Cardiac *Slo2.1* Is Required for Volatile Anesthetic Stimulation of K^+ Transport and Anesthetic Preconditioning

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ABSTRACT

Background: Anesthetic preconditioning (APC) is a clinically important phenomenon in which volatile anesthetics (VAs) protect tissues such as heart against ischemic injury. The mechanism of APC is thought to involve K^+ channels encoded by the *Slo* gene family, and the authors showed previously that *slo-2* is required for APC in *Caenorhabditis elegans*. Thus, the authors hypothesized that a *slo-2* ortholog may mediate APC-induced cardioprotection in mammals.

Methods: A perfused heart model of ischemia–reperfusion injury, a fluorescent assay for K^+ flux, and mice lacking *Slo2.1* (Slick), *Slo2.2* (Slack), or both (double knockouts, *Slo2.x dKO*) were used to test whether these channels are required for APC-induced cardioprotection and for cardiomyocyte or mitochondrial K^+ transport.

Results: In wild-type (WT) hearts, APC improved post-ischemia–reperfusion functional recovery (APC = 39.5 ± 3.7% of pre-ischemic rate × pressure product *vs.* 20.3 ± 2.3% in controls, means ± SEM, $P = 0.00051$, unpaired two-tailed t test, n = 8) and lowered infarct size (APC = 29.0 ± 4.8% of LV area *vs.* 51.4 ± 4.5% in controls, $P = 0.0043$, n = 8). Protection by APC was absent in hearts from *Slo2.1*$^{-/-}$ mice (% recovery APC = 14.6 ± 2.6% *vs.* 16.5 ± 2.1% in controls, $P = 0.569$, n = 8 to 9, infarct APC = 52.2 ± 5.4% *vs.* 53.5 ± 4.7% in controls, $P = 0.865$, n = 8 to 9). APC protection was also absent in *Slo2.x dKO* hearts (% recovery APC = 11.0 ± 1.7% *vs.* 11.9 ± 2.2% in controls, $P = 0.725$, n = 8, infarct APC = 51.6 ± 4.4% *vs.* 50.5 ± 3.9% in controls, $P = 0.855$, n = 8). Meanwhile, *Slo2.2*$^{-/-}$ hearts responded similar to WT (% recovery APC = 41.9 ± 4.0% *vs.* 18.0 ± 2.5% in controls, $P = 0.00016$, n = 8, infarct APC = 25.2 ± 1.3% *vs.* 50.8 ± 3.3% in controls, $P < 0.000005$, n = 8). Furthermore, VA-stimulated K^+ transport seen in cardiomyocytes or mitochondria from WT or *Slo2.2*$^{-/-}$ mice was absent in *Slo2.1*$^{-/-}$ or *Slo2.x dKO*.

Conclusion: Slick (*Slo2.1*) is required for both VA-stimulated K^+ flux and for the APC-induced cardioprotection.

(Anesthesiology 2016; 124:1065-76)

CLINICALLY relevant doses of halogenated volatile anesthetics (VAs) can protect tissues such as the heart from ischemia–reperfusion (IR) injury,[1–3] a phenomenon known as "anesthetic preconditioning" (APC). APC is evolutionarily conserved from *Caenorhabditis elegans*[4] to humans,[3] and current American Heart Association/American College of Cardiology (AHA/ACA) guidelines specifically recommend the use of VAs during cardiac surgery for their protective effects.[5]

The mechanism of APC is complex and involves many of the same effector signaling pathways as ischemic preconditioning (IPC), including protein kinases C[6] and A,[7,8] inhibitory G-proteins,[9] adenosine,[10,11] nitric oxide,[12] and mitochondrial reactive oxygen species (ROS) generation.[13,14] These signals are thought to converge at the level of mitochondria through distinct K^+ channels, but the molecular identity of these channels is a subject of debate.

Several K^+ channel types have been proposed to exist in mitochondria, including adenosine triphosphate (ATP)-sensitive (K_{ATP}),[15] voltage-sensitive (K_V),[16] and large-conductance (BK) channels of the *Slo* gene family[17] as well as a K^+/H^+ exchanger[18] (for review, see the studies reported by Szabo and Zoratti[19] and Szewczyk *et al.*[20]). Current evidence favors an involvement of

What We Already Know about This Topic

- Volatile anesthetics can protect the heart from ischemia–reperfusion injury, a phenomenon known as anesthetic preconditioning (APC)
- The clinical efficacy of APC remains to be determined, and further understanding of the mechanism for APC is critical for the development of drugs and strategies for cardiac protection in the clinic
- Ion channels in the cardiac myocyte and mitochondria play important roles in the mechanism of APC though the full identity of the channels remains unknown

What This Article Tells Us That Is New

- The authors have used novel gene-deleted mice to demonstrate that K^+ flux *via* the K_{Na} Slick channel encoded by the *Slo2.1* gene is required for anesthetic preconditioning in mice
- The identification of the role for Slick in anesthetic preconditioning will drive further development of novel cardiac-protective strategies and drugs for the clinical setting

This article is featured in "This Month in Anesthesiology," page 1A. Corresponding article on page 986. Supplemental Digital Content is available for this article. Direct URL citations appear in the printed text and are available in both the HTML and PDF versions of this article. Links to the digital files are provided in the HTML text of this article on the Journal's Web site (www.anesthesiology.org). Drs. Wojtovich and Smith contributed equally to this work. Data Transparency: All original data used to prepare figures for this article and for statistical analyses are available at: https://dx.doi.org/10.6084/m9.figshare.2062833. Accessed January 28, 2016.

Submitted for publication July 10, 2015. Accepted for publication January 5, 2016. From the Departments of Anesthesiology (A.P.W., W.R.U., Y.T.W., P.S.B.), Biochemistry (C.O.S.), Pharmacology and Physiology (A.P.W., P.S.B., K.N.), and Medicine (K.N.), University of Rochester Medical Center, Rochester, New York; and Department of Anesthesiology, Washington University School of Medicine, St. Louis, Missouri (X.-M.X.).

 Anesthesiology 2016; 124:1065-76

<zdoi;10.1097/ALN.0000000000001046>

mitochondrial K_{ATP} channels in IPC,[21] whereas a mitochondrial *Slo* channel is thought to underlie APC.[7,22,23]

The mammalian *Slo* channel family comprises *Slo1* (*KCNMA1*; BK), *Slo2.1* (*KCNT2,* Slick), *Slo2.2* (*KCNT1,* Slack), and *Slo3* (*KCNU1*).[24] *Slo3* expression is germline restricted,[24,25] but the others are widely expressed. Because pharmacologic Slo1 channel activators can protect the heart against IR injury,[17,26] it was widely assumed that a mitochondrial Slo1 channel (also termed BK, K_{Ca}, or BK_{Ca}) was responsible for APC. However, we recently showed that both *C. elegans* and mice lacking Slo1 channels can still be protected by APC, and mitochondria from these organisms contain a K^+ channel activated by VA.[22]

We have also previously demonstrated that the K_{Ca} channel SLO-2 is required for VA-induced protection of *C. elegans* against IR injury, with *slo-2*-mutant nematodes also lacking VA-stimulated mitochondrial K^+ flux.[22] Thus, we hypothesized that one of the mammalian SLO-2 orthologs, Slick or Slack (which are K_{Na}, not K_{Ca} channels[24,27]), may underlie APC in mammals. Herein, we used novel gene-deleted mice (*Slo2.1*$^{-/-}$, *Slo2.2*$^{-/-}$, and *Slo2.x* double knockout [dKO]) to conclusively demonstrate that *Slo2.1* codes the channel required for APC and for VA-stimulated K^+ flux in cardiomyocytes and mitochondria.

Materials and Methods

Animals

Mice were housed in an Association for Assessment & Accreditation of Laboratory Animal Care-accredited pathogen-free facility with food and water available *ad libitum*. All procedures were approved by the University of Rochester's Committee on Animal Resources (protocol no. 2010–030) in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (2011 revision). A *Slo2.x dKO* strain containing both mutant *Slo2.x* alleles[28] was provided by Dr. Chris Lingle, Ph.D. (Washington University, St. Louis, Missouri). Mice were outcrossed more than six times to a C57BL/6 background, and conventional breeding was used such that experimental wild-type (WT) and knockout mice (males, 8 to 10 weeks old) were littermates. All animals were genotyped by tail-clip polymerase chain reaction (Kapa Biosystems, USA)[29] with the following primers: *Slo2.1* (Slick), C523-24C 5′-AACTTTATGAGTTCCTCTTCCATG-3′, C320-24F 5′-GAGCATCATACTTTGCTTTTTGGG-3′, KO = 269 base pairs (bp), WT = 579 bp; *Slo2.2* (Slack) C311-30 5′-CCCATTCCACACTGCAGCCCTGTCTCTTTC-3′, C315-30 5′-TGTTTACTAGGGTCCAGGGAGAACCTATGA-3′, KO = 200 bp, WT = 607 bp. Due to personnel limitations (*i.e.*, same person handling mice and doing experiments), it was not feasible to blind the experimenter to animal genotype. However, for all experiments, mice of varying genotypes were randomly assigned to experimental groups, with treatments in randomized order across experimental days.

Ex Vivo *Perfused Heart*

Mouse hearts were perfused as previously described.[22,26,29] In brief, after tribromoethanol anesthesia (100 mg/kg ip), the aorta was rapidly cannulated and perfused without pacing at a constant flow of 4 ml/min per 100 mg with Krebs–Henseleit buffer (118 mM NaCl, 4.7 mM KCl, 25 mM $NaHCO_3$, 10 mM D-glucose, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, and 2.5 mM $CaCl_2$, gassed with 95% O_2 and 5% CO_2 at 37°C). Left ventricular (LV) pressure was measured *via* a water-filled transducer-linked low density polyethylene balloon. LV and coronary root pressures were monitored and digitally recorded at 1 kHz (DATAQ, USA). Parameters calculated from the LV balloon throughout perfusion included heart rate, systolic and end-diastolic pressures, LV-developed pressure (systolic – diastolic), rate × pressure product, dP/dT_{MAX} (contraction), and dP/dT_{MIN} (relaxation).

Hearts were equilibrated for 20 min before initiating data acquisition. A total of 163 hearts were analyzed, and none were excluded from the study. A 30-min normoxic perfusion was followed by 30 min of no flow global ischemia and then 60 min of reperfusion (fig. 1). Treatment groups were as follows: (1) control IR injury; (2) IPC comprising three cycles of 5-min ischemia plus 5-min reperfusion to replace the 30-min perfusion; (3) 10-min isoflurane (Henry Schein Animal Health, USA) infusion (100 μM or approximately 0.35 minimum alveolar concentration [MAC] for a C57BL/6 mouse[30,31]); (4) 20-min 7-chloro-3-methyl-4H-1,2,4-benzothiadiazine 1,1-dioxide (diazoxide, a K^+ channel activator[32]; Sigma, USA) infusion (30 μM); and (5) 10-min 2,2'-sulfanediylbis(4,6-dichlorophenol) (bithionol a Slo channel activator[33]; TCI America, USA) infusion (2.5 nM). Pharmacologic agents were delivered *via* syringe pump just above the perfusion cannula for the indicated times, followed by 30-s washout before index ischemia. At the end of IR protocols, hearts were sliced, stained in 1% (wt/vol) 2,3,5-triphenyltetrazolium chloride for 20 min and fixed in 10% neutral buffered formalin for 24 h. Heart slices were imaged and infarct size was analyzed by planimetry as previously described.[22,26,29]

Plasma Membrane Thallium (Tl^+) Flux Assay on Isolated Mouse Cardiomyocytes and Human Embryonic Kidney (HEK)293 Cells

Mouse adult ventricular cardiomyocytes were isolated by collagenase perfusion as previously described.[26] In brief, after anesthesia (tribromoethanol, see *Ex Vivo* Perfused Heart, above), hearts were cannulated and perfused with isolation buffer (IB) at 37°C (IB: 120 mM NaCl, 15 mM KCl, 0.6 mM Na_2HPO_4, 0.6 mM KH_2PO_4, 1.2 mM $MgSO_4$, 10 mM HEPES, 4.6 mM $NaHCO_3$, 30 mM taurine, 5.5 mM D-glucose, and 10 mM 2,3-butanedione monoxime, pH = 7.4) for less than 2 min before switching to digestion buffer (35 ml IB plus $CaCl_2$ 12.5 μM final, trypsin 400 μl at 2.5% [w/v], collagenase A 6.525 units, and collagenase D 15.375 units), and 8 min later placed in 2 to 3 ml of stop buffer (IB plus $CaCl_2$ 12.5 μM, 10% heat-inactivated Fetal Bovine Serum). Cardiomyocytes were dissociated

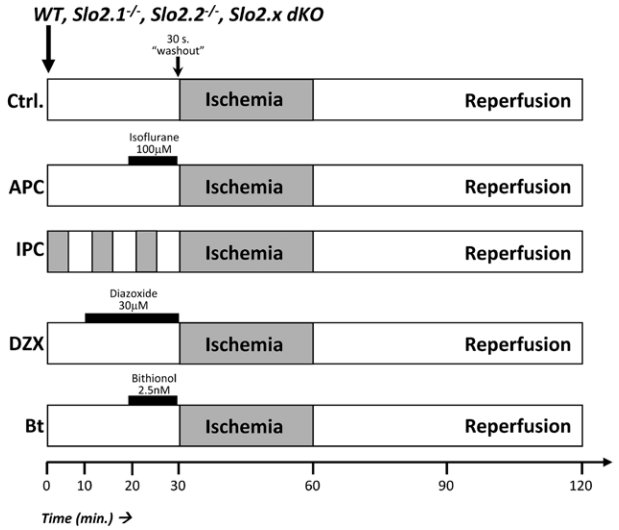

Fig. 1. Experimental protocol. Schematic shows the five perfused heart experimental conditions tested. *Gray shading* represents ischemia; 30-s washout was performed after administration of pharmacologic agents before ischemia. APC = anesthetic preconditioning; Bt = bithionol; dKO = double knockouts; DZX = diazoxide; IPC = ischemic preconditioning; WT = wild type.

and filtered through 75 μm mesh, and then, gravity sedimentation and resuspension were used to bring [Ca^{2+}] stepwise to 1.8 mM, and the final pellet was placed into 1 ml of Minimal Essential Media (GIBCO, USA). Cell viability and yield were determined using Trypan blue and a hemocytometer, and preparations with greater than 85% viable rod-shaped cells were used experimentally. Cells were seeded (2500 cells per well) in a Falcon 96-well plate and incubated at 37°C for 1 h and then loaded with Thallos reagent (2 μM; Teflabs, USA) in 65-μl Hanks buffered salt solution (GIBCO) and monitored for thallium (Tl^+) uptake by addition of 4 mM final Tl_2SO_4 in stimulus buffer (SB: 276 mM Na^+ gluconate, 2.6 mM $CaSO_4$, 1.6 mM $MgSO_4$, 11.2 mM D-glucose, and 40 mM HEPES, pH = 7.3) in a BioTek Synergy plate reader in kinetic mode measuring every 7 s. (λex 488 nm; λem 525 nm) in the presence or absence of channel modulators. *N*-benzyl-*N*-(3-isobutoxy-2-pyrrolidin-1-yl-propyl)aniline (Bepridil; Sigma) is a calcium channel blocker that also inhibits Slo2 channels.[33] Eight measurements were taken before additions. Paired control wells contained Tl^+-free SB, enabling determination of Tl^+-specific fluorescence changes.

HEK293 cells were seeded on glass 12-mm coverslips and transfected using Lipofectamine 3000 (Invitrogen, USA) with the expression vector pKT122 expressing an mCherry:rat Slick complementary DNA fusion in a pcDNA5/TO vector backbone (Invitrogen). After 24 h, cells were incubated in 1 ml of Hanks buffered saline solution containing 2 μM Thallos reagent (TefLabs) for 30 min and then transferred to an open-flow perfusion rig attached to a Nikon TE2000 microscope (Nikon, USA) equipped with a monochromator (TILL Photonics, Germany), charge coupled device camera (PCO-TECH, USA), and appropriate filter sets. Transfected cells were identified *via* mCherry fluorescence and perfused with SB, with or without Slo2 channel modulators, then SB with 4 mM Tl_2SO_4, and images acquired every 5 s (λex 488 nm; λem 535 nm). Changes in fluorescent emission were quantified for all cells in the field of view, both transfected and untransfected, using TILLvisION software (TILL Photonics).

Mitochondrial Thallium (Tl^+) Flux Assay on Isolated Mouse Heart Mitochondria

After anesthesia (tribromoethanol as above), mitochondria were isolated from three pooled mouse hearts, loaded with BTC-AM (Life Technologies, USA) and monitored for Tl^+ uptake (a surrogate for K^+ channel flux) as previously described.[22,29,34]

Isoflurane Dosing

In perfused hearts, isoflurane infusion *via* syringe pump afforded no possibility for volatilization before entering the heart. For C57BL/6 mice, 100 μM isoflurane equates to approximately 0.35 MAC.[30,31] For cell and mitochondrial experiments, incubations generally lasted less than 1 min such that isoflurane volatilization was considered negligible. As such, isoflurane concentrations are denoted as "initial."

Statistical Analysis

Significance was determined by using a two-way ANOVA and when warranted *post hoc* unpaired *t* testing (two tailed) analysis using Bonferroni multiple comparisons correction. *P* values reported in the results are from *t* tests, with *P* value less than 0.05 (following multiple comparisons correction) considered statistically significant. Sample size was determined based on experience and previous studies.

Results

Cardiac APC Requires Slo2.1 (Slick)

In *C. elegans*, SLO-2 is encoded by a single gene and contributes to VA-stimulated mitochondrial K^+ flux and APC.[22] However, in mammals, *slo-2* has diverged into two paralogs (*Slo2.1* and *Slo2.2*) with differing ion sensitivity to the *C. elegans* channel. By using a recently developed mouse strain with the genes coding for Slick (*Kcnt2* or *Slo2.1*) and Slack (*Kcnt1* or *Slo2.2*) both deleted,[28] our goal herein was to determine whether either or both of these mammalian gene products are orthologous to the worm SLO-2 channel and facilitate K^+ flux across the mitochondrial inner membrane in response to VA. First, we verified using genomic polymerase chain reaction that the *Slo2.x* dKO contained the expected lesions in each of the two genes and that each allele was unambiguously detectable (fig. 2, A and B). Next, we used Western blot analysis to query whether the channels themselves were similarly ablated. Slick and Slack are abundantly expressed in neural tissue, and our results demonstrate that we can readily detect both paralogs in brain lysates as well as their absence in the *dKO* background (fig. 2C). Finally, we report that *Slo2.x*

dKO mice were viable and had normal cardiac electrical function (table S1, Supplemental Digital Content 1, http://links.lww.com/ALN/B256).

To interrogate the individual contribution of each *Slo2.x* paralog to APC, the mutant alleles were genetically separated through extensive backcrossing to a C57BL/6 background. *Ex vivo* perfused hearts from WT and *Slo2*-ablated mice (both the *Slo2.x dKO* and single mutant alleles) were then subjected to IR injury with optional APC (isoflurane in perfusion media). Injury was assessed by measuring post-IR functional recovery (fig. 3A) and infarct size (fig. 3B). APC was protective in WT hearts, with a 95% improvement in the post-IR recovery of rate pressure product (RPP; heart rate × left ventricular–developed pressure; $P = 0.0005$) and a 44% reduction in infarct size ($P = 0.0043$) *versus* control hearts. *Slo2.2*$^{-/-}$ mice exhibited a similar degree of protection by APC (132% improvement in recovery of RPP, $P = 0.00016$, 50% reduction in infarct size, $P = 0.000005$) *versus* control hearts. However, APC in *Slo2.1*$^{-/-}$ mice resulted in no protection (12% worse recovery of RPP, $P = 0.057$, 2% reduction in infarct size, $P = 0.817$, compared with IR alone). As expected, APC in *dKO* mice was similarly ineffective (8% worse recovery of RPP, $P = 0.725$, 2% increase in infarct size, $P = 0.854$, compared with IR alone). None of the genotypes exhibited significant differences in baseline susceptibility to ischemia.

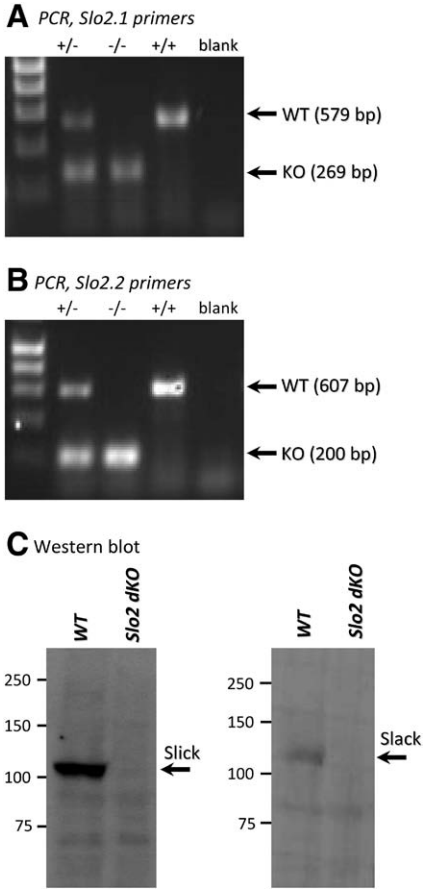

Fig. 2. *Slo2.1* and *Slo2.2* genotyping and protein expression analysis of Slick and Slack in *Slo2.x* double knockout *(dKO)* mice. (*A*) Tail-clip PCR showing wild-type (*WT*) or knockout *Slo2.1* products at the indicated mass, for WT (+/+), knockout (–/–), or heterozygous (+/–) animals. Primers were used as described in the Materials and Methods. Left lane = DNA ladder. (*B*) Similar to that in *A*, but with *Slo2.2* primers. (*C*) Western blot for Slick and Slack (Neuromab antibody; Neuromab, USA) on brain tissue lysates from WT and *Slo2.x dKO* mice.

Cardioprotection by IPC or the mK$_{ATP}$ Channel Opener Diazoxide Are Independent of Slo2.x Channel Genotype

To discount the possibility that lack of protection by APC in the *Slo2.1*$^{-/-}$ or *dKO* hearts was due to an inability of these hearts to be protected at all, we examined two additional cardioprotective treatments that are unrelated to VA: namely IPC (fig. 4) and the mitochondrial K_{ATP} channel opener diazoxide (fig. 5).

The improvement in functional recovery (RPP) induced by IPC for each genotype was as follows: WT 148%, *dKO* 184%, *Slo2.1*$^{-/-}$ 108%, *Slo2.2*$^{-/-}$ 193%, compared with IR alone, $P < 0.025$ for all genotypes (fig. 4A). The reduction in infarct size induced by IPC was as follows: WT 53%, *dKO* 34%, *Slo2.1*$^{-/-}$ 45%, *Slo2.2*$^{-/-}$ 49%, compared with IR alone, $P < 0.0025$ for all genotypes (fig. 4B). Similar to IPC, diazoxide delivered consistent cardioprotection across all genotypes. Namely, the improvement in functional recovery (RPP) induced by diazoxide: WT 98%, *dKO* 171%, *Slo2.1*$^{-/-}$ 125%, *Slo2.2*$^{-/-}$ 143%, compared with IR alone, $P < 0.01$ for all genotypes (fig. 5A). The reduction in infarct size induced by IPC was as follows: WT 37%, *dKO* 45%, *Slo2.1*$^{-/-}$ 55%, *Slo2.2*$^{-/-}$ 48%, compared with IR alone, $P < 0.001$ for all genotypes (fig. 5B). Thus, cardioprotection by either IPC or diazoxide was consistent regardless of genotype, suggesting *Slo2.1*$^{-/-}$ hearts are still capable of being protected by these interventions and that cardioprotection, even *via* mechanisms thought to involve mitochondria, does not invariably require Slick.

In addition to functional recovery measured as RPP, data for dP/dT_{MAX} and dP/dT_{MIN}, LV-developed pressure, and LV end-diastolic pressure, for all genotypes and treatment regimens, are provided in Supplemental Digital Content 1, http://links.lww.com/ALN/B256, tables 2–4, respectively.

VA-stimulated K$^+$ Transport in Cardiomyocytes and Mitochondria Requires Slo2.1 (Slick)

Given the proposed role of a mitochondrial large-conductance K$^+$ channel in APC,[7,22,23] we examined VA stimulation of K$^+$ flux in both primary cardiomyocytes and isolated cardiac mitochondria by using a thallium (Tl$^+$)-based fluorescence assay.[22,34] All four genotypes were studied at the isolated mitochondrial level, but due to our initial results (vide supra) indicating that Slick but not Slack is involved in APC, studies in cardiomyocytes were limited to WT and *Slo2.1*$^{-/-}$.

The VA isoflurane was found to stimulate K$^+$ flux in cardiomyocytes (fig. 6A) as well as mitochondria (fig. 6B) from WT mice, and this effect was blocked by the inhibitor bepridil. Bepridil is thought to target cardiac K_{Na}[35] and other channels[36] and has recently been shown to function as an Ebola

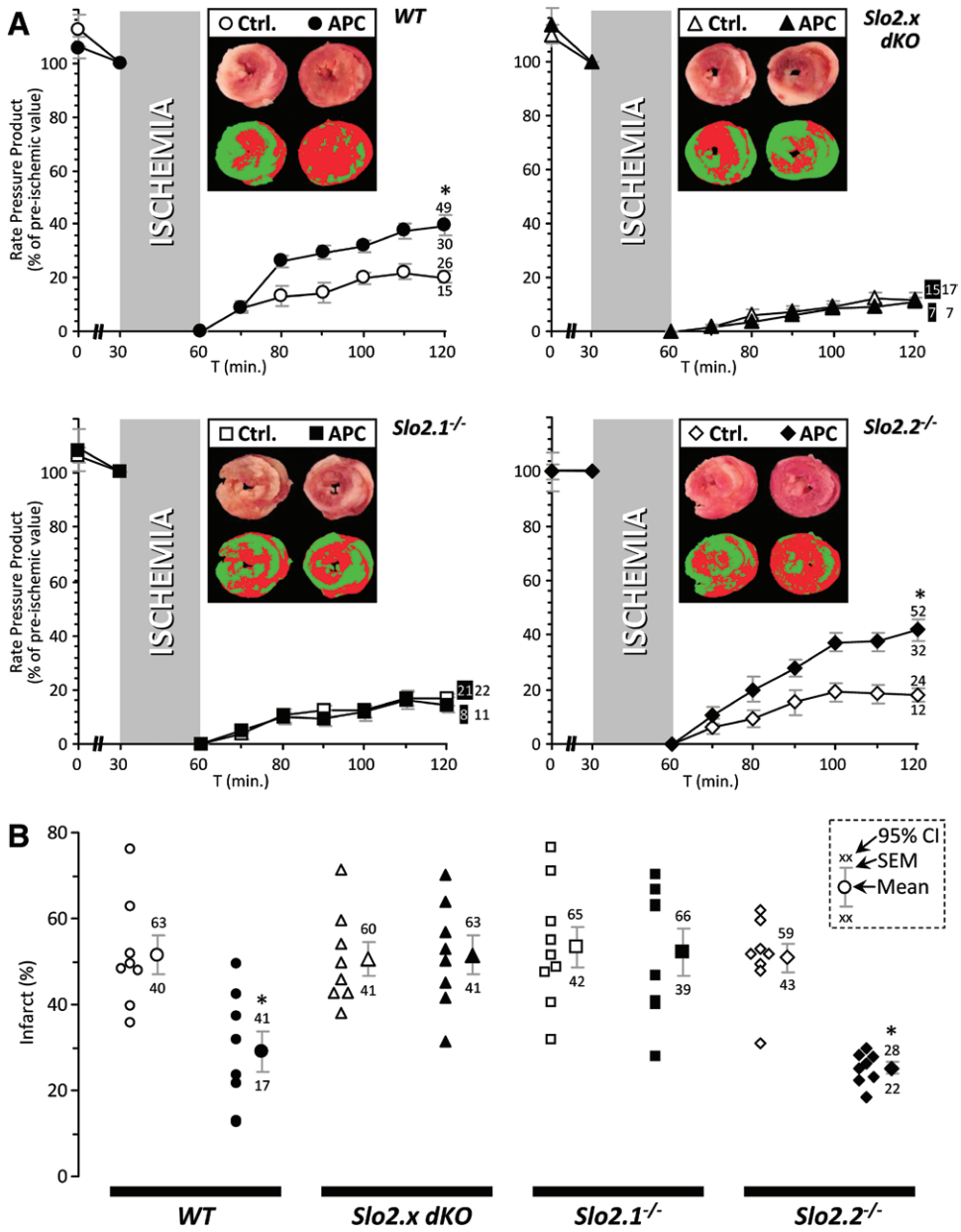

Fig. 3. Anesthetic preconditioning (APC) in wild-type (WT) and *Slo2.x* knockout mouse hearts. Langendorff-perfused hearts from *WT* (*circles*), *Slo2.x* double knockouts (*dKO*) (*triangles*), *Slo2.1*$^{-/-}$ (*squares*), and *Slo2.2*$^{-/-}$ (*diamonds*) were subjected to ischemia–reperfusion (IR) injury alone (*open symbols*) or IR with APC comprising 100 μM isoflurane infusion for 10 min before ischemia (*filled symbols*). (*A*) Cardiac function data (rate pressure product [RPP], product of heart rate × left ventricular developed pressure) throughout perfusion. RPP is expressed as a percentage of the value immediately before ischemia. Data are means ± SEM, with 95% CIs for the 60-min reperfusion time point shown adjacent to *error bars* (*numbers shaded* where appropriate to indicate which data set they belong to—see *inset* to *B*). *Insets*: representative heart cross-sections stained with 2,3,5-triphenyltetrazolium chloride (*upper images*) and threshold pseudo-colored images used to quantify infarct size (*lower images*). (*B*) Infarct size for control IR and APC + IR–treated hearts from each genotype. Within each group, individual data points are on the left, thereby indicating the number of replicates (N). Means ± SEM are on the right, with 95% CIs shown adjacent. *Inset key* shows position of mean, SEM, and 95% CIs on the graphs. *Statistically significant difference between control IR and APC + IR groups at 60 min of reperfusion (ANOVA, with *post hoc* unpaired *t* test).

antiviral.[37] Its use here was motivated by its ability to inhibit Slo2 channels,[33] which we validated using recombinant Slick expressed in HEK293 cells (fig. S1, Supplemental Digital Content 1, http://links.lww.com/ALN/B256). Notably, VA stimulation of K^+ flux was absent in both cardiomyocytes and mitochondria from *Slo2.1*$^{-/-}$ hearts (fig. 6, A and B). At the mitochondrial level, the Tl^+ flux pattern in *Slo2.2*$^{-/-}$ was similar to WT, and the pattern in *dKO* was similar to *Slo2.1*$^{-/-}$. These data indicate that isoflurane induces a *Slo2.1*-dependent K^+ flux in both cardiomyocytes and mitochondria.

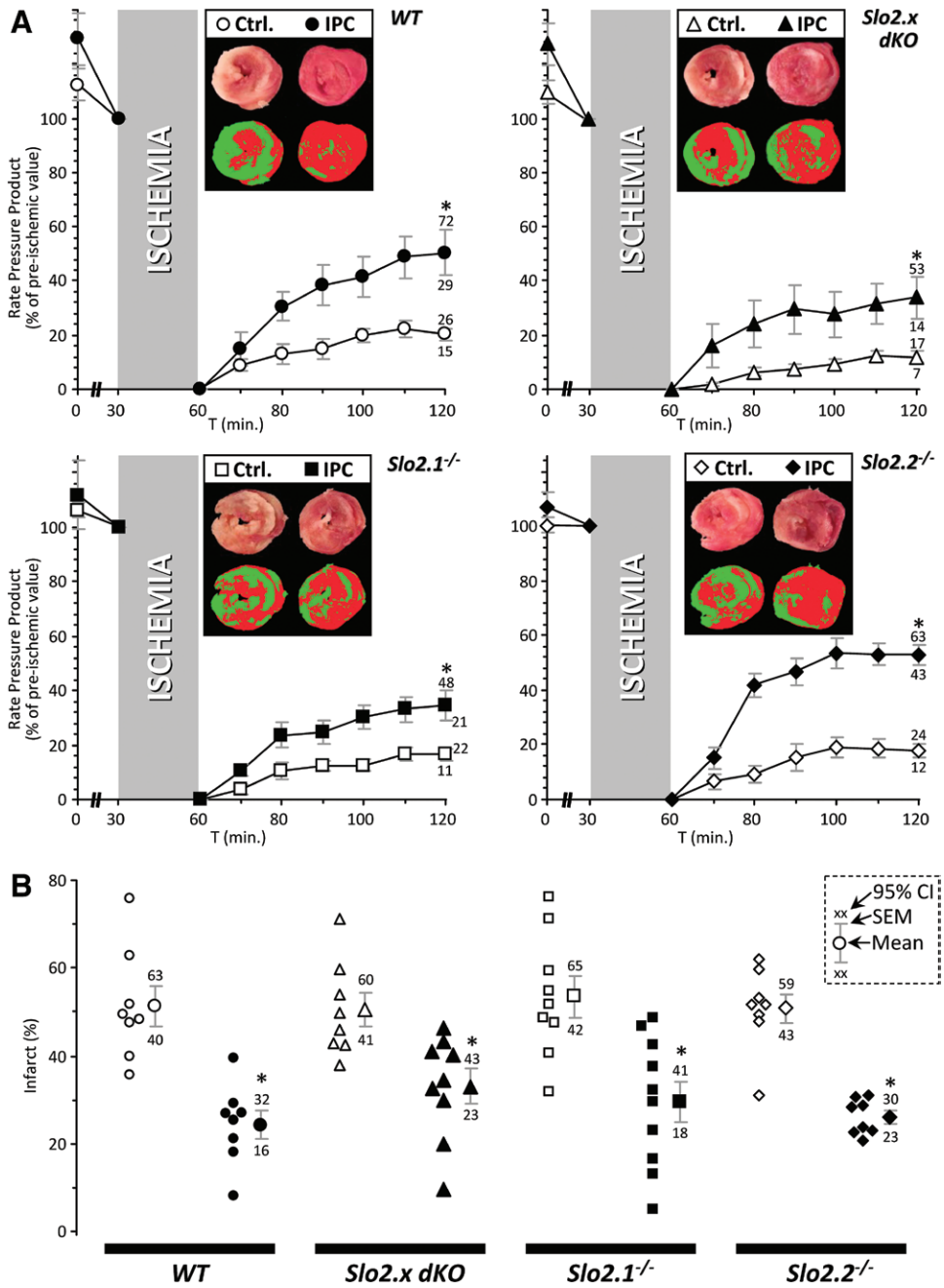

Fig. 4. Cardioprotection by ischemic preconditioning (IPC) in wild-type (WT) and *Slo2.x* knockout mouse hearts. Langendorff-perfused hearts were subjected to control ischemia–reperfusion (IR) injury alone (*open symbols*) or IR + IPC (*filled symbols*). *Symbols* for genotypes are the same as in figure 3. Data for controls (IR alone, no treatment) are reproduced from figure 3 and shown for comparative purposes. (*A*) Cardiac function data (rate pressure product [RPP], product of heart rate × left ventricular developed pressure) throughout perfusion. RPP is expressed as a percentage of the value immediately before ischemia. Data are means ± SEM, with 95% CIs for the 60-min reperfusion time point shown adjacent to *error bars* (see *inset* to *B*). *Insets*: representative heart cross-sections stained with 2,3,5-triphenyltetrazolium chloride (*upper images*) and threshold pseudo-colored images used to quantify infarct size (*lower images*). (*B*) Infarct size for control IR and IPC + IR–treated hearts from each genotype. Within each group, individual data on the left, thereby indicating the number of replicates (N). Means ± SEM are on the right, with 95% CIs shown adjacent. *Inset* key shows position of mean, SEM, and 95% CIs on the graphs. *Statistically significant difference between control IR and IPC + IR groups at 60 min of reperfusion (ANOVA, with *post hoc* unpaired *t* test). dKO = double knockout.

To substantiate the hypothesis that K^+ flux and cardioprotection occur through Slick activation, we turned to a second distinct reagent. Bithionol has been shown to block Slo-type channels[33] and inhibits recombinant Slick expressed in HEK293 cells (fig. S1, Supplemental Digital Content 1, http://links.lww.com/ALN/B256). At the cardiomyocyte

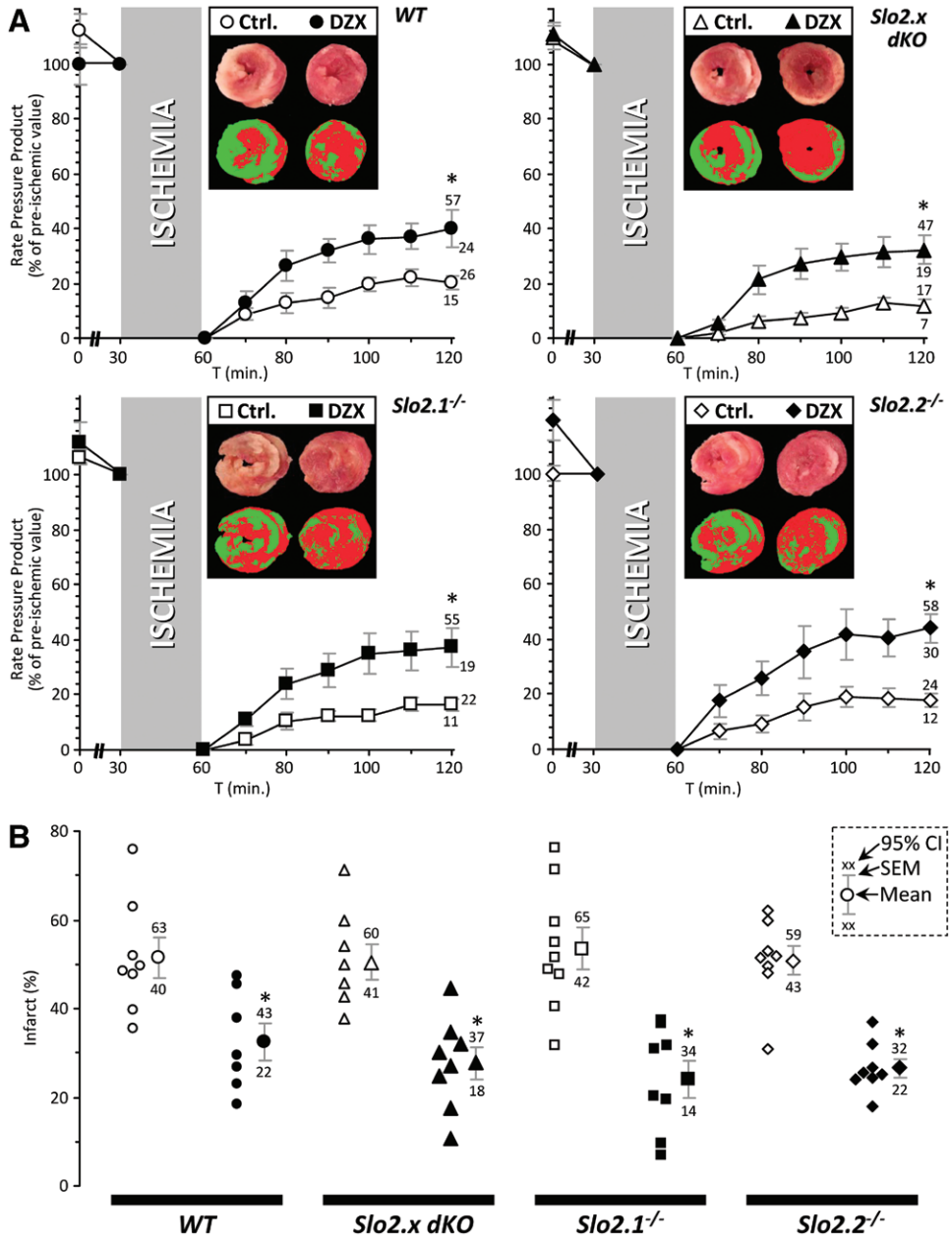

Fig. 5. Cardioprotection by diazoxide (DZX) in wild-type (WT) and Slo2.x knockout mouse hearts. Langendorff-perfused hearts were subjected to control ischemia–reperfusion (IR) injury alone (*open symbols*) or DZX + IR (*filled symbols*). Symbols for genotypes are the same as in figure 3. Data for controls (IR alone, no treatment) are reproduced from figure 3 and are shown for comparative purposes. (*A*) Cardiac function data (rate pressure product [RPP], product of heart rate × left ventricular developed pressure) throughout perfusion. RPP is expressed as a percentage of the value immediately before ischemia. Data are means ± SEM, with 95% CIs for the 60-min reperfusion time point shown adjacent to *error bars* (see *inset* to *B*). *Insets*: representative heart cross-sections stained with 2,3,5-triphenyltetrazolium chloride (*upper images*) and resulting pseudocolored images calculated *via* threshold masks and used to quantify infarct size (*lower images*). (*B*) Infarct size for control IR and DZX + IR–treated hearts from each genotype. Within each group, individual data are shown on the left, thereby indicating the number of replicates (N). Means ± SEM are on the right, with 95% CIs shown adjacent. *Inset key* shows position of mean, SEM, and 95% CIs on the graphs. *Statistically significant difference between control IR and DZX + IR groups (ANOVA, with *post hoc* unpaired *t* test). dKO = double knockout.

level, bithionol induced a bepridil-sensitive K^+ flux in WT cells that was absent in *Slo2.1*$^{-/-}$ cells (fig. 6C). In mitochondria, bithionol induced a bepridil-sensitive K^+ flux in WT that was absent in the *dKO* mitochondria and reduced in both the *Slo2.1*$^{-/-}$ and *Slo2.2*$^{-/-}$ mitochondria (fig. 6D). The cell data fully support our hypothesis. However, the mitochondria data suggest that both Slick and Slack partially contribute to bithionol-stimulated Tl^+ uptake, which is potentially

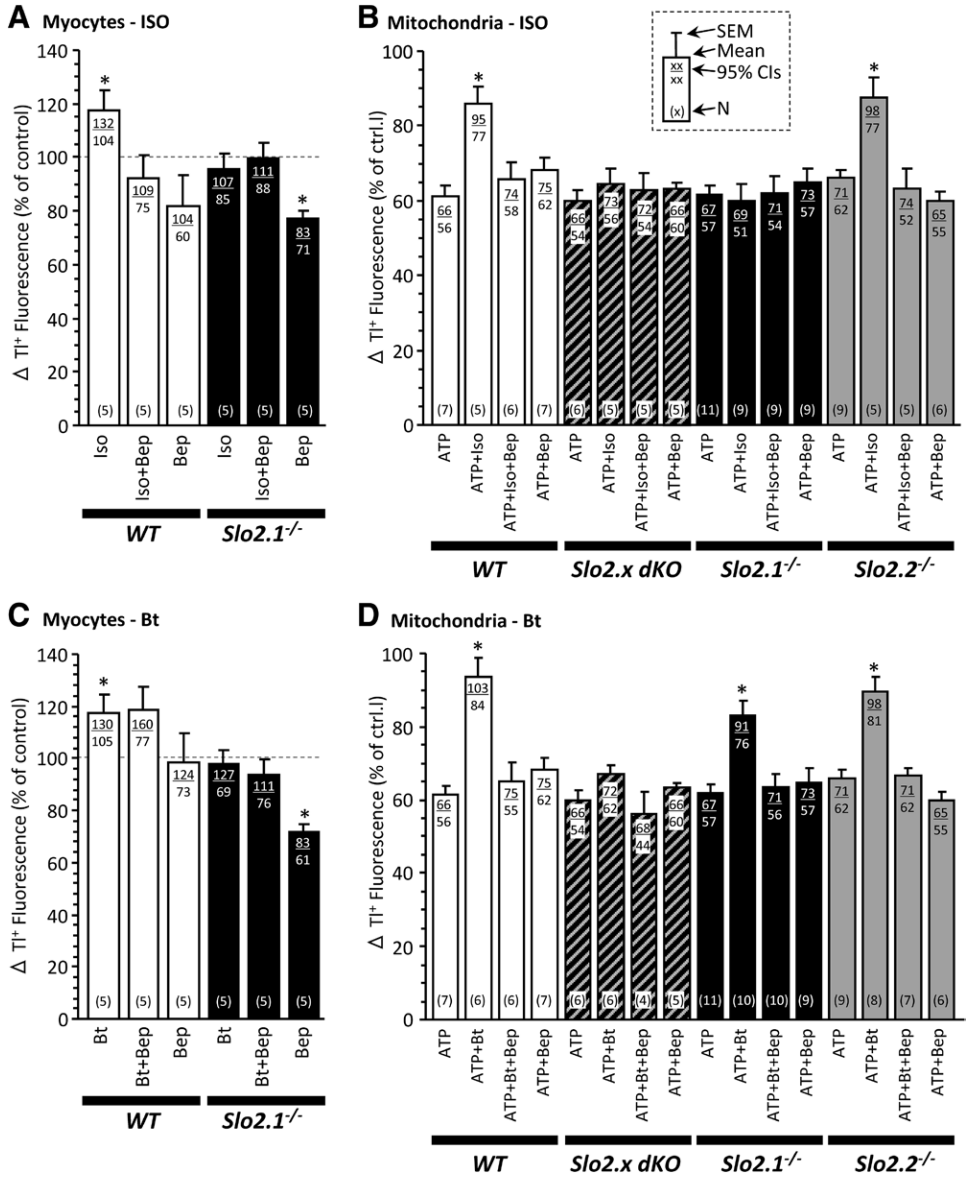

Fig. 6. Thallium (Tl^+) flux in cardiomyocytes and mitochondria from mice of varying Slo2 genotype. (*A*) Cardiomyocytes were isolated from wild-type (WT) (*white bars*) and *Slo2.1*$^{-/-}$ (*black bars*) mice, and surface K^+ channel activity assayed *via* thallium (Tl^+) uptake. Where indicated, isoflurane (Iso, 150 μM initial) and/or bepridil (Bep, 10 μM) were added. Data are normalized to control (no addition, *dashed line*) and are means ± SEM. 95% CIs are shown at top, number of replicates (N) at bottom, of each *graph bar*—see *inset key* in *B*. *Statistically significant difference *versus* control within a given genotype (ANOVA, with *post hoc* unpaired *t* test). (*B*) Mitochondria were isolated from WT (*white bars*), *Slo2.x* double knockout (*dKO*) (*hashed*), *Slo2.1*$^{-/-}$ (*black*), and *Slo2.2*$^{-/-}$ (*gray*) hearts, and K^+ channel activity assayed *via* Tl^+ uptake. Adenosine triphosphate (ATP) was present throughout to block mK_{ATP} channels. Where indicated, isoflurane (Iso, 300 μM initial) and/or bepridil (Bep, 10 μM) were added. Data are normalized to control (no addition) and are means ± SEM. 95% CIs are shown at *top*, number of replicates (N) at *bottom*, of each graph bar—see *inset key*. *Statistically significant difference *versus* ATP condition within a given genotype (ANOVA, with *post hoc* unpaired *t* test). (*C*) Cardiomyocyte Tl^+ flux, as in *A*, but stimulated with bithionol (Bt, 0.25 μM) instead of isoflurane. Data are normalized to control (no addition, *dashed line*) and are means ± SEM. 95% CIs and N as per *inset key* in *B*. *Statistically significant difference *versus* control within a given genotype (ANOVA, with *post hoc* unpaired *t* test). (*D*) Mitochondrial Tl^+ flux as in *B* but stimulated with bithionol (Bt, 2.5 μM) instead of isoflurane. Data are normalized to control (no addition) and are means ± SEM. 95% CIs and N as per *inset key* in *B*. *Statistically significant difference *versus* ATP condition within a given genotype (ANOVA, with *post hoc* unpaired *t* test). DZX = diazoxide.

interesting (see Discussion). However, it is likely that bithionol stimulates other channel activities or even mitochondrial function through unknown mechanisms of action, and very low levels of *Slo2.2* are expressed in the heart.[28]

Finally, additional support for our hypothesis came from the observation that bithionol was cardioprotective in WT hearts (fig. 7, A and B: 129% improvement in RPP recovery, $P = 0.0098$, 57% reduction in infarct size, $P = 0.00008$,

compared with IR alone). This protection was partially lost in *Slo2.1*$^{-/-}$ hearts (bithionol induced 97% improvement in RPP recovery, $P = 0.028$, 14% reduction in infarct size, $P = 0.15$). Overall, these data support a K^+ transport pathway with the genetic and pharmacologic characteristics of Slick, being required for the cardioprotective effects of APC.

Discussion

Herein, we demonstrated that the K_{Na} channel Slick, encoded by the *Slo2.1* gene, is required for cardioprotection by APC in mice. We also showed that Slick is responsible for VA-stimulated K^+ flux at both the cardiomyocyte plasma and mitochondrial membranes and that pharmacologic Slick openers are cardioprotective.

Although SLO-2 is a K_{Ca} channel in *C. elegans*, in mammals it has diverged into two paralogs, Slick and Slack, which are K_{Na} channels.[24,27] These channels may contribute to various physiologic functions attributed to K_{Na} channels, including metabolic sensing and neuronal plasticity,[24,27,38] and although the major focus of K_{Na} channel studies to date has been neuronal, K_{Na} channels are found in cardiac myocytes.[39] Notably, they have not been reported in mitochondria. Although these results cement a cardioprotective role for Slick, the relative contribution of plasma membrane *versus* mitochondrial Slick channels to this effect remains unclear and is a topic of ongoing research.

Many signaling pathways such as APC and IPC are evolutionarily conserved,[22] and there are many redundancies in their signaling cascades. For example, the mechanism of APC and IPC share commonalities such as protein kinase C activation,[40] ROS generation,[14,41–43] and mitochondrial K^+ channels.[44–46] Although the precise mechanism of APC is not well understood, evidence suggests that APC involves both altered mitochondrial function and K^+ channel activity (reviewed in the study by Agarwal *et al.*[47]).

Studies have implicated the mitochondrial ATP-sensitive K^+ channel (mK_{ATP}) in APC largely through the use of nonspecific measures of channel activity (*e.g.*, flavoprotein fluorescence) and drugs that are known to have off-target mitochondrial effects (*e.g.*, diazoxide or 5-hydroxydecanoate).[44–46,48,49] Although these pharmacologic approaches suggest the involvement of the mK_{ATP}, the effects could have also been due to another K^+ channel such as Slick. The current lack of a molecular identity for the mK_{ATP} channel precludes a definitive answer to this dilemma.

Moreover, the data presented herein do not rule out a potential cross talk between the mK_{ATP} and Slick. For example, because both diazoxide- and IPC-mediated cardioprotection were preserved in *Slo2.1*$^{-/-}$ mice, Slick activation may be upstream of mK_{ATP} channel activation. Overall, although there are many similarities between APC and IPC, there is also evidence for distinct signaling pathways,[50] and herein we show that Slick appears to be involved only in APC, not in IPC.

In the field of APC, the canonical BK channel Slo1, which may be present in cardiac mitochondria,[51] emerged as an early candidate effector of APC protection.[7,23] However, the importance of this channel was recently questioned by the evidence that both IPC and APC protection are robust in *Slo1*-ablated organisms.[22] Although the role of Slo1 in the heart remains to be fully elucidated, it is important to emphasize that our data do not preclude coexistence of Slo1 and Slick in cardiomyocytes or cardiac mitochondria. Rather, they conclude that only Slick is required for APC in the heart.

Our results are consistent with the findings in *C. elegans*, which showed that SLO-2 was required for APC.[22] Despite the large sequence similarity (approximately 74% identical)[38] between Slick and Slack, we found no role for Slack in cardiac APC or IPC. This is in agreement with the low expression levels of *Slo2.2* in the heart.[27] However, this does not rule out a role for Slack in protecting other tissues from ischemia. Both *Slo2.1* and *Slo2.2* are highly expressed in the nervous system,[24] and there is evidence that Slick and Slack may coassemble into channels.[52] In this respect, it is intriguing that both Slick and Slack appeared to contribute to bithionol-induced K^+ flux in purified mitochondria (fig. 6D). However, this was the only condition where both Slick and Slack were additive in our hands. Moreover, the mechanism of action of bithionol is unknown, but its activity as an antihelminthic may rely on suppressing succinate oxidation.[53] Hence, it is premature to conclude that Slack contributes to mitochondrial function. Nevertheless, it is possible that in other tissues such as the brain, *Slo2.2* may play a role protecting against stress.

It is also interesting to speculate that Slick did not evolve to be opened by VAs, and given our results, we speculate that it may have a physiologic role in the mitochondrion. Although some mitochondrial K^+ channels are demonstrated to play roles in pathophysiology (*e.g.*, protection from IR injury),[19] most are only reported at the phenomenological level as mitochondrial swelling or K^+ flux sensitive to activators/inhibitors, with very little known about the normal function of these channels. The activation of mitochondrial K^+ channels is hypothesized to modulate mitochondrial matrix volume, Ca^{2+} uptake capacity, and ROS production,[54] and although these events are involved in the pathophysiology of IR injury, they also have effects on regular mitochondrial function and metabolism. For example, Ca^{2+} stimulates oxidative phosphorylation, and matrix volume can determine the efficiency of high-energy phosphate transport between the mitochondrion and cytosol. Ongoing research in our laboratory is aimed at determining the role of Slick in regulating physiologic mitochondrial function.

A potential limitation of this study is the low dose of isoflurane (approximately 0.35 MAC) used to elicit cardioprotection. It is unclear if higher doses, or other VAs (*e.g.*, desflurane, sevoflurane), will exhibit similar *Slo2.1*-dependent cardioprotective effects. Nevertheless, we note that the current widespread clinical use of VAs, and the ACC/AHA recommendations for their use during cardiac surgery,[5] suggests that the identification of Slick as a mediator of APC may drive development of novel cardioprotective drugs targeting this channel.

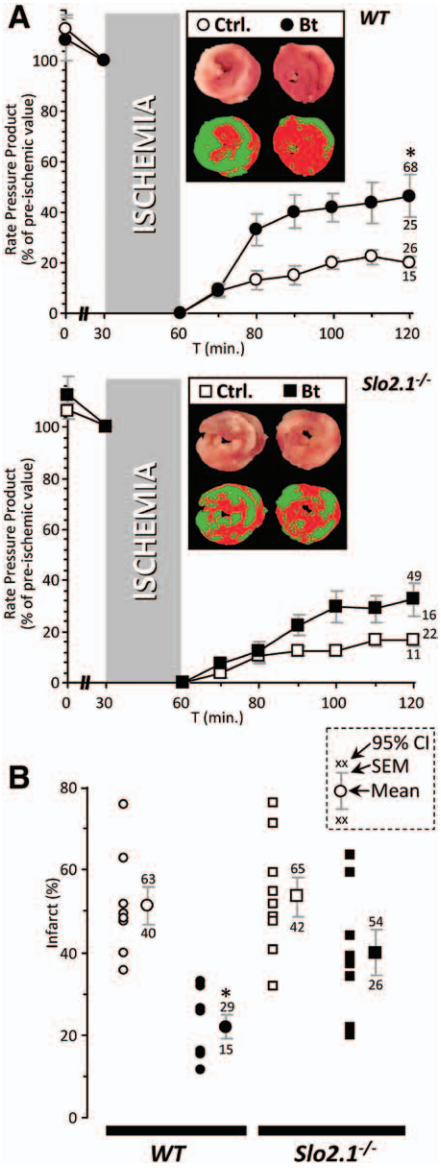

Fig. 7. Effect of bithionol on ischemia–reperfusion (IR) injury outcomes in wild-type (WT) and *Slo2.1* knockout mouse hearts. (*A*) Langendorff-perfused hearts from *WT* (*circles*) and *Slo2.1*$^{-/-}$ (*squares*) were subjected to IR injury alone (*open symbols*) or IR with bithionol (Bt, 2.5 nM for 20 min before the onset of ischemia, *filled symbols*), as detailed in the Materials and Methods and in figures 3 to 5. The control groups are reproduced from figure 3 and are shown here for comparative purposes. RPP is expressed as a percentage of the value immediately before ischemia. Data are means ± SEM, with 95% CIs for the 60-min reperfusion time point shown adjacent to *error bars* (see *inset*, *B*). *Insets*: representative heart cross-sections stained with 2,3,5-triphenyltetrazolium chloride (*upper images*) and resulting pseudo-colored images calculated *via* threshold masks and used to quantify infarct size (*lower images*). (*B*) Infarct size for control IR and Bt + IR–treated hearts from each genotype. Within each group, individual data points are shown on the left, thereby indicating the number of replicates (N). *Inset key* shows position of mean, SEM, and 95% CIs on the graphs. *Statistically significant difference between control IR and Bt + IR groups at 60 min of reperfusion (ANOVA, with *post hoc* unpaired *t* test).

Acknowledgments

The authors thank Chris Lingle, Ph.D. (Department of Anesthesiology, Washington University School of Medicine, St. Louis, Missouri), for providing *Slo2.x* knockout mice.

Supported by the National Institutes of Health (Bethesda, Maryland; grant no. R01-GM087483 to Drs. Brookes and Nehrke).

Competing Interests

The authors declare no competing interests.

Correspondence

Address correspondence to Dr. Nehrke & Brookes: University of Rochester Medical Center, 601 Elmwood Avenue, Rochester, New York 14642. keith_nehrke@urmc.rochester.edu; paul_brookes@urmc.rochester.edu. Information on purchasing reprints may be found at www.anesthesiology.org or on the masthead page at the beginning of this issue. Anesthesiology's articles are made freely accessible to all readers, for personal use only, 6 months from the cover date of the issue.

References

1. Cope DK, Impastato WK, Cohen MV, Downey JM: Volatile anesthetics protect the ischemic rabbit myocardium from infarction. Anesthesiology 1997; 86:699–709
2. Stadnicka A, Marinovic J, Ljubkovic M, Bienengraeber MW, Bosnjak ZJ: Volatile anesthetic-induced cardiac preconditioning. J Anesth 2007; 21:212–9
3. Landoni G, Biondi-Zoccai GG, Zangrillo A, Bignami E, D'Avolio S, Marchetti C, Calabrò MG, Fochi O, Guarracino F, Tritapepe L, De Hert S, Torri G: Desflurane and sevoflurane in cardiac surgery: A meta-analysis of randomized clinical trials. J Cardiothorac Vasc Anesth 2007; 21:502–11
4. Jia B, Crowder CM: Volatile anesthetic preconditioning present in the invertebrate *Caenorhabditis elegans*. Anesthesiology 2008; 108:426–33
5. Hillis LD, Smith PK, Anderson JL, Bittl JA, Bridges CR, Byrne JG, Cigarroa JE, Disesa VJ, Hiratzka LF, Hutter AM, Jr, Jessen ME, Keeley EC, Lahey SJ, Lange RA, London MJ, Mack MJ, Patel MR, Puskas JD, Sabik JF, Selnes O, Shahian DM, Trost JC, Winniford MD: 2011 ACCF/AHA Guideline for Coronary Artery Bypass Graft Surgery. A report of the American College of Cardiology Foundation/American Heart Association Task Force on Practice Guidelines. Developed in collaboration with the American Association for Thoracic Surgery, Society of Cardiovascular Anesthesiologists, and Society of Thoracic Surgeons. J Am Coll Cardiol 2011; 58:e123–e210
6. Ludwig LM, Weihrauch D, Kersten JR, Pagel PS, Warltier DC: Protein kinase C translocation and Src protein tyrosine kinase activation mediate isoflurane-induced preconditioning *in vivo*: Potential downstream targets of mitochondrial adenosine triphosphate-sensitive potassium channels and reactive oxygen species. Anesthesiology 2004; 100:532–9
7. Redel A, Lange M, Jazbutyte V, Lotz C, Smul TM, Roewer N, Kehl F: Activation of mitochondrial large-conductance calcium-activated K^+ channels *via* protein kinase A mediates desflurane-induced preconditioning. Anesth Analg 2008; 106:384–91
8. Yang C, Talukder MA, Varadharaj S, Velayutham M, Zweier JL: Early ischaemic preconditioning requires Akt- and PKA-mediated activation of eNOS *via* serine1176 phosphorylation. Cardiovasc Res 2013; 97:33–43
9. Toller WG, Kersten JR, Gross ER, Pagel PS, Warltier DC: Isoflurane preconditions myocardium against infarction *via* activation of inhibitory guanine nucleotide binding proteins. Anesthesiology 2000; 92:1400–7

10. Kersten JR, Orth KG, Pagel PS, Mei DA, Gross GJ, Warltier DC: Role of adenosine in isoflurane-induced cardioprotection. Anesthesiology 1997; 86:1128–39
11. Ishida T, Yarimizu K, Gute DC, Korthuis RJ: Mechanisms of ischemic preconditioning. Shock 1997; 8:86–94
12. Novalija E, Fujita S, Kampine JP, Stowe DF: Sevoflurane mimics ischemic preconditioning effects on coronary flow and nitric oxide release in isolated hearts. Anesthesiology 1999; 91:701–12
13. Vanden Hoek TL, Becker LB, Shao Z, Li C, Schumacker PT: Reactive oxygen species released from mitochondria during brief hypoxia induce preconditioning in cardiomyocytes. J Biol Chem 1998; 273:18092–8
14. Müllenheim J, Ebel D, Frässdorf J, Preckel B, Thämer V, Schlack W: Isoflurane preconditions myocardium against infarction *via* release of free radicals. Anesthesiology 2002; 96:934–40
15. Garlid KD, Halestrap AP: The mitochondrial K(ATP) channel—Fact or fiction? J Mol Cell Cardiol 2012; 52:578–83
16. Szabò I, Bock J, Jekle A, Soddemann M, Adams C, Lang F, Zoratti M, Gulbins E: A novel potassium channel in lymphocyte mitochondria. J Biol Chem 2005; 280:12790–8
17. Xu W, Liu Y, Wang S, McDonald T, Van Eyk JE, Sidor A, O'Rourke B: Cytoprotective role of Ca^{2+}-activated K^+ channels in the cardiac inner mitochondrial membrane. Science 2002; 298:1029–33
18. Shi GY, Jung DW, Garlid KD, Brierley GP: Induction of respiration-dependent net efflux of K^+ from heart mitochondria by depletion of endogenous divalent cations. J Biol Chem 1980; 255:10306–11
19. Szabo I, Zoratti M: Mitochondrial channels: Ion fluxes and more. Physiol Rev 2014; 94:519–608
20. Szewczyk A, Jarmuszkiewicz W, Kunz WS: Mitochondrial potassium channels. IUBMB Life 2009; 61:134–43
21. Kohro S, Hogan QH, Nakae Y, Yamakage M, Bosnjak ZJ: Anesthetic effects on mitochondrial ATP-sensitive K channel. Anesthesiology 2001; 95:1435–340
22. Wojtovich AP, Sherman TA, Nadtochiy SM, Urciuoli WR, Brookes PS, Nehrke K: SLO-2 is cytoprotective and contributes to mitochondrial potassium transport. PLoS One 2011; 6:e28287
23. Stumpner J, Lange M, Beck A, Smul TM, Lotz CA, Kehl F, Roewer N, Redel A: Desflurane-induced post-conditioning against myocardial infarction is mediated by calcium-activated potassium channels: Role of the mitochondrial permeability transition pore. Br J Anaesth 2012; 108:594–601
24. Salkoff L, Butler A, Ferreira G, Santi C, Wei A: High-conductance potassium channels of the SLO family. Nat Rev Neurosci 2006; 7:921–31
25. Schreiber M, Wei A, Yuan A, Gaut J, Saito M, Salkoff L: Slo3, a novel pH-sensitive K+ channel from mammalian spermatocytes. J Biol Chem 1998; 273:3509–16
26. Wojtovich AP, Nadtochiy SM, Urciuoli WR, Smith CO, Grunnet M, Nehrke K, Brookes PS: A non-cardiomyocyte autonomous mechanism of cardioprotection involving the SLO1 BK channel. PeerJ 2013; 1:e48
27. Bhattacharjee A, Joiner WJ, Wu M, Yang Y, Sigworth FJ, Kaczmarek LK: Slick (Slo2.1), a rapidly-gating sodium-activated potassium channel inhibited by ATP. J Neurosci 2003; 23:11681–91
28. Martinez-Espinosa PL, Wu J, Yang C, Gonzalez-Perez V, Zhou H, Liang H, Xia XM, Lingle CJ: Knockout of Slo2.2 enhances itch, abolishes KNa current, and increases action potential firing frequency in DRG neurons. Elife 2015; 4
29. Wojtovich AP, Urciuoli WR, Chatterjee S, Fisher AB, Nehrke K, Brookes PS: Kir6.2 is not the mitochondrial KATP channel but is required for cardioprotection by ischemic preconditioning. Am J Physiol Heart Circ Physiol 2013; 304:H1439–45
30. Sonner JM, Gong D, Li J, Eger EI II, Laster MJ: Mouse strain modestly influences minimum alveolar anesthetic concentration and convulsivity of inhaled compounds. Anesth Analg 1999; 89:1030–4
31. Franks NP, Lieb WR: Temperature dependence of the potency of volatile general anesthetics: Implications for *in vitro* experiments. Anesthesiology 1996; 84:716–20
32. Garlid KD, Paucek P, Yarov-Yarovoy V, Murray HN, Darbenzio RB, D'Alonzo AJ, Lodge NJ, Smith MA, Grover GJ: Cardioprotective effect of diazoxide and its interaction with mitochondrial ATP-sensitive K^+ channels. Possible mechanism of cardioprotection. Circ Res 1997; 81:1072–82
33. Yang B, Gribkoff VK, Pan J, Damagnez V, Dworetzky SI, Boissard CG, Bhattacharjee A, Yan Y, Sigworth FJ, Kaczmarek LK: Pharmacological activation and inhibition of Slack (Slo2.2) channels. Neuropharmacology 2006; 51:896–906
34. Wojtovich AP, Williams DM, Karcz MK, Lopes CM, Gray DA, Nehrke KW, Brookes PS: A novel mitochondrial K(ATP) channel assay. Circ Res 2010; 106:1190–6
35. Mori K, Saito T, Masuda Y, Nakaya H: Effects of class III antiarrhythmic drugs on the Na(+)-activated K^+ channels in guinea-pig ventricular cells. Br J Pharmacol 1996; 119:133–41
36. Yatani A, Brown AM, Schwartz A: Bepridil block of cardiac calcium and sodium channels. J Pharmacol Exp Ther 1986; 237:9–17
37. Johansen LM, DeWald LE, Shoemaker CJ, Hoffstrom BG, Lear-Rooney CM, Stossel A, Nelson E, Delos SE, Simmons JA, Grenier JM, Pierce LT, Pajouhesh H, Lehár J, Hensley LE, Glass PJ, White JM, Olinger GG: A screen of approved drugs and molecular probes identifies therapeutics with anti-Ebola virus activity. Sci Transl Med 2015; 7:290ra89
38. Kaczmarek LK: Slack, slick and sodium-activated potassium channels. ISRN Neurosci 2013; 2013:pii: 354262
39. Kameyama M, Kakei M, Sato R, Shibasaki T, Matsuda H, Irisawa H: Intracellular Na^+ activates a K^+ channel in mammalian cardiac cells. Nature 1984; 309:354–6
40. Novalija E, Kevin LG, Camara AK, Bosnjak ZJ, Kampine JP, Stowe DF: Reactive oxygen species precede the epsilon isoform of protein kinase C in the anesthetic preconditioning signaling cascade. Anesthesiology 2003; 99:421–8
41. Kevin LG, Novalija E, Riess ML, Camara AK, Rhodes SS, Stowe DF: Sevoflurane exposure generates superoxide but leads to decreased superoxide during ischemia and reperfusion in isolated hearts. Anesth Analg 2003; 96:949–55
42. Novalija E, Kevin LG, Eells JT, Henry MM, Stowe DF: Anesthetic preconditioning improves adenosine triphosphate synthesis and reduces reactive oxygen species formation in mitochondria after ischemia by a redox dependent mechanism. Anesthesiology 2003; 98:1155–63
43. Tanaka K, Weihrauch D, Kehl F, Ludwig LM, LaDisa JF Jr, Kersten JR, Pagel PS, Warltier DC: Mechanism of preconditioning by isoflurane in rabbits: A direct role for reactive oxygen species. Anesthesiology 2002; 97:1485–90
44. Kersten JR, Schmeling TJ, Pagel PS, Gross GJ, Warltier DC: Isoflurane mimics ischemic preconditioning *via* activation of K(ATP) channels: Reduction of myocardial infarct size with an acute memory phase. Anesthesiology 1997; 87:361–70
45. Tanaka K, Weihrauch D, Ludwig LM, Kersten JR, Pagel PS, Warltier DC: Mitochondrial adenosine triphosphate-regulated potassium channel opening acts as a trigger for isoflurane-induced preconditioning by generating reactive oxygen species. Anesthesiology 2003; 98:935–43
46. Kapinya KJ, Löwl D, Fütterer C, Maurer M, Waschke KF, Isaev NK, Dirnagl U: Tolerance against ischemic neuronal injury can be induced by volatile anesthetics and is inducible NO synthase dependent. Stroke 2002; 33:1889–98
47. Agarwal B, Stowe DF, Dash RK, Bosnjak ZJ, Camara AK: Mitochondrial targets for volatile anesthetics against cardiac ischemia-reperfusion injury. Front Physiol 2014; 5:341
48. Hanley PJ, Mickel M, Löffler M, Brandt U, Daut J: K(ATP) channel-independent targets of diazoxide and

5-hydroxydecanoate in the heart. J Physiol 2002; 542(Pt 3):735–41

49. Bantel C, Maze M, Trapp S: Neuronal preconditioning by inhalational anesthetics: Evidence for the role of plasmalemmal adenosine triphosphate-sensitive potassium channels. Anesthesiology 2009; 110:986–95
50. Sergeev P, da Silva R, Lucchinetti E, Zaugg K, Pasch T, Schaub MC, Zaugg M: Trigger-dependent gene expression profiles in cardiac preconditioning: Evidence for distinct genetic programs in ischemic and anesthetic preconditioning. Anesthesiology 2004; 100:474–88
51. Singh H, Lu R, Bopassa JC, Meredith AL, Stefani E, Toro L: MitoBK(Ca) is encoded by the Kcnma1 gene, and a splicing sequence defines its mitochondrial location. Proc Natl Acad Sci U S A 2013; 110:10836–41
52. Chen H, Kronengold J, Yan Y, Gazula VR, Brown MR, Ma L, Ferreira G, Yang Y, Bhattacharjee A, Sigworth FJ, Salkoff L, Kaczmarek LK: The N-terminal domain of Slack determines the formation and trafficking of Slick/Slack heteromeric sodium-activated potassium channels. J Neurosci 2009; 29:5654–65
53. Hamajima F: Studies on metabolism of lung fluke genus Paragonimus. VII. Action of bithionol on glycolytic and oxidative metabolism of adult worms. Exp Parasitol 1973; 34:1–11
54. Facundo HT, Fornazari M, Kowaltowski AJ: Tissue protection mediated by mitochondrial K^+ channels. Biochim Biophys Acta 2006; 1762:202–12

ANESTHESIOLOGY REFLECTIONS FROM THE WOOD LIBRARY-MUSEUM

Blade Bearer to *The Court of Death*: Peale's Warrior … and Colton's Surgeon?

On the right third of *The Court of Death*, a painting by America's Rembrandt Peale (1778–1860), a closer view (*above*) of a lithographic copy reveals Famine (6) and then Pestilence (5) in the wake of the Conflagration (11) of War. In his 100,000 chromolithographs of that oil painting, nitrous oxide pioneer Gardner Q. Colton (1814–1898) publicized how such threats trampled not only the Victim (7) of War, but also the Widow (9) and the Orphan (8). Whether a sword-brandishing Warrior (10) or a scalpel-wielding surgeon or dentist, figures brandishing blades were associated by artist Peale with death and by Colton with the near-death to which he subjected his patients with laughing gas. Remarkably, despite smothering his patients with up to two minutes of 100% nitrous oxide while watching their faces turn blue and then gray from lack of oxygen, Colton recorded over 190,000 such anesthetics without one anesthetic death. (Copyright © the American Society of Anesthesiologists, Inc.)

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