Altered PKC Regulation of Pulmonary Endothelial Store- and Receptor-Operated Ca^{2+} Entry Following Chronic Hypoxia

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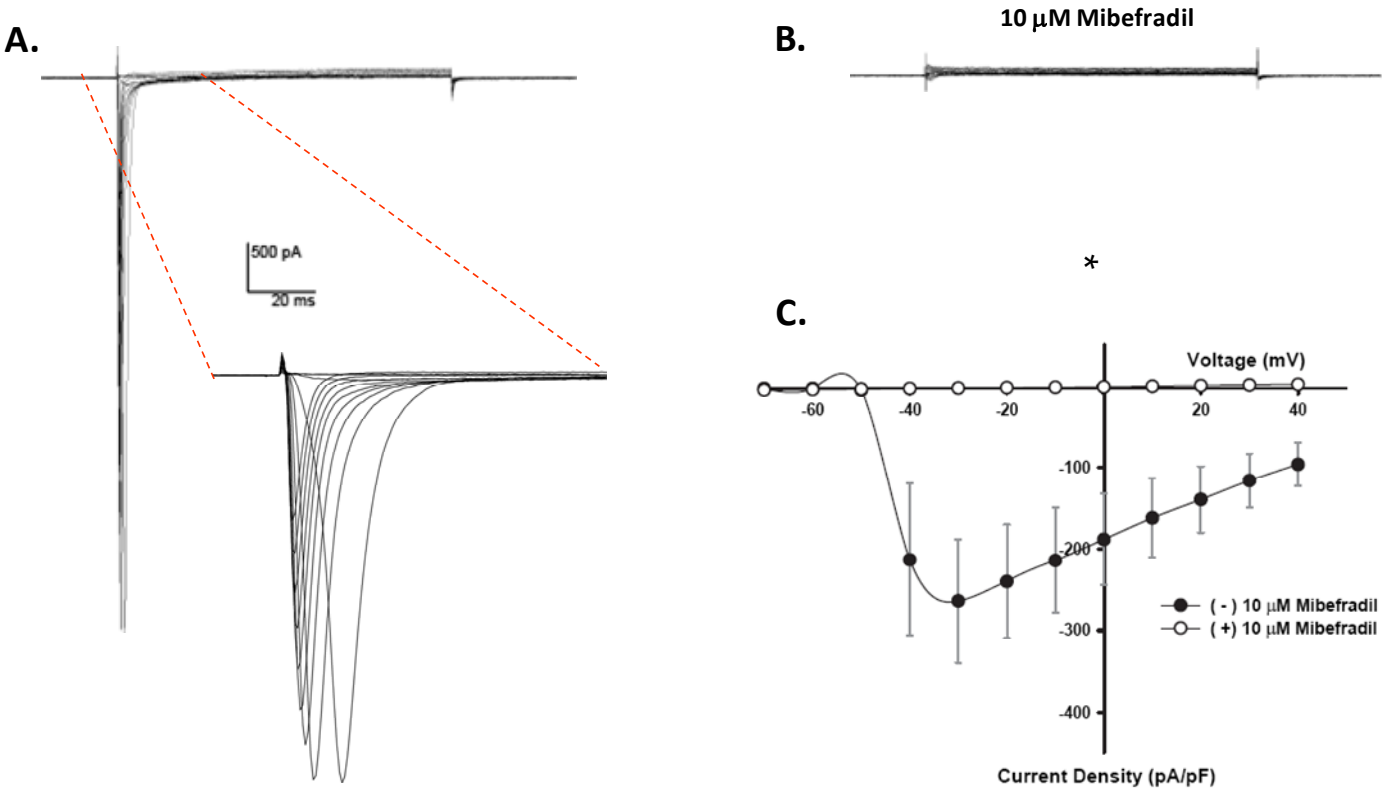
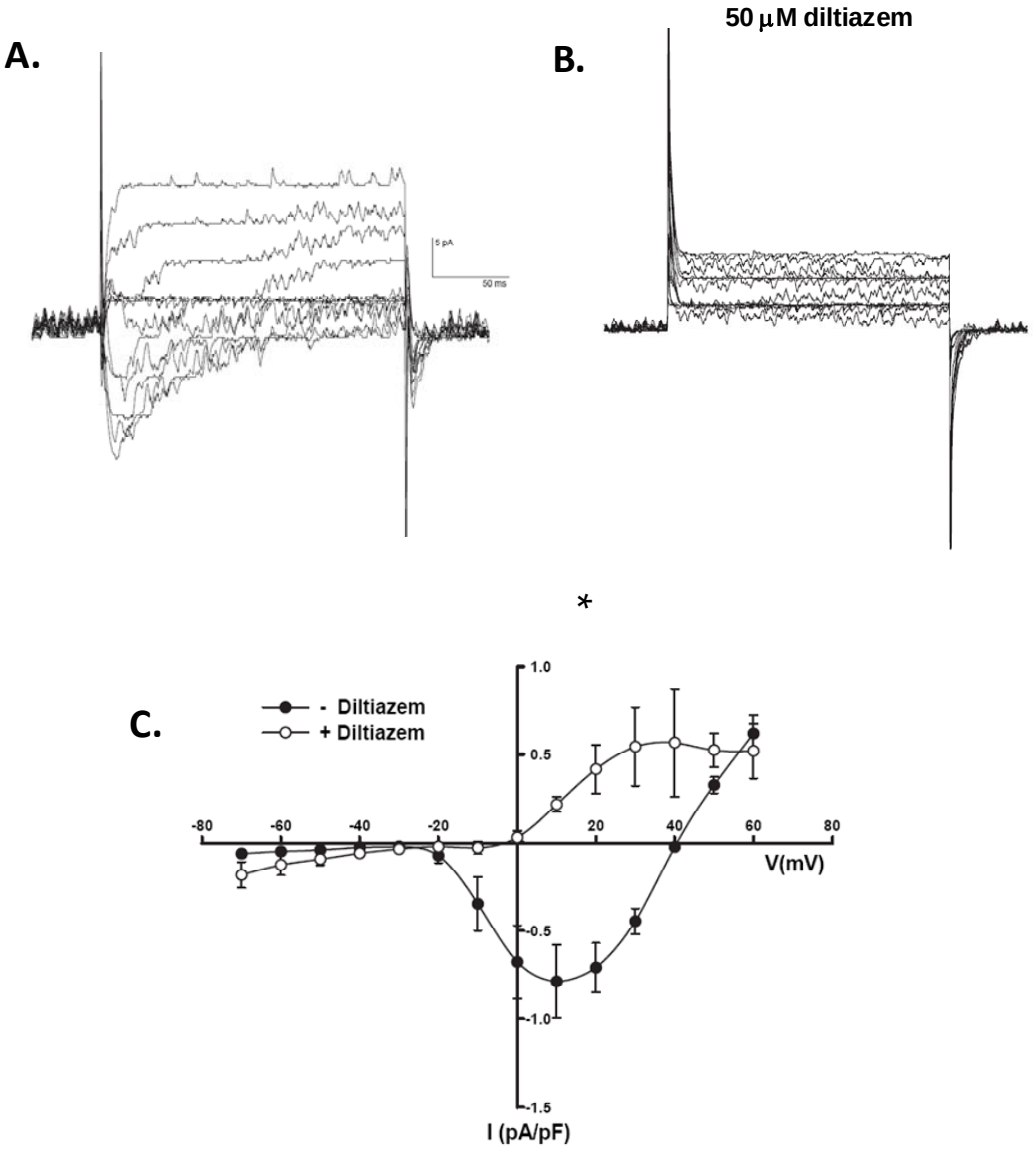
Supplemental Figure 1



Supplemental Figure 2



Supplemental Figure Legends

Supplemental Figure 1. T-type voltage-gated Ca^{2+} channel (VGCC) inhibition with mibefradil in neonatal rat ventricular myocytes (NRVM). The whole cell-attached patch clamp method was used to examine the relative characteristics of native NRVM T-type VGCCs. A) Voltage-dependent Ca^{2+} currents ($I_{Ca^{2+}}$) were evoked in NRVM by a voltage pulse protocol and demonstrated relatively rapid activation-inactivation profiles *(see inset)*. Scale bars set for inset blow-up panel. B) Mibefradil (10 μM) completely abolished $I_{Ca^{2+}}$ in NRVMs. C) Current-voltage relationship illustrating a low threshold voltage-dependent activation of mibefradil-sensitive $I_{Ca^{2+}}$. Summary expressed as mean ± SEM (n = 4). $^{*}P \leq 0.05$ from -40 to +40 mV repeated-measures ANOVA.

Supplemental Figure 2. Inhibition of L-type VGCCs with diltiazem in freshly isolated pulmonary artery smooth muscle cells. Whole-cell ($I_{Ca^{2+}}$) evoked from depolarizing voltage steps depict a voltage-dependent inward current (A) which is inhibited with 50 μM diltiazem (B). Summary data (C) illustrate a peak inward current at +10 mV *(closed circles)* and inhibition with diltiazem *(open circles)*, both indicative of L-type VGCCs. Summary expressed as mean ± SEM (n = 4). $^{*}P \leq 0.05$ from -10 to +40 mV repeated-measures ANOVA.

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Supplemental Methods

Ventricular Myocyte and Smooth Muscle Cell Isolation

Six day old rat pups (Sprague-Dawley) were euthanized by decapitation and the ventricular myocardium rapidly removed and placed in ice-cold Ca^{2+} and Mg^{2+}-free rodent Ringer solution containing (in mM): 155 NaCl. 5 KCl, 11 glucose, 20 taurine, 10 HEPES adjusted with NaOH to pH 7.4. Ventricles were minced and enzymatically digested at 37°C for 1 hr with the addition of collagenase type IA (1 mg/ml). Freshly dispersed myocytes were centrifuged (300 x g) and the remaining pellet was re-suspended in a 2:1 mixture of Dulbecco's modified Eagle-HAMS F-12 medium containing 10% FBS. Neonatal rat ventricular myocytes (NRVM) were subsequently passed through a 70 μm cell strainer (BD Biosciences) and allowed to seed for 24 hrs prior to measuring Ca^{2+} currents.

The left lungs from adult male Sprague-Dawley rats were rapidly excised following a lethal injection of sodium pentobarbital (200 mg kg^{-1} i.p.) and placed in HEPES buffered saline solution (HBSS). Intrapulmonary arteries were rapidly dissected cut into 2 mm segments and placed in an ice-cold Ca^{2+}-free solution of the following composition (in mM): 60 NaCl, 85 sodium glutamate, 5.6 KCl, $MgCl_2$, glucose, HEPES, NaOH to pH 7.4. After a 10 min equilibration (37°C), artery segments were placed in Ca^{2+}-free isolation solution (37°C) containing 1 mg/ml albumin, 0.7 mg/ml papain and 1 mg/ml DTT. After 40 min exposure to papain, artery segments were placed for 10–15 min in a second isolation solution containing 0.1 mM $CaCl_2$ and a type II collagenase and hyaluronidase mixture (1 mg/ml each). The tissue was subsequently washed twice (10 min each) in Ca^{2+}-free isolation solution and triturated with a polished wide-bore pipet. Pulmonary artery smooth muscle cells (PASMC) were stored on ice and used the same day.

Electrophysiological Recordings of Voltage-Dependent I_{Ca}

Voltage-dependent Ca^{2+} currents were examined in acute (24 hr) primary NRVM cultures or freshly isolated PASMCs using the conventional whole-cell patch clamp technique. Extracellular recording solution contained the following (in mM): 125 NaCl, 6 CsCl, 10 $CaCl_2$, 5 HEPES, 10 TEA-Cl, 5 sucrose, NaOH to pH 7.4. Electrodes with tip resistances of 4-6 MΩ were filled with an intracellular recording solution (in mM): 130 CsCl, 2 Mg-ATP, 1 $MgCl_2$, 5 HEPES, 5 EGTA, CsOH to pH 7.2. After obtaining successful whole-cell configuration, NRVM or PASMC E_m was clamped at a holding potential of –90 mV. Voltage pulses from -70 mV to +60 mV were generated using pClamp software (version 8.6) integrated with an Axopatch200B amplifier (Molecular Devices). Whole-cell capacitance and leak currents were compensated prior to initiating voltage pulse protocols. Because NRVMs are tetrototoxin insensitive (Nuss and Marban, 1994) and abundantly express T-type VGCC (Horiba et al., 2008) we utilized these cells to assess the specificity of the putative inhibitor, mibefradil (10 uM). In addition, freshly isolated PASMCs are known express L-type VGCCs and were utilized to assess the inhibitory action of the recognized L-channel blocker diltiazem. All data were analyzed off-line using Clampfit software (version 9.0)

References Cited

Nuss HB, Marban (1994) Electrophysiological properties of neonatal mouse cardiac myocytes in primary culture. *J. Physiol.* **479**:265-79.

Horiba M, Muto T, Ueda N, Opthof T, Miwa K, Hojo M, Lee JK, Kamiya K, Kodama I, Yasui K **(**2008) T-type Ca2+ channel blockers prevent cardiac cell hypertrophy through an inhibition of calcineurin-NFAT3 activation as well as L-type Ca2+ channel blockers. *Life Sci.* **11-12**:554-60.