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Materials and Methods

Isoproterenol infusion and isolation of ENDO and EPI ventricular myocytes

We used wild type (C57BL/6J), NFATc3-null, β1 null, and NFAT-luc mice. For some experiments mice were infused (7 days) with saline (control) or ISO delivered at a rate of 2 mg/kg/day [1, 2]. Preparation and implantation of these pumps was performed as described elsewhere [3, 4]. Myocytes were obtained from the inner-most (ENDO) and outer-most (EPI) regions of the left ventricular free wall — thus discarding the mid-myocardial layer — as previously described using standard enzymatic methods [5]. Cells were dissociated separately from the EPI and ENDO and maintained at room temperature (25 °C) until used. After dissociation, cells were maintained in a Ringer's solution until used.

Electrophysiology and $[Ca^{2+}]_i$ measurements

Currents were recorded using an Axopatch 200B. K^+ currents were measured using a pipette solution containing (in mM): 110 K-Aspartate, 30 KCl, 10 HEPES, 5 ATP-Mg and 10 EGTA (pH = 7.2). With this solution, the patch electrodes had resistances that ranged from 0.8 to 1.2 MΩ. The external solution used to measure K^+ currents contained (in mM): 140 N-methyl-D-glucamine, 5 KCl, 10 HEPES, 10 glucose, 0.1 $CaCl_2$, 2 $MgCl_2$, and 0.01 nifedipine (pH = 7.4). To this solution, we added 4-aminopyridine (4-AP; 50 μM) and tetraethylammonium chloride (TEA; 25 mM) to pharmacologically isolate the I_{to} (defined here as the difference between the peak and the sustained current measured at the end of the 1 s pulse). This approach has been used by others [6-9] to isolate I_{to} from other voltage-gated Kv currents (e.g. I_{Kslow}) in mouse ventricular

myocytes. It is important to note that Brunet et al. [10] demonstrated that the slow I_{to} (or $I_{to,s}$) is not detectable in mouse left ventricular myocytes. Thus, we are confident the I_{to} records from left ventricular EPI and ENDO cells correspond to the fast I_{to} (or $I_{to,f}$). For experiments measuring Ca^{2+} currents (I_{Ca}) cells were superfused with solution containing (in mM): 140 NaCl, 5 CsCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 Glucose, 10 HEPES, 0.010 TTX. The pipette solution used in these experiments contained (in mM) 130 CsCl, 10 TEA-Cl, 5 Mg-ATP and 10 HEPES.

All electrophysiological and imaging experiments were performed at 22-25 °C. Normalization of ventricular K^+ currents was performed by dividing the current amplitudes by the capacitance of the cells from which they were recorded. For $[Ca^{2+}]_i$ measurements cells were loaded with the fluorescent Ca^{2+} indicator Fluo-4 AM as previously described [11]. $[Ca^{2+}]_i$ recordings were performed with a Nikon TE2000 inverted microscope coupled to an IonOptix photometry system.

Real Time and Conventional RT-PCR

Total RNA was isolated from ventricular tissue from either the endo- or epicardium using the TRIzol reagent® (Life Technologies, Gaithrsburg, MD) following the manufacturers' instructions. Following isolation, total RNA was cleaned and DNase treated using the RNeasy mini kit (Qiagen, Valencia, CA). Total RNA was extracted from ENDO or EPI isolated cardiac myocytes in culture using the RNeasy Micro Kit (Qiagen, Valencia, CA) as per the manufacturer's protocol. All qPCR was performed using a one-step protocol with the TaqMan One-Step RT-PCR Master Mix Reagent (Applied Biosystems, Foster City, CA). The final

concentrations of primers and probes were 100 nM each per reaction. The level of Kv4 and luciferase gene products was normalized to the relative expression of endogenous standard (β-actin, NM_007393) in each sample. Primers and probes for real time RT-PCR were as follows: Kv4.2 (GenBank accession no. NM_019697) sense nt 1769-1792; anti-sense nt 1823-1840, and probe nt 1793-1822; Kv4.3 (GenBank accession no. NM_019931) sense nt 85-104; anti-sense nt 132-151, and probe nt 106-128; β-actin (GenBank accession no. NM_007393) sense nt 1035-1055; anti-sense nt 1090-1110, and probe nt 1060-1086. KChIP2 (GenBank accession no. NM_145704) sense nt-131; anti-sense nt-182, and probe nt-137. Conventional RT-PCR was performed using standard techniques [4, 11]. Primers for conventional RT-PCR were as follows: *P. pyralis* (firefly) luciferase (GenBank accession no. M15077) sense nt-520; antisense nt-1009; β-actin (GenBank accession no. V01217); sense nt-2384 and antisense nt-3071.

Western blot analysis

ENDO and EPI tissue from wild mice was ground under liquid nitrogen and homogenized for 30-60 seconds in RIPA buffer (1% Titon-X 100, 0.1% SDS, 0.15M NaCl, 0.01 M sodium phosphate) with protease inhibitor cocktail and phosphatase inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). The homogenate was cleared of cellular debris by centrifugation at 10,000 x g at 4°C, for 10 min. Protein concentration of the supernatant was determined using the bicinchronic acid method with bovine serum albumin (BSA) as a standard. 30 μg of total protein was loaded on a 4-20% Tris-HCl polyacrylamide gel and run with an appropriate molecular weight standard at 100 V for 1 hour. Fractionated protein was transferred to a polyvinylidene difluoride (PVDF) membrane using a Mini-trans Blot Cell (Bio-Rad Laboratories, Hercules, CA) at 100 V for 1h or 80 V for 40min (for phospholamban) at 4 °C.

The blots were blocked in TBS-Tween (25mmol/L Tris pH 7.3, 150 mmol/L NaCl, and 0.1% Tween-20) with 5% nonfat milk or 5% BSA (for P-PLB) for 1h at 25°C. After this, blots were incubated with primary antibodies specific to the β1 adrenergic receptor (AbCam, Cambridge, MA), PKA RI (BD Biosciences, San Jose, CA), PKA RII α (Santa Cruz Biotechnology, Santa Cruz, CA), phosphorylated Ser16 of phospholamban ($Ser16^{P}$-PLB; Badrilla Ltd., Leeds, UK), and Actin (Millipore, Billerica, MA) in TBS-Tween with 5% non-fat milk or 5% BSA (for P-PLB) at 25°C for 1h. After incubating with the primary antibody, blots were washed 3x with TBS-tween for 10 minutes each time and incubated with a horseradish peroxidase (HRP) conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted in TBS-Tween with 5% nonfat milk or 5% BSA (for P-PLB) for 40min at 25°C. Horseradish peroxidase bound to immunoblot was visualized with Amersham ECL enhanced chemiluminescence (GE Healthcare, Buckinghamshire, UK) and Amersham ECL chemiluminescence film. Protein was quantified using densitometry.

Calcineurin assay

For calcineurin measurements, protein extracts were purified from freshly dispersed ENDO and EPI myocytes and quantified using the Bradford Assay[12]. Calcineurin activity in these cells was quantified using a Calcineurin Assay Kit (Promega, Madison, WI) as per the manufacturers instructions.

Statistics

Data are presented as mean ± standard error of the mean (SEM). Two-sample comparisons were made using Student's t-test. Multi-group comparisons were performed using an ANOVA, which,

if necessary, was followed by a Tukey test. A p value of less than 0.05 was used as an indicator of significance.

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