggered neurotransmitter release. Furthermore, such actions on calcium channels may direct the development of Ros derivatives as new therapeutic agents. We used patch clamp recordings to characterize mechanisms that underlie the agonist effects of Ros on unitary N-type calcium channel gating. We found that N-type channels normally gate with either a short or long mean open time, that Ros significantly prolonged the mean open time of the long gating component and increased the probability of observing channels that gated with a long open time, but had no effect on single channel conductance. Using Monte Carlo simulations of a single channel kinetic model and Ros interactions, we were able to reproduce our experimental results and investigate the model's microscopic dynamics. In particular, our simulations predicted that the longer open times generated by Ros were due to the appearance of a long open state combined with an increased amount of time spent in transitions between open states. Our results suggest a mechanism for agonist effects of Ros at the level of single channels, and provide a mechanistic explanation for previously reported agonist effects on whole cell calcium currents.

Key words: voltage-gated calcium channel, patch clamp, roscovitine, single channel current, channel kinetics, conductance.

(R)- and (S)-Roscovitine, together with a structurally similar compound olomoucine, inhibit cyclin-dependent kinases (cdks). Of these compounds, (R)-Roscovitine (Ros) in particular also has been shown to have cdk-independent effects on calcium (Ca^{2+}) channels (Yan et al., 2002; Buraei et al., 2005, 2007; Cho and Meriney, 2006). The effect of Ros on P/Q- and N-type Ca^{2+} channels (Ca_v 2.1 and Ca_v2.2) manifests itself at the population level by slowing deactivation kinetics (Yan et al., 2002; Tomizawa et al., 2002; Buraei et al., 2005, 2007). This action prolongs Ca^{2+} tail currents and has been reported to increase transmitter release at CNS synapses (Yan et al., 2002; Tomizawa et al., 2002) and the frog neuromuscular junction (Cho and Meriney, 2006). With increasing concentrations, Ros also displays Ca^{2+} current antagonist activity, albeit with a slower onset than observed for agonist effects (Buraei et al., 2007). Recently Buraei and Elmslie (2008) have begun to elucidate the molecular pharmacologic interactions that might underlie differences between agonist and antagonist activities of Ros on Ca^{2+} channels.

Aside from the use of Ros derivatives to study Ca^{2+} channel gating and the regulation of transmitter release, such compounds might also be developed as potential therapeutic agents that selectively target N- and P/Q-type Ca^{2+} channels. Despite recent work documenting effects on whole cell currents, it is not yet known how Ros affects single channel gating. Thus, to characterize these effects, we performed cell-attached patch clamp recordings using a cell line that stably expresses mammalian N-type Ca^{2+} channels. We show that these channels gate with distinct short or long mean open times. Ros significantly lengthened the longer mean open time component, and increased the probability of observing the longer openings. On the other hand, we did not detect any effect of Ros on single channel conductance. These results are reminiscent of the selective effects of BayK 8644 and FPL 64176 on L-type Ca^{2+} channels (Schramm et al., 1983; Kokubun and Reuter, 1984; Hess et al., 1984; Nowycky et al., 1985; Zheng et al., 1991; Kunze and Rampe, 1992; Lauven et al., 1999; Tavalin et al., 2004). We also propose a kinetic scheme for Ros modulation of voltage-gated calcium channels (modified from Buraei et al., 2005), constrained by our new single channel data and a previous estimate of the probability that N-type Ca^{2+} channels open during an action potential (Poage and Meriney, 2002; Wachman et al., 2004; King and Meriney, 2005; Luo et al., 2009). Our results provide a mechanistic explanation for the previously reported agonist effects of Ros on whole cell calcium currents.

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Abbreviations: Ca^{2+}, calcium; cdk, cyclin-dependent kinase; DMEM, Dulbecco's modification of Eagle's medium; DMSO, dimethyl sulfoxide; EDTA, ethylene diamine tetraacetic acid; EGTA, ethylene glycol tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid; MCell, Monte Carlo simulator of cellular microphysiology; P_o, probability of opening; Ros, (R)-roscovitine; TEA, tetraethylammonium.

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EXPERIMENTAL PROCEDURES

tsA201 cells expressing N-type calcium channels

We used a tsA201 cell line (kindly provided by Dr. Diane Lipscombe, Brown University; see Lin et al., 2004) that stably expresses all of the subunits of the N-type Ca^{2+} channel splice variant predominantly present in mammalian brain and spinal cord: $Ca_v2.2$ rn$\alpha_{1B\text{-}c}$ (Ca_v 2.2 e[24a,Δ31a]), $Ca_v\beta_3$ and $Ca_v\alpha_2\delta_1$. The cells were maintained in DMEM supplemented with 10% fetal bovine serum, 25 μg/ml zeocin, 5 μg/ml blasticidin, and 25 μg/ml hygromycin.

Whole-cell patch clamp recordings

Whole-cell currents through Ca^{2+} channels were recorded as previously described (White et al., 1997; Yazejian et al., 1997; Pattillo et al., 1999). Briefly, the pipette solution consisted of (mM): 135 CsCl, 4 $MgCl_2$, 10 HEPES, 1 EGTA, 1 EDTA, pH 7.4. The culture was bathed in a solution consisting of (mM): 130 ChCl, 10 TEA–Cl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, pH 7.4. In some experiments the cells were bathed in a high barium solution (100 mM $BaCl_2$, 10 mM HEPES, 10 mM TEA–Cl, pH 7.4). Patch pipettes were fabricated from borosilicate glass and had an average access resistance of 4.3±2.9 MΩ (mean±SD, n=37), compensated for by 85% (lag setting=10 μs). Capacitive currents and passive membrane responses to voltage commands were subtracted using four waveforms of reverse polarity, each 1/4 the size of the full waveform. Ca^{2+} currents were amplified by an Axopatch 200B amplifier, filtered at 5 kHz, and digitized at 10 kHz for subsequent analysis using pClamp software (Axon Instruments/Molecular Devices, Sunnyvale, CA, USA). Liquid junction potentials of −7 and −9 mV (for calcium and barium containing solutions respectively) were accounted for in the plotting of data. Fitting of tail current deactivation was begun 50 μs after the peak of the tail current. All experiments were carried out at room temperature (22 °C).

Cell-attached patch clamp recordings

Cell-attached recordings of single Ca^{2+} channel currents were performed using a bath saline that collapsed the membrane potential (mM): 140 K-aspartate, 10 HEPES, 5 EGTA, 5 $MgCl_2$, pH 7.4 with KOH. The pipette solution included barium as the major charge carrier to increase the size of current through Ca^{2+} channels (mM): 100 $BaCl_2$, 10 HEPES, 10 TEA–Cl, pH 7.4. For data acquisition and analysis we used the pClamp software package (Axon Instruments/Molecular Devices). Single channel currents were amplified by an Axopatch 200B amplifier with an actively cooled capacitive head stage, filtered at 5 kHz, and digitized at 10 or 20 kHz for subsequent analysis. Before analysis, unitary currents were digitally filtered offline at 2 kHz. Patch pipettes were fabricated from quartz (1.5 mm outside diameter; 0.5 mm inside diameter; Sutter Instrument Co., Novato, CA, USA) using a laser-based pipette puller (P-2000, Sutter Instrument Co.) to provide low noise recordings. Capacitive and leak currents were subtracted off-line using null sweeps. A liquid junction potential of −20 mV was accounted for in the plotting of data. Unitary event transitions were recognized using a 50% threshold method, and transitions shorter than 0.2 ms were ignored (consistent with our filtering conditions).

Reagents

(R)-roscovitine (a gift of Dr. Laurent Meijer, CNRS, Roscoff, France) was dissolved in DMSO as a 100 mM stock and stored at −20 °C. For whole-cell recordings, aliquots were diluted on the day of use into extracellular saline at a final concentration of 100 μM, and bath applied via a delivery pipette in a ~0.3 ml chamber using a rapid perfusion system running at ~1 ml/min. Control recordings performed with 0.1% DMSO alone added to the drug delivery pipette solution revealed no significant effects on whole cell Ca^{2+} currents. For cell-attached recordings, aliquots were diluted into the patch pipette solution at the same final concentration. All other salts and chemicals were obtained from Sigma-Aldrich chemical company (St. Louis, MO, USA).

Kinetic modeling of channel gating

Calcium channel kinetics were simulated with MCell (Monte Carlo Cell; Stiles et al., 1996; Stiles and Bartol, 2001), which in its latest revision (MCell version 3) allows continuous-time stochastic simulation of voltage-dependent state transitions and ion flux (Kerr et al., 2008). Our model consisted of one or more Ca^{2+} channels embedded in a surface that represented a cell membrane. During simulations, the channel(s) underwent stochastic transitions between closed and open states according to the kinetic scheme shown in Fig. 8, with voltage-dependent rate constants assigned to each transition except Ros binding and unbinding. Voltage-dependent rate constants were calculated from $K_x=A_x\ \exp((V-V_c)z_xF/RT)$, where A_x is the rate amplitude at the characteristic voltage V_c=10 mV, z_x is the charge moved, and F, R, and T are the Faraday constant, ideal gas constant, and temperature, respectively. For the sake of computational efficiency, Ros binding at a constant concentration (100 μM) was simulated using a pseudo-first order rate constant for each binding transition (the pseudo-first order rate constants were obtained from the product of a bimolecular binding rate constant and the constant Ros concentration).

Each time a channel entered a new state, or at times when the membrane potential changed, a random number was used to choose a lifetime from the exponential distribution of possible lifetimes for the new state and/or voltage. Whenever the current lifetime elapsed, another random number was used to decide which transition would occur and a subsequent lifetime was generated for the destination state. Production of Ca^{2+} ions from open states was handled in a similar stochastic fashion, using rate constants calculated from the membrane voltage, reversal potential, and single channel conductance (2.6 pS in 2 mM extracelluluar calcium; Church and Stanley, 1996). Whenever an event was scheduled to occur from an open state, the channel could either make a transition to an adjacent state, or produce a calcium ion and remain in the original open state until the next event occurred. Since the next event also might produce a calcium ion (and might even occur during a given simulation timestep), it was possible for multiple ions to be produced prior to exit from an open state.

Simulation output included the times of all transitions induced by a step depolarization, but to be consistent with experimental (steady-state) patch clamp data, we analyzed simulated lifetime distributions only after the channel had equilibrated at the new holding potential (the last 20 ms of each simulation; see Fig. 8D, E). Mean open times were calculated from 10,000 single channel simulations, each of which used a different random number seed and a 50 ms step depolarization from the resting potential (−60 mV) to −20 mV (the same step size used in experimental recordings). As with our experimental data, we omitted open times <0.2 ms from analysis (including such short events changed calculated time constants by less than 0.1 ms). Final model parameters were determined by an extensive parameter sweep, adjusting inputs until the model yielded good agreement with the experimental open time distribution while maintaining microscopic reversibility for the cyclic part of the kinetic scheme. The same final model then was used to simulate whole cell currents using a population of 10,000 channels.

RESULTS

Roscovitine effects on whole cell currents

Whole cell currents (Fig. 1A) were recorded from tsA201 cells expressing N-type Ca^{2+} channels $Ca_v2.2$e[24a, Δ31a] together with $Ca_v\beta_3$ and $Ca_v\alpha_2\delta_1$. Using 2 mM Ca^{2+}