HIV protease inhibitors elicit volume-sensitive Cl^- current in cardiac myocytes via mitochondrial ROS

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ONLINE SUPPLEMENT

METHODS

1. *Ventricular myocytes isolation*

Myocyte isolation was based on our previously described method [1]. Hearts excised from anesthetized New Zealand white rabbits (~3 kg), were mounted on a Langendorff column, perfused at 37ºC with Tyrode solution containing (in mM): 130 NaCl, 5 KCl, 1.8 $CaCl_2$, 4 KH_2PO_4, 3 $MgCl_2$, 5 HEPES, 15 taurine, 5 creatine, 10 glucose, pH 7.25 (adjusted with NaOH), and the right an left ventricles were vented. Perfusate then was switched to Ca-free Tyrodes in which 0.1 mM Na_2-EGTA replaced $CaCl_2$ and, finally, to enzyme solution containing collagenase (0.45 mg/ml; Cls 4, Worthington) and pronase (0.015 mg/ml, Type XIV, Sigma-Aldrich) in nominally Ca^{2+}-free Tyrode solution without EGTA. Isolated myocytes were washed twice and stored in a modified Kraft–Brühe (KB) solution until use. The modified KB myocyte storage solution contained (in mM): 120 K-glutamate, 10 KCl, 10 KH_2PO_4, 0.5 K_2EGTA, 10 taurine, 1.8 $MgSO_4$, 10 HEPES, 20 glucose, 10 mannitol, pH 7.2 (adjust with KOH). Rod-shaped quiescent cells with clear striations and no membrane blebs or other morphological irregularities were studied within 10 h of isolation.

2. *HL-1 myocytes for flow cytometry*

HL-1 cardiac myocytes, an immortalized mouse atrial cell line [2], were used for flow cytometry studies because they provide a more homogeneous cell population than freshly isolated, enzymatically dissociated myocytes, which unavoidably included damaged cells. Tissue culture flasks first were coated with gelatin (0.02%)/fibronectin (0.5%) (2 ml in T25 or 6 ml in T75 flask) and incubated at 37°C overnight. HL-1 cells were cultured in pre-coated flasks using Claycomb medium (JRH Biosciences, Lenexa, KS) supplemented with 10% fetal bovine serum (Sigma-Aldrich), 100 μg/ml penicillin/streptomycin, 0.1 mM norepinephrine (Sigma-Aldrich) and 2 mM L-glutamine (Invitrogen). The cells were kept at 37°C in 5% CO2 and 95%

air at ~95% relative humidity. Once the HL-1 cells reached confluence, the cells were passaged by splitting 1 to 2. To split the cells, the myocytes were rinsed briefly with DPBS, then 0.05% trypsin/EDTA, and incubated with 0.05% trypsin/EDTA at 37°C for 1 – 2 min until the cells were dislodged. Equal amount of soybean trypsin inhibitor (25 mg/100 ml DPBS) was added directly to the cells, and cells were transferred into a 15 ml centrifuge tube and centrifuged at 1100 rpm for 5 min. The supernatant was removed by aspiration, and the pellet was gently suspended in 3 ml of supplemented Claycomb medium. Finally, cells were transferred into gelatin/fibronectin-coated flasks for culturing or 15 ml centrifuge tube for flow cytometry.

3. Solutions and drugs

For studies of membrane currents, bath and pipette solutions were designed to isolate anion currents, and $I_{Cl,swell}$ is essentially time-independent under these conditions [3]. Bath solution contained (in mM): 90 *N*-methyl-D-glucamine (NMDG)-Cl, 3 $MgCl_2$, 10 HEPES, 10 glucose, 5 CsCl, 0.5 $CdCl_2$ pH 7.4 (adjusted with CsOH), 91.8 mannitol. Mannitol was omitted in hyposmotic solutions to reduce osmolarity to 0.7-times (0.7T) that in isosmotic solutions. Pipette solution contained: 110 Cs-aspartate, 20 CsCl, 2.5 Mg-ATP, 8 Cs_2-EGTA, 0.1 $CaCl_2$, 10 HEPES, pH 7.1 (adjusted with CsOH; liquid junction potential, −15 mV). For action potential recordings, bath solution contained: 140 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 0.5 $MgCl_2$, 0.33 NaH_2PO_4, 5 HEPES (pH 7.4), and pipette solution contained: 110 K-aspartate, 20 KCl, 5 Mg-ATP, 5 NaH_2PO_4, 1 $MgCl_2$, 0.2 Na_2GTP, 5 HEPES (pH 7.2). Solution osmolarities were adjusted with mannitol and verified by freezing-point depression.

Ritonavir, lopinavir, amprenavir, nelfinavir, and raltegravir (all 50 mM) from the NIH AIDS Research and Reference Reagent Program (http://www.aidsreagent.org) (for structures, see Fig. S1), rotenone (20 mM) from Sigma-Aldrich, and ebselen (15 mM) from Calbiochem were prepared as stock solutions in DMSO at the indicated concentrations and kept frozen at –20°C in small aliquots until use. DCPIB (20 mM) from Tocris was prepared in ethanol and refrigerated (−4°C), and apocynin (500 mM) from Sigma was dissolved in DMSO and stored at room temperature. 2,4-dinitrophenol (DNP) from MP Biomedicals was added at a final concentration of 0.3 mM. A membrane-permeant fusion peptide inhibitor of NADPH oxidase, gp91ds-tat, was synthesized [4]. The inhibitor is an 18-mer comprising a 9-mer, CSTRIRRQL, that blocks NADPH oxidase assembly by mimicking the NOX2 $gp91^{phox}$ docking site for $p47^{phox}$ joined to a tat 9-mer, RKKRRQRRR, that drives transmembrane uptake. Peptide stocks (1.2 mg/ml) were made in 150 mM NaCl plus 10 mM acetic acid and frozen (20ºC) in aliquots until use.

4. Electrophysiology

Whole-cell recordings were made at room temperature (22 − 23°C). Pipettes were pulled using 7740 thin-walled borosilicate capillary tubing (Sutter) and fire-polished to give a resistance in bath solution of 2 – 3 MΩ. Myocytes were placed in a glass-bottomed chamber and suprafused with bath solution at ~2 ml/min. Junction potentials were corrected, and a 3-M KCl-agar bridge served as the ground electrode. Typical seal resistances were 5–30 GΩ, and myocytes were dialyzed for at least 10 min before data were collected. Currents were recorded with an Axoclamp 200B and Digidata 1322A under pClamp 9. After low-pass filtering (Bessel, 2 kHz), currents were digitized (5 kHz) and were refiltered (Bessel, 500 Hz) in pClamp for presentation. Successive 500-ms steps were made at 1-s intervals from −60 mV to test potentials between −100 and +60 mV in +10-mV increments, and current-voltage (I–V) relationships were plotted from the late current. Mean currents are expressed as current density (pA/pF) to account for differences in myocyte surface membrane area.

For action potential recordings, myocytes were stimulated at 1 Hz with 1-ms pulses, and the membrane potential was digitized (10 KHz, Bessel filtered at 2 KHz). At least 30 action potentials were recorded to verify that action potential duration (APD) was stable, and the last 10 action potentials were averaged. APD at 50% and 90% repolarization (APD_{50}, APD_{90}) were calculated from the averaged record.

5. Flow cytometry

ROS production was detected in HL-1 myocytes with C-H_2DCFDA-AM [6-carboxy-2',7'-dichlorodihydrofluorescein diacetate di(acetoxymethyl ester)] (Invitrogen), which is converted into a non-fluorescent derivative, carboxy-H_2DCF, by intracellular esterases. Carboxy-H_2DCF is retained in the cytoplasm and is oxidized to fluorescent carboxy-DCF by intracellular ROS, primarily H_2O_2. Confluent cells were incubated with C-H_2DCFDA-AM (2.5 or 5 μM) for 30 min at 37°C, washed with DPBS, and then isolated using the splitting procedure. Single myocyte suspensions in DPBS were analyzed using an EPICS XL flow cytometer (Beckman Coulter). The geometric means of gated fluorescence distributions (excitation: 488 nm; emission: 525 ± 5 nm) were calculated using EXPO32 software (Beckman Coulter). About 30,000 cells contributed to each fluorescence histogram (1024 bins), and histograms were filtered and replotted (5 point running average, 512 bins) for display.

JC-1 [5,5'6,6'tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine] (Invitrogen) was used to detect mitochondrial inner membrane potential ($\Delta\Psi_m$) [5]. HL-1 myocytes were incubate

with 1 μM JC-1 for 90 min (37°C) to maximize J-aggregate formation and washed. $\Delta\Psi_m$ depolarization elicits an increase in monomer (green) and decreases in J-aggregate (orange-red) fluorescence (excitation: 488 nm; emission 525 ± 5 and 575 ± 5 nm). JC-1-loaded cells were assayed as described above (EPICS XL; Beckman Coulter), and the ratio of the geometric means of green:orange-red fluorescence (FL1/FL2) was plotted.

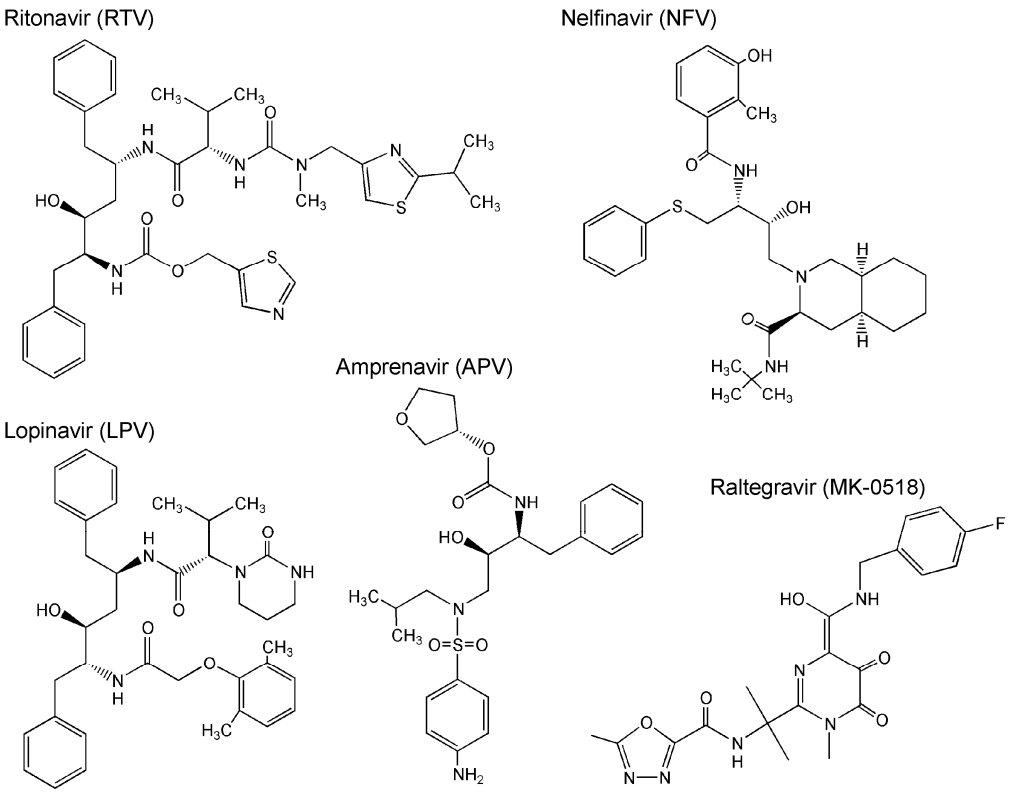

Fig. S1. Structures of HIV-protease inhibitors (ritonavir, RTV; lopinavir, LPV; nelfinavir, NFV; amprenavir, APV) and the integrase inhibitor raltegravir (MK-0518). Agents were employed at concentrations typically achieved in clinical application. Clinically, RTV and LPV are often given in combination.

References

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