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Histamine H_4-Receptors Inhibit Mast Cell Renin Release in Ischemia/Reperfusion via Protein Kinase Cε-Dependent Aldehyde Dehydrogenase Type-2 Activation

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ABSTRACT

Renin released by ischemia/reperfusion (I/R) from cardiac mast cells (MCs) activates a local renin-angiotensin system (RAS) causing arrhythmic dysfunction. Ischemic preconditioning (IPC) inhibits MC renin release and consequent activation of this local RAS. We postulated that MC histamine H_4-receptors (H_4Rs), being $G\alpha_{i/o}$-coupled, might activate a protein kinase C isotype–ε (PKCε)–aldehyde dehydrogenase type-2 (ALDH2) cascade, ultimately eliminating MC-degranulating and renin-releasing effects of aldehydes formed in I/R and associated arrhythmias. We tested this hypothesis in ex vivo hearts, human mastocytoma cells, and bone marrow–derived MCs from wild-type and H_4R knockout mice. We found that activation of MC H_4Rs mimics the cardioprotective anti-RAS effects of IPC and that protection depends on the sequential activation of PKCε and ALDH2 in MCs, reducing aldehyde-induced MC degranulation and renin release and alleviating reperfusion arrhythmias. These cardioprotective effects are mimicked by selective H_4R agonists and disappear when H_4Rs are pharmacologically blocked or genetically deleted. Our results uncover a novel cardioprotective pathway in I/R, whereby activation of H_4Rs on the MC membrane, possibly by MC-derived histamine, leads sequentially to PKCε and ALDH2 activation, reduction of toxic aldehyde-induced MC renin release, prevention of RAS activation, reduction of norepinephrine release, and ultimately to alleviation of reperfusion arrhythmias. This newly discovered protective pathway suggests that MC H_4Rs may represent a new pharmacologic and therapeutic target for the direct alleviation of RAS-induced cardiac dysfunctions, including ischemic heart disease and congestive heart failure.

Introduction

Activation of the renin-angiotensin system (RAS) in the heart (Dzau, 1987; Baker et al., 1992; Dostal and Baker, 1999), which culminates in the local formation of angiotensin II, sympathetic overactivity, and excessive norepinephrine (NE) release, is a common cause of arrhythmias in myocardial ischemia, congestive heart failure, and hypertension (Hirsch et al., 1990; Barlucchi et al., 2001; Varagic and Frohlich, 2002; Dilaveris et al., 2005). We showed that cardiac mast cells (MCs) are a critical source of renin (Silver et al., 2004). Released by ischemia/reperfusion (I/R), MC renin activates a local RAS (Mackins et al., 2006). Indeed, angiotensinogen and angiotensin-converting enzyme are present in cardiac interstitial fluid in concentrations sufficient to ultimately produce angiotensin II (Dell'italia et al., 1997; Dostal and Baker, 1999; Bader et al., 2001), which then acts on angiotensin II type-1 receptors on sympathetic nerve endings, promoting excessive NE release, thus causing severe arrhythmic dysfunction (Mackins et al., 2006).

We have recently reported that ischemic preconditioning (IPC) inhibits MC renin release and RAS activation during subsequent I/R (Koda et al., 2010). This action is not attributed to depletion of MC renin during preconditioning or to a hypothetical angiotensin II type-2 receptor–mediated cardioprotective effect. Rather, it involves a signaling cascade initiated by adenosine, which triggers a protein kinase C isotype-ε (PKCε)–mediated activation of mitochondrial aldehyde dehydrogenase type-2 (ALDH2) in cardiac MCs (Koda et al., 2010). Activated ALDH2 eliminates toxic aldehydes that accumulate in the ischemic heart (Eaton et al., 1999; Chen et al., 2008) and are known to degranulate MC (Koivisto et al., 1999; Kawano

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ABBREVIATIONS: 4MeH, 4-methylhistamine; A_{2b}R and A_3R, adenosine A_{2b}- and A_3-receptor; ALDH2, aldehyde dehydrogenase type-2; β-HEX, β-hexosaminidase; BMMC, bone marrow–derived mast cells; ECG, electrocardiogram; GTN, glyceryl trinitrate; HMC-1, a human mastocytoma cell line; H_4R, histamine H_4-receptor; $H_4R^{-/-}$, histamine H_4-receptor-deleted mice; IPC, ischemic preconditioning; I/R, ischemia/reperfusion; KH, Krebs-Henseleit; MAO, monoamine oxidase; MC, mast cells; NE, norepinephrine; PC12-H_3, a rat pheochromocytoma cell line transfected with the human H_3R; PKCε, protein kinase C isotype-ε; PMA, phorbol 12-myristate 13-acetate; RAS, renin-angiotensin system; VT/VF, ventricular tachycardia/ventricular fibrillation; WT, wild type.

et al., 2004), thus preventing renin release and its dysfunctional consequences (Koda et al., 2010).

Adenosine A_3-receptor (A_3R) activation on the MC surface appears to play an important role in this cardioprotective anti-RAS role of adenosine (Koda et al., 2010), which is produced by various cardiac cells during I/R (Headrick, 1996). Inasmuch as A_3Rs are coupled to the inhibitory G protein $G\alpha_{i/o}$ (Linden, 2001), which is known to promote the sequential activation of PKCε and ALDH2 (Koda et al., 2010), we questioned whether other $G\alpha_{i/o}$-coupled receptors might share in this cardioprotective anti-RAS effect. We focused our attention on histamine H_4-receptors (H_4Rs), because they are $G\alpha_{i/o}$ coupled (Nijmeijer et al., 2012); are expressed by hematopoietic cells, including MCs (Liu et al., 2001; Zhu et al., 2001); and could easily be activated in an autocrine mode by MC histamine, which we have shown to be released in I/R (Imamura et al., 1994; Hatta et al., 1997).

To test this hypothesis, we used both pharmacological and gene-deletion approaches in ex vivo hearts, human mastocytoma cells (HMC-1), and bone marrow–derived MCs (BMMCs) from wild-type (WT) and H_4R knockout ($H_4R^{-/-}$) mice. We report that activation of H_4Rs on the MC membrane mimics the cardioprotective anti-RAS effects of IPC, an effect that depends on the sequential activation of PKCε and ALDH2, culminating in the reduction of toxic aldehyde-induced MC degranulation and renin release and ultimately alleviation of reperfusion arrhythmias. These cardioprotective effects are lost when H_4Rs are pharmacologically blocked or genetically deleted.

Materials and Methods

Ex Vivo Guinea Pig Hearts. Hearts were isolated and perfused as previously described (Koda et al., 2010). In brief, guinea pigs (male Hartley, 300–350 g; Charles River Laboratories, Kingston, NY) were anesthetized with CO_2 and humanely killed by stunning while under anesthesia (Institutional Animal Care and Use Committee approved). Isolated hearts were perfused at constant pressure with oxygenated Ringer at 37°C in a Langendorff apparatus. After equilibration, all hearts were subjected to 20-minute global ischemia followed by 30-minute reperfusion. For IPC, hearts were subjected to 2 × 5-minute cycles of ischemia, each followed by 5-minute reperfusion. For the pharmacologic prevention of IPC, antagonists were perfused for 20 minutes with glyceryl trinitrate (GTN) for 30 minutes before and during IPC, and then washed out for 15 minutes before I/R. For pharmacologic preconditioning, given agents were perfused for 2 × 5-minute cycles and then washed out for 5 minutes before I/R. For prevention of pharmacologic preconditioning, antagonists were perfused for 20 minutes (GTN, 30 minutes) before and during pharmacologic preconditioning and then washed out for 15 minutes before I/R. Coronary flow was measured every 2 minutes; samples were assayed for renin and NE. Surface ECG was recorded and analyzed using Power Laboratory/8SP (ADInstruments; Colorado Springs, CO).

Ex Vivo Murine Hearts. Hearts were isolated from male C57BL/6 WT and $H_4R^{-/-}$ mice and perfused as previously described (Mackins et al., 2006). $H_4R^{-/-}$ mice were generated by Lexicon Genetics (Woodlands, TX) as previously described (Hofstra et al., 2003) and were provided by Janssen Research & Development, L.L.C. (San Diego, CA). In brief, mice were anesthetized with CO_2 vapor and humanely killed by cervical dislocation while under anesthesia (Institutional Animal Care and Use Committee approved). Hearts were quickly excised and cooled in ice-cold Krebs-Henseleit (KH) solution containing pyruvic acid (0.5 mM) and equilibrated with 95% O_2 + 5% CO_2. Hearts were then perfused in a Langendorff apparatus (Radnoti, Monrovia, CA) (pressure: 100 cm H_2O) with KH buffer. Two needle electrodes were attached to the surface of the right atrium and left ventricular apex for ECG recordings. ECG was recorded online (sample frequency of 1 kHz) and analyzed using Powerlab/8SP (ADInstruments). Coronary flow was measured by timed collections of the effluent. After stabilization, hearts were subjected to 30-minute ischemia (glucose- and pyruvic acid–free KH buffer, 95% N_2 + 5% CO_2, and sodium dithionite) followed by 30-minute reoxygenation (reperfusion) with KH buffer. Onset and duration of reperfusion arrhythmias were recorded and quantified. Some hearts were pretreated with the selective H_4R antagonist A943931 (Cowart et al., 2008) (300 nM, 20 minutes) and then subjected to IPC (i.e., 2 × 5-minute cycles of ischemia each followed by 5-minute perfusion with A943931), followed by a 15-minute drug-free washout before I/R. Other hearts were perfused with the selective H_4R agonist 4-methylhistamine (4MeH) (1 μM) (Lim et al., 2005) for 2 × 5-minute cycles, each followed by a 5-minute washout before I/R. Other hearts were perfused with the nonisotype selective PKC agonist phorbol 12-myristate 13-acetate (PMA) (LC Laboratories, Woburn, MA) (0.05 nM) (Koda et al., 2010) for 2 × 5-minute cycles, each followed by a 5-minute washout before I/R.

HMC-1 Culture. HMC-1 cells were maintained in suspension culture as previously described (Koda et al., 2010).

Western Blot. Total lysates from a rat pheochromocytoma cell line transfected with the human H_3R (PC12-H_3) and HMC-1 and BMMC were prepared with cell lysis buffer 1× (Cell Signaling Technology, Danvers, MA). A human erythroleukemia cell line (HEL 92.1.7) lysate was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Samples of PC12-H_3, HMC-1, BMMC, and HEL 92.1.7 for electrophoresis (40 μg per lane) and Western blot were prepared as previously described (Koda et al., 2010). Polyvinylidene fluoride membranes were probed with antihistamine receptor 3 (anti-H_3R) (Santa Cruz Biotechnology), anti-human histamine receptor 4 (anti-H_4R) (Alpha Diagnostic Intl. Inc.; San Antonio, TX), and anti-mouse H_4R (Abcam, Cambridge, MA) at a dilution of 1:1000. Anti-rabbit IgG horseradish peroxidase-linked secondary antibodies (Cell Signaling Technology, Beverly, MA) were used at a dilution of 1:3000 for both primary antibodies. Proteins of interest were detected using Immobilon Western chemiluminescent horseradish peroxidase substrate (EMD Millipore Corporation, Billerica, MA).

Norepinephrine Assay. NE overflow into the coronary effluent of ex vivo hearts was measured by high-performance liquid chromatography with electrochemical detection as previously described (Koda et al., 2010).

***β*-Hexosaminidase and Renin Assay.** β-Hexosaminidase (β-HEX) and renin release were measured as previously described (Koda et al., 2010). In brief, pooled confluent flasks of HMC-1 and 1×10^5 of BMMC per sample were washed and resuspended in Ringer buffer (pH 7.4). Same volumes of cells were measured in aliquots in Eppendorf tubes (HMC-1) or in 96-well plates (BMMC) and incubated with gentle oscillation at 37°C with the given agents (4MeH, A943931, $\varepsilon V_{1\text{-}2}$) for 10 minutes (preceded or not by a 30-minute incubation with GTN). Acetaldehyde (Sigma-Aldrich, St Louis, MO) was subsequently added to the cells for 20 minutes. At the end of the incubation, samples were placed in ice and centrifuged at 500*g* for 5 minutes. Supernatants were collected and used to measure the β-HEX content and renin release. For the renin assay, human and porcine angiotensinogen were used for HMC-1 and BMMC samples, respectively. Cell pellets were lysed with 0.5% Triton X-100, and total lysates were used to determine total β-HEX content and total protein concentration by Bio-Rad DC Protein Assay kit (Bio-Rad Laboratories, Hercules, CA). All results were normalized and expressed as percentage above control.

Translocation of PKCε. Following incubation of HMC-1 and BMMC with PMA, 4MeH, or A943931 for 10 minutes at 37°C, cytosolic and membrane fractions were prepared as previously described (Koda et al., 2010). Translocation of PKCε was determined by Western blot analysis using a PKCε antibody at a dilution of 1:1000 (Santa Cruz Biotechnology Inc.). Methods for Western blot analysis

were as previously described (Koda et al., 2010). PKCε translocation was expressed as the ratio between PKCε in the membrane and PKCε in the cytosol.

ALDH2 Enzymatic Activity Assay. Enzymatic activity of ALDH2 in HMC-1 cells and BMMCs was determined spectrophotometrically by monitoring the reductive reaction of NAD^+ to NADH at 340 nm as previously described (Koda et al., 2010). The assay was carried out at 25°C in 50 mM sodium pyrophosphate buffer, pH 9.0. To start the reaction, 10 mM acetaldehyde was added to 300 μg of HMC-1 cell lysate or 500 μg of BMMC total lysate and 2.5 mM NAD. NADH accumulation was recorded for 3 minutes with measurements taken every 15 seconds. ALDH2 reaction rates were calculated as micromoles of NADH per minute per milligram of proteins. HMC-1 or BMMCs were incubated with a given agent (Alda-1, 4MeH, A943931, εV_{1-2}, or PMA) for 10 minutes at 37°C (preceded or not by a 30-minute incubation with GTN). NADH production was expressed as percent increase from control.

Mast Cell Differentiation from Murine Bone Marrow. Murine bone marrow mast cells were obtained as previously described (Aldi et al., 2014). In brief, C57BL/6 WT and $H_4R^{-/-}$ mice were anesthetized with CO_2 vapor and killed by cervical dislocation while under anesthesia without awakening (Institutional Animal Care and Use Committee approved). Femurs and tibiae were removed, and bone marrow was flushed out. Bone marrow–derived cells were cultured in RPMI 1640 medium (Invitrogen Life Technologies, Carlsbad, CA) containing antibiotics (10 U/ml penicillin/streptomycin), 10% heat-inactivated fetal calf serum, 2-mercaptoethanol (55 μM), recombinant murine IL-3, and stem cell factor (both 20 ng/ml; PeproTech, Rocky Hill, NJ). Bone marrow–derived cells were counted and placed in culture at a density of 0.5×10^6 cells/ml. Cell medium was changed every 3 to 4 days, and nonadherent cells were transferred to a new culture well. Mature bone marrow–derived MCs were obtained after 4 or 5 weeks when >90% of cells were double positive for c-kit/CD117 and high-affinity IgE receptor (FcεRI) (Aldi et al., 2014).

Results

IPC Prevents the Activation of a Local Cardiac RAS and Alleviates Arrhythmic Dysfunction: Activation of H_4Rs Mimics the Cardioprotective Anti-RAS Effect of IPC. Spontaneously beating Langendorff-perfused guinea pig hearts were subjected to 20-minute global ischemia followed by 30-minute reperfusion (I/R). I/R caused large increases in renin and NE overflow (i.e., ~3- and ~200-fold, respectively), and severe ventricular arrhythmias: ventricular tachycardia/ventricular fibrillation (VT/VF) that lasted ~8 minutes (Fig. 1). We had previously shown that I/R results in MC degranulation, MC renin release, and activation of a local RAS, culminating in angiotensin II–induced NE release and arrhythmias (Mackins et al., 2006; Koda et al., 2010).

When I/R was preceded by IPC (i.e., 2 × 5-minute cycles of ischemia, each followed by 5-minute reperfusion), the overflow of renin and NE and the duration of VT/VF were greatly reduced (by ~60, 65, and 90%, respectively) (Fig. 1), clearly indicating a cardioprotective anti-RAS effect of IPC. It is noteworthy that the finding that IPC markedly attenuated the I/R-induced release of renin and NE was not to the result of depletion of renin and NE pools during preconditioning (Koda et al., 2010).

The cardioprotective effects of IPC involve cardiac MC stabilization prior to I/R (Koda et al., 2010), and MC express histamine H_4-receptors (Figs. 2 and 5A). H_4R activation has been shown to protect the liver from I/R-induced injury (Adachi et al., 2006). Thus, we next assessed whether selective activation of H_4Rs mimics the IPC-mediated attenuation of renin release in

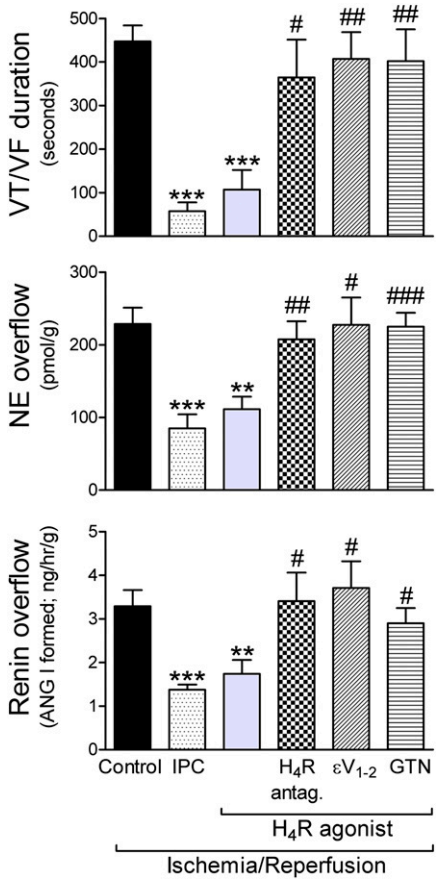

Fig. 1. IPC reduces renin and NE release and shortens arrhythmias caused by I/R in guinea pig hearts ex vivo. This cardioprotective anti-RAS effect is mimicked by activation of histamine H_4R with 4-methylhistamine (2 × 5-minute cycles, 1 μM). The IPC-like effect of 4MeH is prevented by H_4R blockade with compound A943931 (100 nM), inhibition of PKCε with εV_{1-2} (1 μM), or ALDH2 desensitization with glyceryl trinitrate (2 μM, 30 minutes). Coronary overflow of renin (expressed as angiotensin I formed) and NE, and duration of reperfusion arrhythmias (VT/VF) in I/R (control, $n = 8$), I/R preceded by IPC ($n = 7$), I/R preceded by 4MeH ($n = 7$), or I/R in the presence of A943931 (H_4R antagonist; $n = 7$), εV_{1-2} (PKCε inhibitor; $n = 7$), or GTN (ALDH2 desensitizer; $n = 8$). Bars represent means ± S.E.M. of overflows during the first 2 minutes of reperfusion or duration of VT/VF. $^{**}P < 0.01$; $^{***}P < 0.001$; respectively, from I/R. $\#P < 0.05$; $\#\#P < 0.01$; and $\#\#\#P < 0.001$ from H_4R agonist, respectively, by unpaired t test.

hearts subjected to I/R. We found that activation of H_4Rs with 4-methylhistamine (Lim et al., 2005) (4MeH; 1 μM, for 2 × 5 cycles) mimicked the cardioprotective anti-RAS effects of IPC and that this effect was prevented by selective blockade of H_4Rs with compound A943931 (Cowart et al., 2008) (100 nM, 30 minutes) (Fig. 1).

Translocation of PKCε Mediates the IPC-Like Cardioprotective Anti-RAS Effects of H_4R Activation. Given that PKCε translocation/activation is involved in the cardioprotective anti-RAS effects of IPC (Koda et al., 2010), we next investigated the role of PKCε in the cardioprotective anti-RAS effects of H_4R activation. We found that selective inhibition of PKCε with εV_{1-2} (Johnson et al., 1996) (1 μM) prevented the IPC-like cardioprotective anti-RAS effects of H_4R activation (i.e., εV_{1-2} abolished the 4MeH-induced inhibition of renin and NE release as well as the alleviation of VT/VF; Fig. 1). Thus, PKCε activation appears to be required for the genesis of the IPC-like cardioprotective anti-RAS effects of H_4R activation.

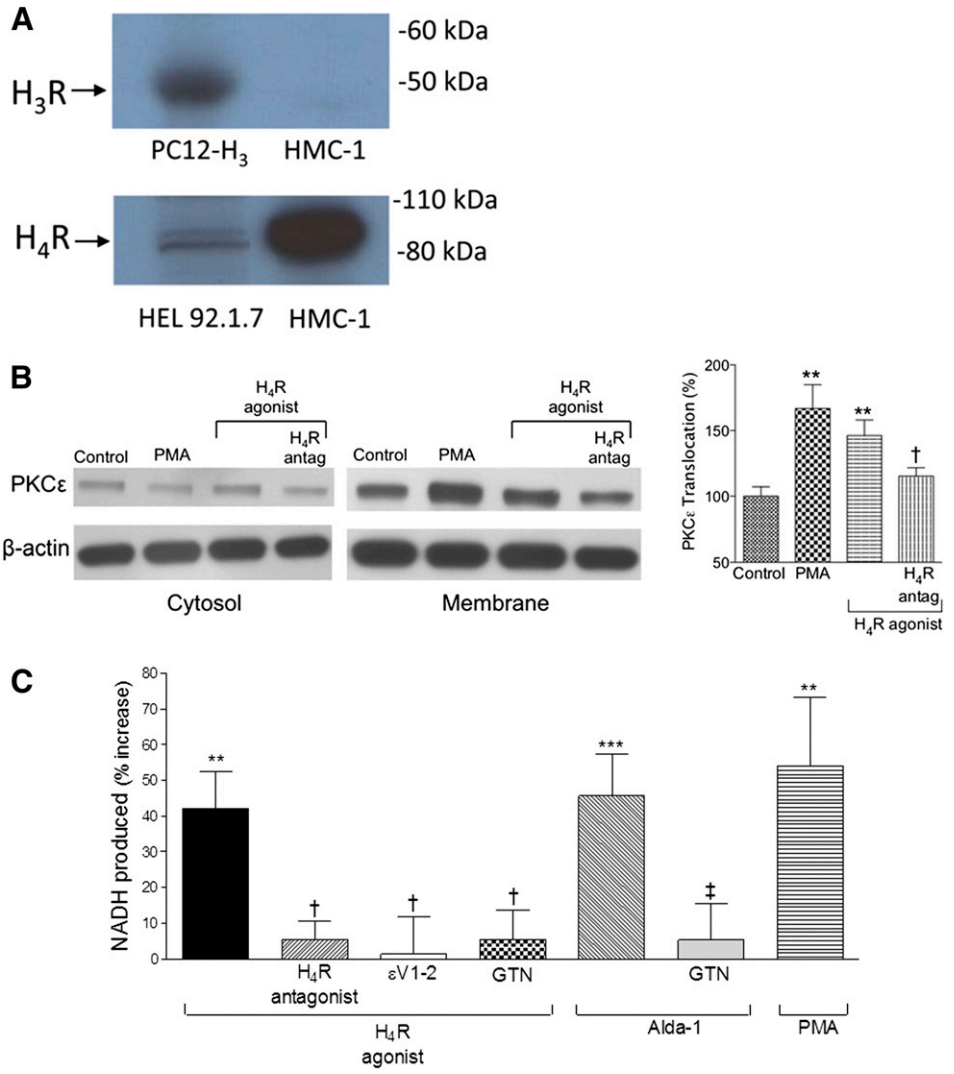

Fig. 2. H_4Rs are expressed in mast cells and their activation translocates/activates PKCε and enhances ALDH2 activity. (A) Top row: Western blot of PC12 cells stably transfected with H_3R (PC12-H_3; positive control) and HMC-1 total lysate probed with anti-Rat H_3R antibody. Lower row: Immunoblot of HEL 92.1.7 cells (positive control) and HMC-1 total lysate probed with anti-human H_4R antibody (40 μg protein/lane). (B) Left: Control, HMC-1 were incubated with sodium Ringer buffer for 10 minutes to obtain PKCε basal activity. HMC-1 incubation with the general PKC activator phorbol ester myristate (300 nM, 10 minutes; positive control) or the H_4R agonist (4MeH, 20 μM, 10 minutes) causes an increase in PKCε translocation from cytosol to membrane. (B) Right: Quantification of PKCε translocation from cytosol to membrane expressed as % of control. Bars are means ± S.E.M. (n = 6–8) normalized over β-actin. $^{**}P < 0.01$ from control; $^{\dagger}P < 0.05$ from H_4R agonist by unpaired t test. (C) Incubation of HMC-1 cells with the ALDH2 activator Alda-1 (100 μM, 10 minutes), the PKC activator PMA (300 nM, 10 minutes), or the H_4R agonist 4MeH (20 μM, 10 minutes) elicits an increase in ALDH2 activity (measured as the rate of NADH production at 340 nm). Pretreatment with H_4R antagonist A943931 (300 nM, 20 minutes), the PKCε-selective inhibitor εV_{1-2} (1 μM, 20 minutes), or GTN (2 μM, 30 minutes) prevents the effects of the H_4R agonist. Pretreatment with GTN prevents the effect of Alda-1. Bars are mean percentage increases from control (±S.E.M.; n = 8–22). Basal NADH production was 3.50 ± 0.21 μmol/min/mg protein. $^{**}P < 0.01$; $^{***}P < 0.001$ from control; $^{\dagger}P < 0.05$ from H_4R agonist; $^{\ddagger}P < 0.05$ from Alda-1 by unpaired t test.

Activation of ALDH2 Is Pivotal for the Cardioprotective IPC-Like Anti-RAS Effects of H_4R Activation. Because the PKCε-mediated cardioprotective anti-RAS effects of IPC depend on phosphorylation of mitochondrial ALDH2 (Koda et al., 2010), we next assessed whether the anti-RAS effect of H_4R activation also involves ALDH2 activation. For this, we investigated whether inhibition/inactivation of ALDH2 would abolish the anti-RAS effects of H_4R activation. We found that GTN, perfused for 30 minutes at a concentration (2 μM) that is known to inactivate ALDH2 (Chen et al., 2008), prevented the IPC-like cardioprotective anti-RAS effects of H_4R activation (i.e., GTN abolished the 4MeH-induced inhibition of renin and NE release as well as the alleviation of VT/VF; Fig. 1). Collectively, these findings suggest that ALDH2 activation by PKCε is a crucial mechanistic step in the development of the IPC-like anti-RAS effects of H_4R activation.

Mast Cells Are the Site of the IPC-Like Cardioprotective Anti-RAS Effects of H_4R Activation: Roles of PKCε and ALDH2. Given the pivotal role that MCs play in the activation of RAS in the heart (Mackins et al., 2006), cardiac MCs are likely to be the site at which the IPC-like anti-RAS effects of H_4R activation develop. To substantiate this hypothesis we first ascertained that MCs indeed express H_4Rs. For this, we used human MCs in culture (HMC-1) and bone marrow–derived murine MCs. Using Western immunoblot analysis, we found that HMC-1 and BMMCs both express H_4Rs but neither expresses H_3R protein (Figs. 2A and 5A).

We next ascertained that MC PKCε is translocated/activated upon H_4R activation. By use of Western analysis in cytosolic and membrane fractions of HMC-1 cells, we found that the phorbol ester PMA (300 nM, 7 minute; positive control) markedly increased (~70%) the translocation of PKCε from cytosol to membrane (i.e., a hallmark of PKCε activation) (Fig. 2B). Incubation of HMC-1 cells with the H_4R agonist (4MeH, 20 μM, 10 minutes) also enhanced PKCε translocation from cytosol to membrane (~50%; Fig. 2B), an action that was prevented by the H_4R antagonist A943931 (300 nM, 20 minutes).

Our findings in guinea-pig hearts ex vivo and in cultured MCs suggested that IPC-like effects result from the activation of H_4Rs expressed on cardiac MCs and the consequent PKCε-dependent activation of mitochondrial ALDH2. We tested this postulate by measuring ALDH2 activity in HMC-1 cells in response to the H_4R agonist 4MeH. We found that 4MeH (20 μM, 10 minutes) elicited an ~40% increase in ALDH2 enzymatic activity (i.e., NADH production) equivalent to that induced by the

specific ALDH2 activator Alda-1 (100 μM, 10 minutes) (Budas et al., 2009) and the general PKC activator PMA (300 nM, 10 minutes) (Fig. 2C).It is noteworthy that the H_4R-induced increase in ALDH2 activity was prevented by the H_4R antagonist A943931 (300 nM, 20 minutes) and the PKCε-selective inhibitor εV_{1-2} (1 μM, 20 minutes), whereas the selective ALDH2 desensitizer GTN (2 μM, 30 minutes) prevented the ALDH2-enhancing effects of either Alda-1 or 4MeH (Fig. 2C).

We next investigated the role of ALDH2 in MC degranulation and renin release elicited by acetaldehyde, a prototypic toxic compound that accumulates during I/R (Chen et al., 2008). Incubation of HMC-1 cells with acetaldehyde (30–300 μM, 20 minutes), elicited a concentration-dependent increase in the release of β-HEX (~4 to 15%; an indication of MC degranulation) and renin (~15 to 25%) (Fig. 3, A–F). Notably, preincubation of HMC-1 cells with the H_4R agonist 4MeH (20 μM, 10 minutes) prevented the degranulating and renin-releasing effects of acetaldehyde (Fig. 3, A–F); this action was blocked by the selective H_4R antagonist A943931 (300 nM, 20 minutes) (Fig. 3, A and D), by the PKCε inhibitor εV_{1-2} (1 μM, 20 minutes) (Fig. 3, B and E), and by the ALDH2 desensitizer GTN (2 μM, 30 minutes) (Fig. 3, C and F). Collectively, these findings indicate that activation of H_4Rs on the MC membrane leads sequentially to PKCε translocation and ALDH2 activation, which prevents the degranulating effects of acetaldehyde, known to be produced in I/R.

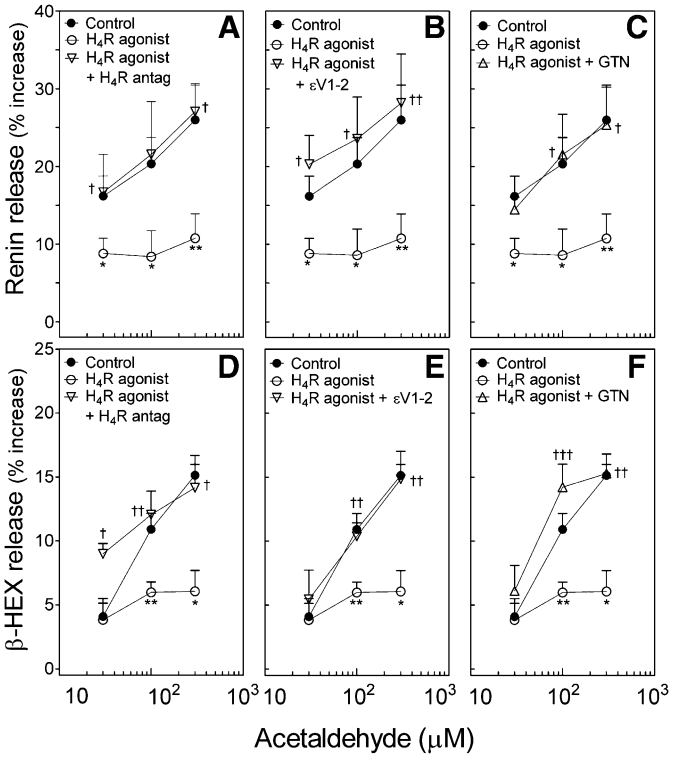

Fig. 3. Mast cell degranulation and renin release induced by acetaldehyde. Incubation of HMC-1 cells with acetaldehyde (30–300 μM, 20 minutes) elicits a concentration-dependent degranulation (i.e., an increased β-HEX release) and renin release (A–F). Preincubation of HMC-1 with H_4R agonist (4MeH, 20 μM, 10 minutes) attenuates both degranulation and renin release, an effect prevented by incubation with the H_4R antagonist A943931 (300 nM, 20 minutes). Pretreatment of HMC-1 with the ALDH2 desensitizer GTN (2 μM, 30 minutes) or the PKCε antagonist εV_{1-2} (1 μM, 20 minutes) also prevents the effect of the H_4R agonist. Basal β-HEX release was 4.56 ± 0.2 (n = 29). Basal renin activity (i.e., angiotensin I formed, was 19.4 ± 2.9 ng/h/mg protein; n = 26). Means ± S.E.M. (n = 4–22)., , *$P < 0.05$; **$P < 0.01$, from control. $^{\dagger}P < 0.05$; $^{\dagger\dagger}P < 0.01$; and $^{\dagger\dagger\dagger}P < 0.001$ from H_4R agonist, by unpaired t test.

Lack of IPC- and H_4R-Mediated Anti-RAS Cardioprotection in H_4R-Deleted Mouse Hearts. We next established a mouse heart model of I/R and I/R preceded by IPC. Spontaneously beating Langendorff-perfused wild-type C57BL/6J mouse hearts were subjected to ischemia (i.e., 30-minute perfusion with glucose- and pyruvic acid–free KH buffer bubbled with 95% N_2 + 5% CO_2, and containing the reducing agent sodium dithionite, 0.25 mM) followed by 30-minute reoxygenation with normal KH buffer (I/R; see *Materials and Methods*) (Mackins et al., 2006). I/R in the ex vivo WT mouse heart resulted in large increases in renin and NE overflow (i.e., ~3- and ~11-fold, respectively) and severe ventricular arrhythmias (VT/VF) that lasted ~100 seconds (Fig. 4). We had previously shown in both cavian and murine hearts that enhanced NE overflow and arrhythmias result from the activation of a local RAS by renin released from cardiac MCs (Mackins et al., 2006).

When I/R was preceded by IPC (i.e., 2 × 5-minute cycles of ischemia, each followed by 5-minute reoxygenation), the overflow of renin and NE and the duration of VT/VF were greatly reduced (by ~55, 60, and 80%, respectively) (Fig. 4), clearly indicating a protective anti-RAS effect of IPC in the murine heart. As observed in the guinea pig heart (Koda et al., 2010), the finding that IPC markedly attenuated the I/R-induced release of renin and NE was not attributed to depletion of renin and NE pools during preconditioning.

It is noteworthy that when WT mouse hearts were pretreated with the selective H_4R antagonist A943931 (300 nM, 20 minutes) and then subjected to IPC (i.e., 2 × 5-minute cycles of ischemia each followed by 5-minute perfusion with A943931), followed by a 15-minute drug-free washout before I/R, the cardioprotective anti-RAS effects of IPC were abolished (Fig. 4). Other WT mouse hearts were perfused with the selective H_4R agonist 4MeH (1 μM) for 2 × 5-minute cycles, each followed by a 5-minute washout before I/R. Similar to what was observed in guinea pig hearts (see Fig. 1), activation of H_4R with 4MeH mimicked the cardioprotective anti-RAS effects of IPC (Fig. 4). Perfusion with the general PKC activator PMA (2 × 5-minute cycles, 0.05 nM) also mimicked the anti-RAS cardioprotective effects of IPC.

Similar experiments were performed in ex vivo hearts isolated from H_4R knockout C57BL/6J mice ($H_4R^{-/-}$). Exposure of these hearts to I/R elicited ~200- and ~130-fold increases in renin and NE overflow, as well as VT/VF that lasted ~2 minutes. Importantly, in contrast with WT hearts, IPC and 4MeH each failed to exert anti-RAS protection, demonstrating the pivotal cardioprotective relevance of H_4Rs. In contrast, PKC activation with PMA provided similar anti-RAS effects in both WT and $H_4R^{-/-}$ hearts (Fig. 4), indicating that H_4R deletion does not affect the efficiency of the protective signaling cascade downstream of H_4R.

Lack of H_4R-Mediated PKCε and ALDH2 Activation in BMMC from $H_4R^{-/-}$ Mice. Similar to HMC-1 (see Fig. 2A), BMMCs express H_4R but not H_3R protein (Fig. 5A). Also similar to HMC-1 (see Fig. 2B), incubation of BMMCs from WT mice with 4MeH (1 μM, 10 minutes) markedly increased (i.e., 2-fold) PKCε translocation from cytosol to membrane (Fig. 5B). In contrast, 4MeH failed to significantly affect PKCε translocation in BMMCs from $H_4R^{-/-}$ mice, whereas PMA

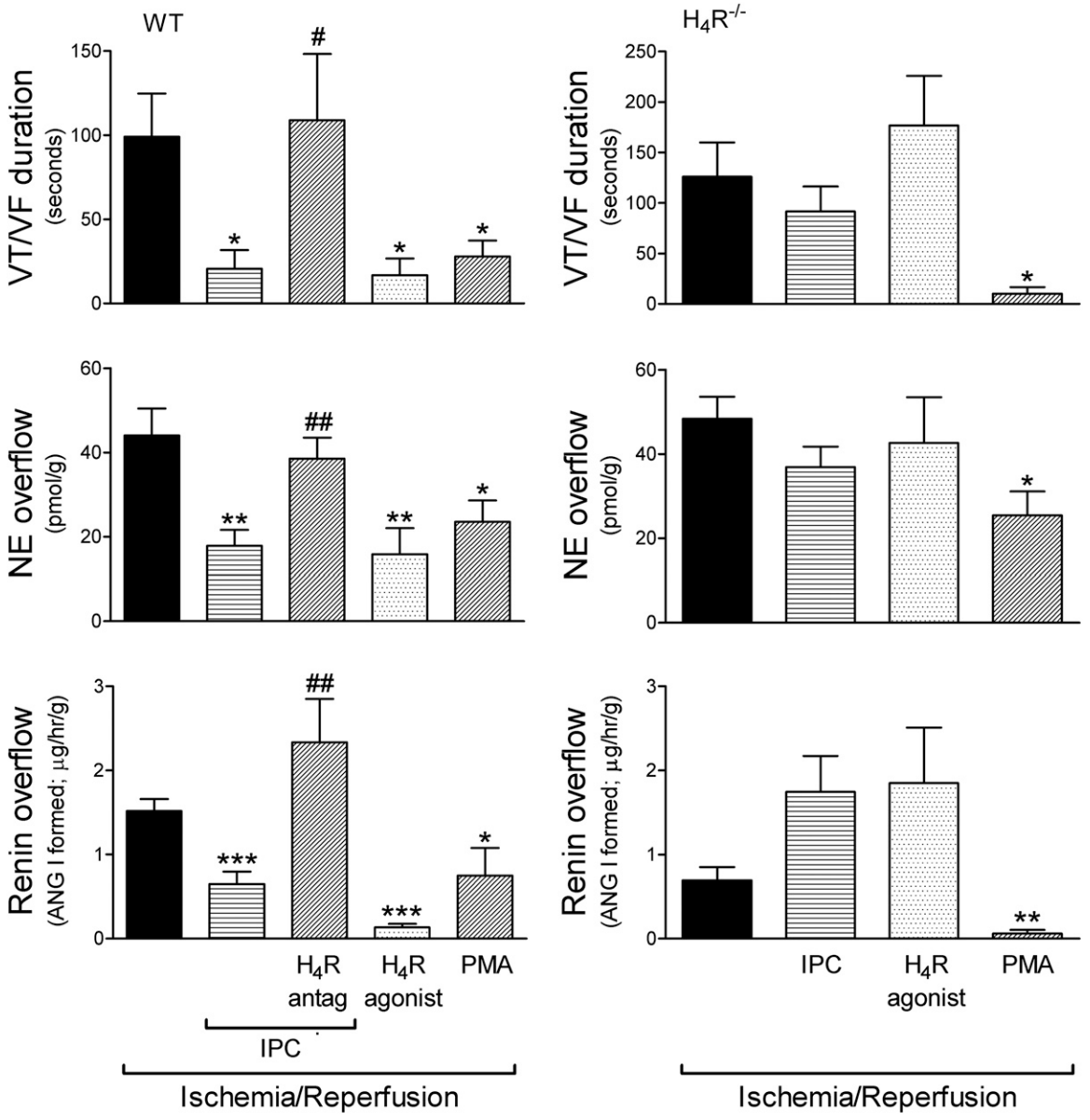

Fig. 4. IPC reduces renin and NE overflow, and shortens arrhythmias caused by I/R in murine hearts ex vivo. H_4R activation mimics the cardioprotective anti-RAS effect of IPC, whereas H_4R blockade or H_4R gene deletion prevents IPC-induced cardioprotection. Coronary overflow of renin and NE, and duration of reperfusion arrhythmias (VT/VF) in control ischemia (i.e., 30-minute perfusion with glucose- and pyruvic acid-free KH buffer, 95% N_2 + 5% CO_2) followed by 30-minute reoxygenation with normal KH buffer (WT, $n = 11$; $H_4R^{-/-}$, $n = 7$). Other hearts were subjected to I/R preceded by IPC (i.e., 2 × 5-minute cycles of ischemia, each followed by 5-minute reoxygenation; WT, $n = 9$; $H_4R^{-/-}$, $n = 9$). Other hearts were pretreated with the selective H_4R antagonist A943931 (300 nM, 20 minutes), and then subjected to IPC (i.e., 2 × 5-minute cycles of ischemia each followed by 5-minute perfusion with A943931), followed by a 15-minute drug-free washout before I/R ($n = 7$). Other hearts were perfused with the selective H_4R agonist 4MeH (1 μM) for 2 × 5-minute cycles, each followed by a 5-minute washout before I/R (WT, $n = 7$; $H_4R^{-/-}$, $n = 6$). Other hearts were perfused with the general PKC activator PMA (0.05 nM) for 2 × 5-minute cycles, each followed by a 5-minute washout before I/R (WT, $n = 7$; $H_4R^{-/-}$, $n = 5$).*$P < 0.05$; **$P < 0.01$; and ***$P < 0.001$ versus control I/R. $^{\#}P < 0.05$; $^{\#\#}P < 0.01$ versus IPC, by unpaired t test.

still enhanced it 2–3-fold (Fig. 5B). Likewise, as observed in HMC-1 cells (see Fig. 2C), incubation of BMMCs from WT mice with 4MeH (1 μM, 10 minutes) elicited an ~50% increase in ALDH2 activity, compared with an ~90% increase induced by PMA (0.05 nM, 10 minutes) (Fig. 5C). In contrast, 4MeH failed to significantly affect ALDH2 activity in BMMCs from $H_4R^{-/-}$ mice, whereas PMA still enhanced ALDH2 activity by ~70% (Fig. 5C). Collectively, these findings in the ex vivo hearts and BMMCs from $H_4R^{-/-}$ mice (see Figs. 4 and 5), established that H_4R deletion does not affect the efficiency of the PKCε-ALDH2 signaling cascade downstream of H_4R.

Lack of H_4R-Mediated Prevention of Acetaldehyde-Induced Degranulation and Renin Release in BMMC from $H_4R^{-/-}$ Mice. Analogous to what we observed in HMC-1 cells (See Fig. 3), incubation of BMMCs with acetaldehyde (100 μM, 20 minutes) caused degranulation (i.e., an ~20% increase in β-HEX release) and an ~35% increase in renin release (Fig. 6). When BMMCs from WT mice were incubated with 4MeH (1 μM, 10 minutes), the degranulating and renin-releasing effects of acetaldehyde were markedly reduced and prevented, respectively (Fig. 6). In contrast, 4MeH failed to affect the acetaldehyde-induced degranulation and renin

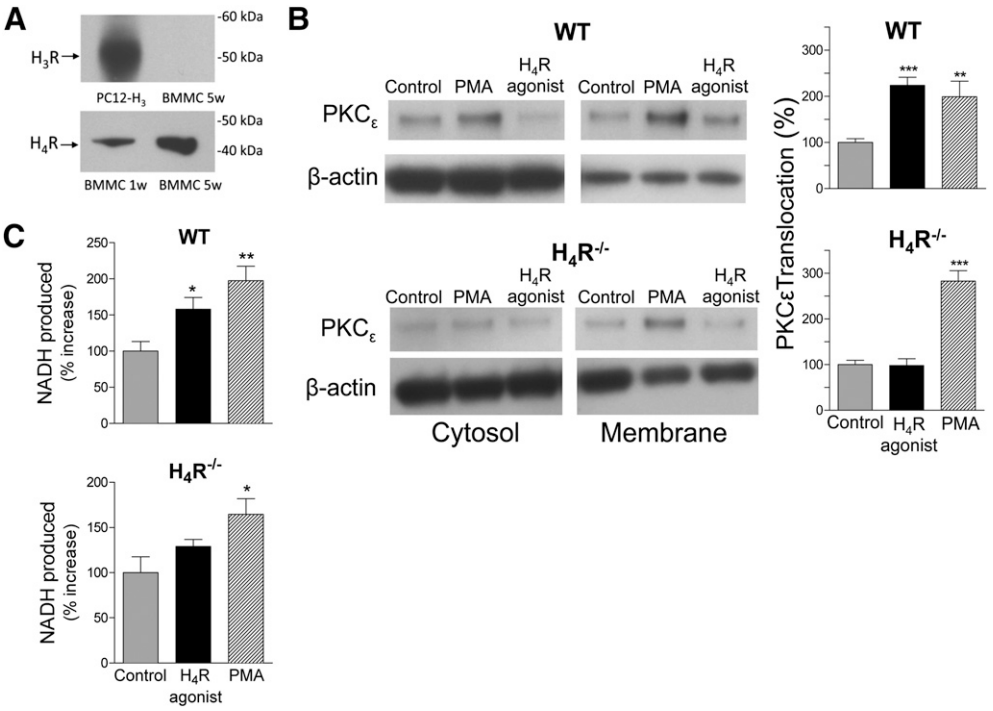

Fig. 5. H_4Rs are expressed in BMMCs and their activation enhances PKCε translocation and ALDH2 activity. (A) Western blot of total lysate of BMMC (1- and 5-week culture) and PC12 cells (stably transfected with H_3R; PC12-H_3; positive control) probed with anti-Rat H_3R antibody and anti-murine H_4R antibody (40 μg of protein/lane). (B) PKCε translocation in BMMCs. Incubation of WT and $H_4R^{-/-}$ BMMC with H_4R agonist (4MeH, 1 μM, 10 minutes) enhances PKCε translocation from cytosol to membrane in WT but not in $H_4R^{-/-}$ BMMCs. Incubation with PMA (10 nM, 10 minutes; positive control) enhances PKCε translocation in both WT and $H_4R^{-/-}$ BMMCs. Bars are means ± S.E.M. (WT and $H_4R^{-/-}$, n = 4–10 each). $^{**}P < 0.01$; $^{***}P < 0.001$ from control, by unpaired t test. (C) ALDH2 enzymatic activity in BMMCs. Incubation of WT and $H_4R^{-/-}$ BMMCs with H_4R agonist (4MeH, 1 μM, 10 minutes) enhances ALDH2 activity in WT but not in $H_4R^{-/-}$ BMMCs. Incubation with PMA (0.05 nM, 10 minutes; positive control) enhances ALDH2 activity in both WT and $H_4R^{-/-}$ BMMCs. Bars are means ± S.E.M. (WT and $H_4R^{-/-}$, n = 5–8 each); basal NADH production was 2.2 ± 0.3 μmol/min/mg protein in WT and 1.6 ± 0.3 μmol/min/mg protein in $H_4R^{-/-}$. $^{*}P < 0.5$; $^{**}P < 0.01$ from control, by unpaired t test.

release in BMMCs from $H_4R^{-/-}$ mice (Fig. 6), demonstrating the crucial role of MC H_4Rs in initiating the abolition of the degranulating and renin-releasing effects of reactive aldehydes produced in I/R.

Discussion

Our results clearly show that activation of H_4Rs on the MC membrane during I/R affords cardioprotective anti-RAS effects, which similar to IPC include a reduction of renin and NE release and alleviation of reperfusion arrhythmias (See Fig. 7).

MC density is known to increase in ischemic canine and human hearts and so does their content of histamine, tryptase, and chymase (Frangogiannis et al., 1998; Patella et al., 1998). MCs also synthesize renin, which is released together with histamine in I/R and hypersensitivity reactions (Imamura et al., 1994; Hatta et al., 1997; Aldi et al., 2014). It is noteworthy that when released in I/R, MC renin initiates the activation of a local RAS, culminating in VT/VF (Mackins et al., 2006) (see Fig. 7). In the absence of cardiac MCs (i.e., c-Kit knockout mice), renin immunoreactivity disappears and I/R fails to elicit VT/VF (Mackins et al., 2006).

Having previously uncovered an MC-directed cardioprotective anti-RAS action of adenosine, mediated in part by $G\alpha_{i/o}$-coupled A_3Rs (Koda et al., 2010), we questioned whether other $G\alpha_{i/o}$-coupled receptors expressed on MCs might provide similar protective IPC-like effects in I/R. We focused on histamine H_4Rs because they are $G\alpha_{i/o}$-coupled (Nijmeijer et al., 2012) and conceivably activatable in an autocrine mode by their natural endogenous ligand (i.e., histamine), which we had shown to be present in cardiac MCs (Silver et al., 2004) and to be released during I/R (Imamura et al., 1994; Hatta et al., 1997). In support of this hypothesis, we found that either pharmacologic blockade of H_4Rs with the selective antagonist A943931 (Cowart et al., 2008) or their genetic deletion prevents the protective effects of IPC in murine hearts, indicating that functional H_4Rs are indispensable for the development of the anti-RAS effects of IPC. This notion is corroborated by the discovery that pharmacologic activation of H_4Rs in both cavian and murine hearts mimics the anti-RAS effects of IPC, as shown by a marked reduction of renin and NE release and alleviation of arrhythmias. An MC involvement was proven by the finding that pharmacologic activation of H_4Rs in cultured human and murine MCs prevented degranulation and renin release by acetaldehyde, a characteristic endogenous product of I/R (Chen et al., 2008). It is noteworthy that this protective effect was prevented by pharmacologic H_4R blockade and was totally absent in MCs isolated from $H_4R^{-/-}$ mice.

Intrigued by these H_4R-mediated protective effects, we investigated their signaling mechanisms. Given that $G\alpha_{i/o}$-coupled receptors are known to translocate/activate PKCε (Inagaki et al., 2006), probably via phospholipase C-diacylglycerol activation (Hofstra et al., 2003), we asked whether PKCε inhibition would preclude the effects of H_4R activation. We found that PKCε inhibition with compound εV_{1-2} (Johnson et al., 1996) not only abolished the IPC-like

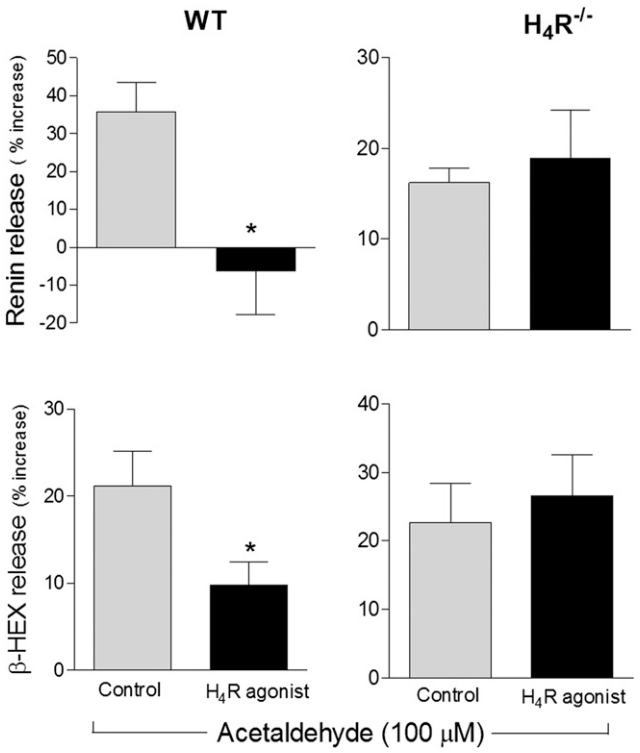

Fig. 6. H_4R activation in BMMCs attenuates acetaldehyde-induced degranulation and renin release. Incubation of BMMCs with acetaldehyde (100 μM, 20 minutes) elicits degranulation (i.e., release of β-HEX) and renin release in cells isolated from WT and $H_4R^{-/-}$ mice. Preincubation with the H_4R agonist 4MeH (1 μM, 10 minutes) inhibits degranulation and renin release in WT but not in $H_4R^{-/-}$ BMMC. Bars are means (±S.E.M.; $n = 4$–9). Basal β-HEX release was 1.95 ± 0.37 and 2.34 ± 0.80 in WT and $H_4R^{-/-}$ BMMCs ($n = 7$ and 4), respectively. Basal renin release (i.e., angiotensin I formed) was 1.1 ± 0.49 and 2.88 ± 0.41 μg/h/mg ($n = 4$ and 4) in WT and $H_4R^{-/-}$, respectively. *$P < 0.05$ from control (i.e., acetaldehyde 100 μM).

anti-RAS effects of H_4R activation with 4MeH (Lim et al., 2005) in guinea pig hearts but also prevented 4MeH from attenuating the degranulating and renin-releasing effects of acetaldehyde in cultured human MCs, clearly indicating an involvement of PKCε in the anti-RAS effects of 4MeH. Indeed, 4MeH translocated/activated PKCε from cytosol to membrane in HMC-1 cells, confirming that the cardioprotective effects of H_4R activation depend on the translocation/activation of PKCε. PKCε is known to phosphorylate mitochondrial ALDH2 in MCs (Chen et al., 2008), and ALDH2 has been shown to prevent the MC-degranulating and renin-releasing effects of reactive oxygen species and toxic aldehydes (Koda et al., 2010). Hence, to uncover the relevance of ALDH2, we pre-empted its action with an excess of GTN (Koda et al., 2010). Similar to PKCε blockade, ALDH2 inhibition abolished the protective anti-RAS effects of IPC in ex vivo hearts and prevented 4MeH from attenuating the degranulating and renin-releasing effects of acetaldehyde in cultured human MCs. It is noteworthy that PKCε blockade prevented ALDH2 activation by 4MeH. Collectively, these findings indicate that the IPC-like H_4R-mediated inhibition of I/R-induced cardiac MC degranulation and renin release results from an initial translocation of PKCε and subsequent phosphorylation of ALDH2, culminating in the elimination of the MC-degranulating effects of acetaldehyde and other toxic species produced during I/R (see Fig. 7). Although we did not measure translocation of PKCε to cardiac mitochondria, work by the Mochly-Rosen laboratory has demonstrated that PKCε activates the intramitochondrial enzyme ALDH2 in an ex vivo model of myocardial infarction. This protection coincides with the translocation of PKCε to cardiac mitochondria, where it associates with ALDH2 (Chen et al., 2008; Churchill et al., 2009). Importantly, we found that in hearts and BMMCs from $H_4R^{-/-}$ mice, no protective anti-RAS effects of IPC occurred, nor H_4R-mediated PKCε translocation and ALDH2 activation, even though the protective PKCε-ALDH2 pathway downstream of H_4R was still activatable with PMA.

We observed that renin and NE release, as well as VT/VF duration, were similar in H_4R-deleted and WT hearts exposed to I/R. Pharmacologic H_4R antagonism, similar to H_4R deletion, also failed to increase renin and NE release and VT/VF duration. Given the anti-RAS role of H_4Rs, one might have expected renin and NE release and arrhythmia to be enhanced when H_4Rs were pharmacologically blocked or deleted. An explanation of why this did not occur is probably found in the very low constitutive activity of H_4Rs in the mouse (Schnell et al., 2011). That constitutive activity plays an important role in this case is proven by our previous findings with H_3Rs, which are located on sympathetic nerve endings in the heart, where they inhibit NE release in I/R (Imamura et al., 1994). Unlike H_4Rs, H_3Rs have a very high constitutive activity (Schnell et al., 2011). Indeed, we had found that when hearts from H_3R-deleted mice were exposed to I/R, NE release was greatly enhanced (Koyama et al., 2003), which substantiates the functional relevance of receptor constitutive activity.

Our finding that anti-RAS IPC was completely abolished in H_4R-deleted hearts indicates that H_4Rs are indispensable for the anti-RAS effects of IPC. This appears to dismiss the idea that other mechanisms previously advocated for "classical" IPC (Murphy and Steenbergen, 2008) play a role in anti-RAS IPC. In fact, "classical" preconditioning focuses on infarct size reduction, recovery of contractility, etc., whereas our IPC paradigm involves an attenuation of MC renin release and prevention of a local RAS activation and its dysfunctional consequences. Indeed, we had reported that the cardioprotective anti-RAS effects of IPC are unaffected by K_{ATP} channel inhibition (Koda et al., 2010), a procedure known to prevent "classical" IPC. On the other hand, we previously demonstrated that adenosine A_{2b}Rs and A_3Rs cooperate in mediating anti-RAS IPC and showed that this effect involves the same PKCε-ALDH2 pathway (Koda et al., 2010), which mediates the H_4R anti-RAS effects of IPC. Thus, if adenosine and histamine played two independent roles, one would have expected adenosine to still be able to afford some anti-RAS protection in H_4R-deleted hearts. Yet, H_4R deletion completely abolished IPC. One possible explanation is that H_4Rs are essential for $A_{2b}R/A_3R$ signaling. In fact, G protein–coupled receptors can physically interact with each other and operate via interdependent mechanisms (Vischer et al., 2011). Further investigation will be necessary to explore this possible interaction.

Conclusions

Our collective evidence delineates a novel cardioprotective chain of events in I/R, whereby an autocrine activation of H_4Rs on the MC membrane by MC-derived histamine leads sequentially to PKCε and ALDH2 activation, reduction of

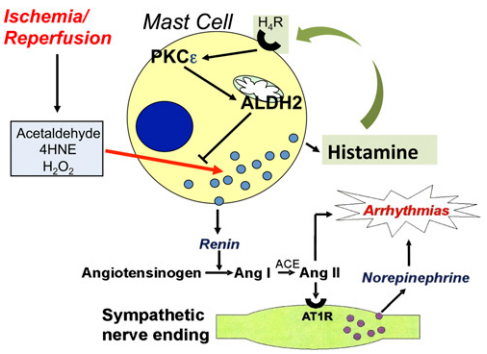

Fig. 7. Proposed mechanisms for the cardioprotective anti-RAS effects of H_4R activation.

toxic aldehyde-induced MC degranulation, decreased renin release, prevention of RAS activation, reduction of NE release, and ultimately alleviation of reperfusion arrhythmias (see Fig. 7). Aside from the physiologic and pathophysiologic relevance of this newly discovered protective pathway, our findings suggest that MC H_4Rs may represent a new pharmacologic and therapeutic target for the direct alleviation of RAS-induced cardiac dysfunctions, including ischemic heart disease and congestive heart failure.

It is noteworthy that H_4Rs could also grant cardioprotection by additional mechanisms. We recently ascertained the presence of H_4Rs in cardiac sympathetic nerve endings where, similar to H_3Rs, H_4Rs inhibit NE release (Chan et al., 2012). This effect could complement the reduction of NE release ultimately resulting from H_4R-mediated actions at the MC level. Yet, we had found that when hearts of MC-deleted mice are exposed to I/R, renin release and VT/VF are abolished (Mackins et al., 2006). This supports the notion that MCs, and MC-expressed H_4Rs, are essential for the anti-RAS cardioprotective effects of H_4R activation.

In addition, mast cells store and release serotonin and express serotonin receptors (Kushnir-Sukhov et al., 2007; Ahern, 2011) and monoamine oxidase (MAO)-B (Vitalis et al., 2003). Resident cardiac mast cells could take up serotonin released from platelets in I/R, leading to MAO activation (Shimizu et al., 2002). Furthermore, it was recently reported that a combination of MAO activation and ALDH2 inhibition by small interfering RNA generates H_2O_2 and toxic aldehydes, leading to mitochondrial dysfunction and cell death in heart failure (Kaludercic et al., 2010, 2014). Accordingly, H_4R-induced ALDH2 activation could play an important cardioprotective mechanism by decreasing MAO-produced toxic aldehydes and preventing myocardial damage.

Moreover, an H_4R-mediated PI3K activation has been reported (Desai and Thurmond, 2011), and PI3K is known to phosphorylate/activate ALDH2 (Lagranha et al., 2010). Thus, H_4Rs may activate ALDH2 independently of PKCε (Lagranha et al., 2010). It is also conceivable that being $G\alpha_{i/o}$-coupled (Nijmeijer et al., 2012), H_4Rs might diminish MC degranulation by decreasing adenylyl cyclase activity, intracellular cAMP level, PKA activity, and thus, Ca^{2+} availability (Hua et al., 2007). Finally, recent evidence suggests that H_4R activation leads to β-arrestin recruitment and consequent mitogen-activated protein kinase/extracellular signal-regulated kinase signaling (Nijmeijer et al., 2012). Mitogen-activated protein kinase/extracellular signal-regulated kinase pathway has been associated with IPC (Ping et al., 1999). All in all, these findings draw attention to H_4Rs as potential cardioprotective new targets.

Lastly, in addition to the heart, H_4R-mediated protective mechanisms may impact other organs (e.g., brain, liver, and kidney) that have renin-containing MCs (Reid et al., 2007; Biran et al., 2008; Veerappan et al., 2008), can suffer ischemic episodes (Kaneko et al., 1998; Guo et al., 2004; Chen et al., 2009), and have been shown to be protected by IPC (Nandagopal et al., 2001; Adachi et al., 2006; Chan et al., 2012; Kukreja, 2012).

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Authorship Contributions

Participated in research design: Aldi, Takano, Tomita, Koda, Chan, Marino, Salazar-Rodriguez, Levi.

Conducted experiments: Aldi, Takano, Tomita, Koda, Chan, Marino, Salazar-Rodriguez.

Contributed new reagents or analytic tools: Thurmond.

Performed data analysis: Aldi, Takano, Tomita, Koda, Chan, Marino, Salazar-Rodriguez, Levi.

Wrote or contributed to the writing of the manuscript: Aldi, Takano, Tomita, Koda, Chan, Marino, Salazar-Rodriguez, Thurmond, Levi.

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