

***Pkd2* haploinsufficiency alters intracellular calcium regulation in vascular smooth muscle cells**

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Autosomal-dominant polycystic kidney disease is a multiorgan disease and its vascular manifestations are common and life-threatening. Despite this, little is known about their pathogenesis. Somatic mutations to the normal *PKD* allele in cystic epithelia and cyst development associated with the unstable *Pkd2*^{WS25} allele suggest a two-hit model of cystogenesis. However, it is unclear if this model can account for the cardiovascular pathology or if haploinsufficiency alone is disease-associated. In the present study, we found a decreased polycystin-2 (PC2, protein encoded by *Pkd2* gene) expression in *Pkd2*^{+/-} vessels, roughly half the wild-type level, and an enhanced level of intracranial vascular abnormalities in *Pkd2*^{+/-} mice when induced to develop hypertension. Consistent with these observations, freshly dissociated *Pkd2*^{+/-} vascular smooth muscle cells have significantly altered intracellular Ca²⁺ homeostasis. The resting [Ca²⁺]_i is 17.1% lower in *Pkd2*^{+/-} compared with wild-type cells ($P=0.0003$) and the total sarcoplasmic reticulum Ca²⁺ store (emptied by caffeine plus thapsigargin) is decreased ($P<0.0001$). The store operated Ca²⁺ (SOC) channel activity is also decreased in *Pkd2*^{+/-} cells ($P=0.008$). These results indicate that inactivation of just one *Pkd2* allele is sufficient to significantly alter intracellular Ca²⁺ homeostasis, and that PC2 is necessary to maintain normal SOC activity and the SR Ca²⁺ store in VSMCs. Based on these findings, and the fact that [Ca²⁺]_i signaling is essential to the regulation of contraction, production and secretion of extracellular matrix, cellular proliferation and apoptosis, we propose that the abnormal intracellular Ca²⁺ regulation associated with *Pkd2* haploinsufficiency is directly related to the vascular phenotype.

INTRODUCTION

Autosomal-dominant polycystic kidney disease (ADPKD) is characterized by progressive renal cystic disease, often resulting in renal failure. The disease, however, is systemic with extrarenal cysts and cardiovascular abnormalities (1). The vascular manifestations of ADPKD are common, including saccular intracranial aneurysms (ICA) and dolichoectasias, aortic root dilatation, dissection of the thoracic aorta and cervicocephalic arteries, and coronary artery aneurysms (1,2). The prevalence of ICA and the incidence of aneurysmal rupture in ADPKD are five to 10 times higher than in the general population and aneurysmal rupture carries a combined severe morbidity–mortality rate of ~50% (3). Familial clustering of

ICA has been observed in some ADPKD families (4,5). ADPKD is caused by mutations to either of two genes, *PKD1* and *PKD2* (6,7). The *PKD1* and *PKD2* encoded proteins, polycystin-1 and -2 (PC1 and PC2), are membrane-associated (8,9) and PC2 is a Ca²⁺-permeable channel that interacts with, and may be regulated by, PC1 (10–13). Somatic mutations to the normal *PKD* allele in cystic epithelia and cyst development associated with the unstable *Pkd2*^{WS25} allele suggest a two-hit model of cystogenesis (14,15). However, it is unclear if this model can account for the cardiovascular pathology (16), which is poorly understood, or if the heterozygous state is disease-associated. Both *PKD1* and *PKD2* mutations have been associated with ICAs (17–19). Expression of the polycystins in vascular smooth muscle cells (VSMCs) and vascular leakage

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and multiple focal hemorrhages in mouse embryos homozygous for *Pkd1* or *Pkd2* null mutations support a direct role for the polycystins in ADPKD associated vascular disease (16,20–23). The reduced life expectancy in *Pkd2*^{+/-} mice and early onset hypertension in *Pkd1*^{del34/+} mice without cystic renal disease also suggest that haploinsufficiency may play a role in some extrarenal manifestations of ADPKD (16,24). In the present study, we demonstrate that *Pkd2*^{+/-} mice have an increased susceptibility to intracranial vascular injury and *Pkd2*^{+/-} VSMCs have significantly altered intracellular Ca²⁺ homeostasis compared with wild-type.

RESULTS

Pkd2^{+/-} mice are more susceptible to intracranial vascular complications

To determine whether animals heterozygous for a null *Pkd2* mutation (truncation at exon 1) are more prone to develop intracranial vascular complications, 19 adult mice (10 wild-type and 9 *Pkd2*^{+/-}) underwent unilateral carotid ligation and induction of hypertension (see Methods for details). All animals tolerated the surgical procedure without immediate mortality, and blood pressures, measured at 2.5 months following the surgery, were similar in both groups (systolic blood pressure = 168 ± 12 mmHg by the tail cuff method). However, three *Pkd2*^{+/-} mice died at 1.5–2.5 months after the induction of hypertension (autopsies were not possible), while none of the wild-type mice died before sacrifice. All surviving animals were sacrificed at 3 months after the onset of hypertension. As shown in Figure 1 and Table 1, five of the six *Pkd2*^{+/-} mice had detectable cerebral arterial lesions on gross examination. Only one wild-type animal had minimal middle cerebral artery dilatation with no change in the vessel wall thickness. These results indicate that the *Pkd2*^{+/-} mice have a significantly increased risk of intracranial vascular complications compared with wild-type animals when placed under hemodynamic stress.

Pkd2^{+/-} vascular smooth muscle has decreased PC2 expression

PC2 is expressed in VSMCs (21). To ascertain the level of PC2 expression in vessels from *Pkd2*^{+/-} mice, quantitative western analysis was carried out using proteins isolated from freshly dissected aortic tunica media layer. PC2 was detected as a 110 kDa band using a polyclonal antibody YCB, as previously reported (9). *Pkd2*^{+/-} vascular smooth muscles were found to express roughly half the amount of PC2 compared to wild-type vessels, consistent with their hemizygous state ($n = 3$, Fig. 2A and B). From the same blots, analysis of calponin, a marker of the contractile phenotype, and smooth muscle α -actin confirmed the vascular smooth muscle phenotype (Fig. 2C). To confirm the decreased PC2 expression in *Pkd2*^{+/-} vascular smooth muscle at the mRNA level, real-time PCR was carried out using tunica media layers from thoracic aortas isolated from wild-type and *Pkd2*^{+/-} littermates. Consistent with the results from the quantitative western analysis, the *Pkd2* mRNA levels in *Pkd2*^{+/-} vessels (1.53 ± 0.23 , *Pkd2* normalized to cyclophilin,

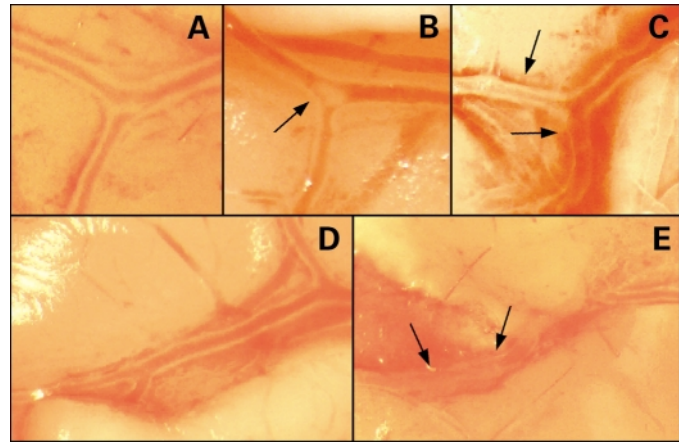


Figure 1. Junctions of the anterior, middle and posterior cerebral arteries from a wild-type (A) and two *Pkd2*^{+/-} mice (B and C). Note the junctional dilatation (B) (arrow) and the luminal dilatation and the irregular thickening and thinning of the arterial wall (C, arrow) in the *Pkd2*^{+/-} vessels. Anterior cerebral arteries from wild-type (D) and a *Pkd2*^{+/-} (E) mice. Note the thinning of the wall, the dilatation of the lumen and the local hemorrhage in the *Pkd2*^{+/-} vessel (arrows).

Table 1. Vascular abnormalities in *Pkd2*^{+/-} mice

ID no.	ACA	MCA	PCA	Junction, ACA/PCA	Ant/post communicant
1	WT, LD		WT		WT, LD
2	LD	WT	WT		
3	WT		WT	LD	
4	WT, LD				WT, LD
5			WT, LD		WT

ACA, MCA and PCA, anterior, medial and posterior cerebral arteries. WT, wall thickening and/or thinning; LD, luminal dilation.

$n = 6$) were about one-half of those measured in wild-type vessels (4.58 ± 0.72 , $n = 6$).

Pkd2^{+/-} VSMCs have decreased resting [Ca²⁺]_i and the capacity of the SR Ca²⁺ store

PC2 has been located in the endoplasmic reticulum (ER) membrane and the overexpression of PC2 in PKD2-transfected LLCPK cells caused an increase in the vasopressin-induced Ca²⁺ release from the ER (12). To determine whether a reduced expression of PC2 in VSMCs is associated with altered intracellular Ca²⁺ regulation, the resting [Ca²⁺]_i and the capacity of the sarcoplasmic reticulum (SR) Ca²⁺ store in freshly dissociated *Pkd2*^{+/-} and wild-type VSMCs were examined. Freshly dissociated cells were chosen because VSMCs in adult animals have an extremely low turnover rate and remain in the contractile phenotype. Fresh dissociation avoids the changes of cellular phenotype (from contractile to proliferative) inevitably associated with primary culture (25). The results represented here therefore most closely reflect their function *in vivo*.

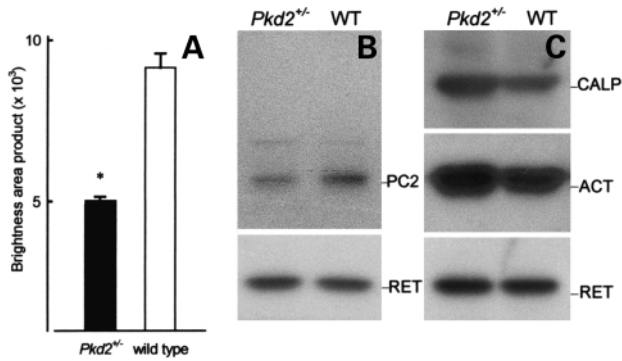


Figure 2. Densitometric analysis ($n = 3$, **A**) and quantitative western blots of polycystin 2 (PC2, **B**), calponin (CALP, **C**), smooth muscle α -actin (ACT, **C**) using protein preparations from $Pkd2^{+/-}$ and wild-type aortic smooth muscles. A calreticulin (RET) antibody was used to verify equal loading of the samples. The expression of PC2 in $Pkd2^{+/-}$ vessels is approximately half of that in wild-type vessels. The expression of calponin and α -actin was slightly increased in $Pkd2^{+/-}$ smooth muscle.

Immunostaining for calponin and/or α -actin and 4',6-diamidino-2-phenylindole (DAPI) nuclear staining were performed in aliquots of dissociated cells prior to experiments (Fig. 3A) and a high purity (99%) of VSMCs was demonstrated. Ruptured cells, determined by positive α -actin/calponin and negative nuclear staining, were rare (less than 1%), indicating that the vast majority of dissociated cells were structurally intact. $Pkd2^{+/-}$ and wild-type VSMCs were loaded with the Ca^{2+} indicator fluo 3 AM in Tyrodes solution (see Methods for its composition) containing 2 mM Ca^{2+} for the resting $[\text{Ca}^{2+}]_i$ measurement. As shown in Figure 3B, the resting $[\text{Ca}^{2+}]_i$ was 17.1% lower in $Pkd2^{+/-}$ ($n = 78$) than in wild-type cells ($n = 64$), a highly significant difference ($P = 0.0003$). The functional integrity of these cells was verified by their responsiveness to caffeine or cyclopiazonic acid stimulation after the resting $[\text{Ca}^{2+}]_i$ measurement (data not shown).

To assess the effect of $Pkd2$ hemizyosity on the capacity of the SR Ca^{2+} store, VSMCs were stimulated with caffeine (5 mM) plus thapsigargin (1 μM) in zero Ca^{2+} perfusate. These concentrations are used routinely by many groups including ours to deplete the SR Ca^{2+} store in VSMCs (26,27). As shown in Figure 4A and B, the total SR Ca^{2+} store emptied by caffeine plus thapsigargin was significantly lower in the $Pkd2^{+/-}$ ($n = 72$) than in the wild-type VSMCs ($n = 48$, $P < 0.0001$). These results indicate that the $Pkd2$ haploinsufficiency has a profound effect on the resting $[\text{Ca}^{2+}]_i$ and the capacity of the SR Ca^{2+} store.

$Pkd2^{+/-}$ VSMCs have reduced SOC channel activity

PC2 is known to directly associate with the transient receptor potential channel 1 (TRPC1) (28), a plasma membrane-spanning subunit of the SOC channel in VSMCs (29). Adequate store-operated calcium (SOC) channel activity is critical to the SR Ca^{2+} repletion and store (30,31). To determine whether the decreased SR Ca^{2+} store observed in $Pkd2^{+/-}$ cells is due to reduced SOC activity, we first depleted

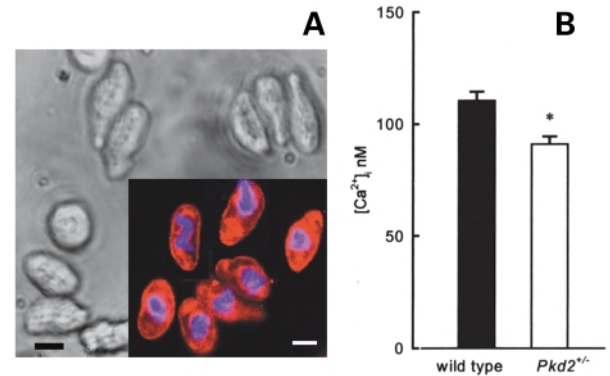


Figure 3. (A) Freshly dissociated VSMCs from the tunica media layer of $Pkd2^{+/-}$ aortas. The insert shows VSMCs stained with α -smooth muscle actin to confirm the VSMC phenotype and DAPI nuclear staining to confirm structural integrity. Bars represents 10 μm . (B) Resting $[\text{Ca}^{2+}]_i$ in wild-type ($n = 64$) and $Pkd2^{+/-}$ VSMCs ($n = 78$). The results are expressed as means \pm SEM. The mean resting $[\text{Ca}^{2+}]_i$ was 17.1% lower in the $Pkd2^{+/-}$ VSMCs (91.0 ± 3.4 versus 109.8 ± 4.0 nM, $P = 0.0003$).

the SR Ca^{2+} store by perfusing the cells with caffeine (5 mM) plus thapsigargin (1 μM) in zero Ca^{2+} Tyrodes containing nifedipine (1 μM). After the SR was depleted, Ca^{2+} (2 mM) was then added into the perfusate. SOC activity was detected as an increase in $[\text{Ca}^{2+}]_i$. As shown in Figure 5A and B, $Pkd2^{+/-}$ VSMCs ($n = 15$) exhibited a significant decrease in SOC activity compared to wild-type cells ($n = 12$, $P = 0.008$). These results indicate that the decreased PC2 expression is associated with abnormal SOC channel activity and this abnormality is probably responsible for the decreased capacity of the SR Ca^{2+} store in $Pkd2^{+/-}$ VSMCs.

DISCUSSION

This study demonstrates that inactivation of just a single $Pkd2$ allele is associated with the expected dosage reduction of PC2 and significantly alters intracellular Ca^{2+} homeostasis. The level of PC2 also alters the SOC channel activity and the characteristics of the SR Ca^{2+} store in VSMCs.

PC2, in addition to being localized in the ER membrane, has recently been localized to the plasma membrane (32,33) and primary cilia (34). In this location, Ca^{2+} channel activity has been demonstrated in response to mechanosensation, which could be blocked using a specific PC2 antibody (34). In the present study, we showed that in VSMCs a reduction of PC2 expression resulted an decreased SOC channel activity, consistent with the reported finding that PC2 interacts with a SOC channel protein (28). This interaction could occur either via a conformational coupling between PC2 in the SR membrane and SOC channel protein in the adjacent plasma membrane (analogous to the coupling between inositol 1,4,5-triphosphate receptor in the ER/SR and TRPC in the plasma membrane) (35,36) and/or via an interaction with both located in the plasma membrane. Using primary cultured wild-type VSMCs, we found that PC2 is mainly localized in the SR membrane by immunohistochemistry and cell surface biotinylation (37), although a plasma membrane fraction of PC2 below the level of detection by these techniques could not be

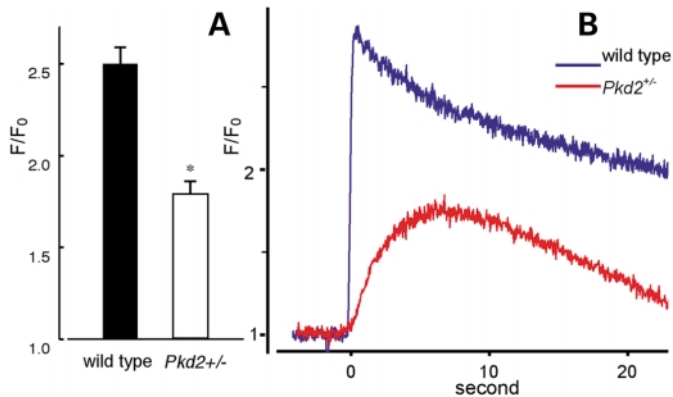


Figure 4. Caffeine plus thapsigargin induced SR Ca^{2+} release in wild-type ($n=48$) and $Pkd2^{+/-}$ ($n=72$) VSMCs in zero Ca^{2+} Tyrodes perfusate. (A) Peak F/F_0 (the ratio of the maximum increment $[\text{Ca}^{2+}]_i$ -dependent fluorescent intensity over pre-stimulus fluorescent intensity) following caffeine (5 mM) plus thapsigargin (1 μM) stimulation (wild-type 2.50 ± 0.61 , $Pkd2^{+/-}$ 1.79 ± 0.07 , $P < 0.00001$). The results are expressed as means \pm SEM. (B) Representative tracings of $[\text{Ca}^{2+}]_i$ responses to caffeine plus thapsigargin in wild-type and $Pkd2^{+/-}$ VSMCs.

excluded. Because SOC channel activity is critical to the Ca^{2+} repletion of the SR (30,38), it is likely that the reduced SOC channel activity associated with the decreased level of PC2 results in the reduced capacity of the SR Ca^{2+} store.

Ca^{2+} signaling is essential to the regulation of contraction, production and secretion of extracellular matrix, cellular proliferation and apoptosis (39,40). $Pkd2$ haploinsufficiency and its associated abnormal intracellular Ca^{2+} regulation in VSMCs observed in this study are probably linked to the vascular phenotype in ADPKD. Elucidating the precise mechanism by which reduced PC2 levels resulted in these fundamental changes in Ca^{2+} homeostasis will help the understanding of the vascular lesions in ADPKD and the general role of PC2.

MATERIALS AND METHODS

$Pkd2^{+/-}$ mice and genotyping

The $Pkd2$ gene targeting strategy and the generation of $Pkd2^{+/-}$ mice with a true null $Pkd2$ allele has been reported (15). In this study $Pkd2^{+/-}$ mice were crossed with C57BL/6 mice to generate wild-type and $Pkd2^{+/-}$ animals. DNA was isolated from a small section of tail using the QIAamp tissue kit (Qiagen) following the protocol by the manufacturer. Genomic DNA was analyzed by PCR with two pairs of specific primers that amplify the wild-type and mutant alleles as previously reported (15).

Induction of intracranial vascular lesions

Six- to 9-month-old, littermate wild-type (five male, five female) and $Pkd2^{+/-}$ (five male, four female) mice underwent a right carotid artery ligation, left nephrectomy and subcutaneous implantation of a deoxycorticosterone acetate (DOCA) tablet (50 mg, 3 months sustained release, Innovative Research of America, Sarasota, FL, USA). Postoperatively, the animals were fed regular chow and given 1% sodium chloride solution

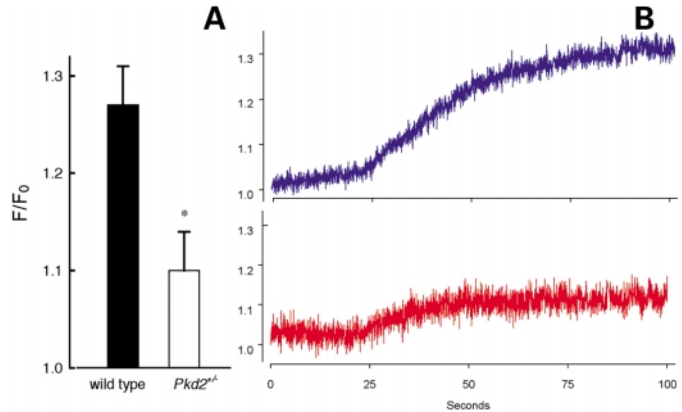


Figure 5. Ca^{2+} uptake via store operated channels (SOCs) after SR Ca^{2+} depletion in wild-type ($n=12$) and $Pkd2^{+/-}$ ($n=15$) VSMCs. (A) Maximal F/F_0 after reintroducing 2 mM extracellular Ca^{2+} following the depletion of SR by caffeine plus thapsigargin (wild-type 1.27 ± 0.05 vs $Pkd2^{+/-}$ 1.10 ± 0.04 , $P=0.008$). The L-type channels were blocked by nifedipine (1 μM). The results are expressed as means \pm SEM. (B) Representative tracings of Ca^{2+} intake in wild-type (blue) and $Pkd2^{+/-}$ (red) VSMCs.

to drink. All surviving animals were sacrificed 3 months later. Their brains and circles of Willis were dissected for examination. Contrary to rats, wild-type mice do not develop prominent intracranial vascular lesions with this protocol (41,42).

Western analysis

The aortic tunica media smooth muscle layer from wild-type and $Pkd2^{+/-}$ mice was minced into small pieces and homogenized using an Eberbach 114 V homogenizer in buffer containing (in mM) 150 NaCl, 5 EDTA, 50 Tris-HCl, pH 7.4, 1% Triton X-100, and an EDTA-free protease inhibitor mix at 4°C for 10 min. Post nuclear supernatants were obtained by spinning the homogenized whole cell lysates (1500g \times 10 min) and protein contents determined by a microtiter Lowry assay (Bio-Rad, Hercules, CA, USA). Five to 20 μg of protein were denatured in the sample buffer (125 mM Tris pH 6.8, 5% β -mercaptoethanol, 6% SDS, 20% glycerol and 0.2% bromophenol blue) at 65°C and subjected to SDS-PAGE on Tris-acetate 3–8% gradient gels (Invitrogen). Fractionated protein was electrotransferred to a PVDF membrane (Invitrogen) and detected with the appropriate antibodies using ECL chemiluminescence (Santa Cruz). The PC2 antibody (YCB9) was used to detect PC2 (9). All the primary and secondary antibodies were diluted to 1:2500. For the quantitative western analysis, the proteins from the wild-type and $Pkd2^{+/-}$ smooth muscles were prepared and fractionated in parallel under an identical experimental protocol and transferred onto a single blot. Hence, the ECL exposure times are identical.

Quantitative real-time PCR measurements

Total RNA was extracted from the tunica media of thoracic aortas using TRIzol reagent (Invitrogen). A total of 12 aortas, from six wild-type and six $Pkd2^{+/-}$ littermates, were used for this experiment. A 1.5 μg aliquot of total RNA was reverse-

transcribed using SuperScript First-Strand Synthesis System (Invitrogen) at 37°C for 1 h. The amount of *Pkd2* mRNA per vessel was quantified using the real-time PCR SYBR Green assay. The 50 µl PCR reaction contained 2 µl of diluted (10-fold) cDNA, 1× SYBR Green JumpStart Tag ReadyMix (Sigma), and 200 nM cyclophilin specific primers (5'-GCTGTC-TCTTTTCGCCGC-3', 5'-CCGTGATGTCGAAGAACACG-3') and *Pkd2* primers (5'-TGTGGTCAGGTTATTGGCGGAGTT-3', 5'-GACATAGCGGATCAGTTTTACAGG-3'). PCR reactions were performed in the DNA Engine Opticon System (MJ Research) with 40 cycles at 95°C for 40 s and 60°C for 1 min. All data were represented as relative to the Cyclophilin level.

Dissociation of VSMCs from the thoracic aorta

The age- and sex-matched (6–8 weeks) wild-type and *Pkd2*^{+/-} mice were anesthetized with a mixture of ketamine (110 mg/kg) and xylazine (10 mg/kg; i.m.); the aorta was removed and placed in ice-cold, oxygenated Hanks' balanced salt solution (HBSS), buffered with 10 mM HEPES (pH 7.4). A total of 19 mice (10 *Pkd2*^{+/-} and nine wild-type littermates with C57/Blk6 background) were used for all the calcium experiments. Isolated aortic tunica media layers were finely minced after scraping off the intima and adventitia layers. VSMCs were isolated using a commercial kit (Papain dissociation system, Worthington Biochemical) modified from the protocol suggested by the manufacturer. Briefly, the minced smooth muscle was suspended in Earle's balanced salt solution (EBSS) containing papain (10 U/ml) and Dnase (1000 U/ml) and incubated at 37°C × 10 min. Elastase (0.05 mg/ml) and collagenase (0.5 mg/ml) were added and the mixture was gently triturated and bubbled with oxygen. The dissociated VSMCs were collected 8 and 10 min later and plated onto the collagen-coated glass cover-slip for study.

Immunofluorescent staining and confocal microscopy

Cells seeded on collagen coated glass slides were fixed with 2.5% freshly made paraformaldehyde (pH 7.6) and permeabilized when indicated with methanol and acetone (at 3:1 ratio) for 10 min. After washing with PBS (3×), primary antibodies (in PBS with 10% goat serum) were added and incubated for 45 min. The slides were washed (3× PBS) and FITC or TRD-conjugated secondary antibodies added (Santa Cruz). Controls included cells handled in the same fashion except stained with only primary or only secondary antibodies. The cells were examined using a confocal immunofluorescent microscope.

Global [Ca²⁺]_i measurements and recording techniques

VSMCs, plated on cover-slip, were loaded with the cytoplasmic Ca²⁺ indicator fluo 3-AM (5 µM, Molecular Probes) for 45 min at 37°C in EBSS. After rinsing in normal Tyrodes medium containing (in mM): 145 NaCl, 4 KCl, 1 MgCl₂, 2 CaCl₂, 10 HEPES (pH 7.4). The cover-slip was mounted on the stage of a Nikon Diaphot inverted microscope and perfused with normal Tyrodes at 4 ml/min at room temperature. The calibration and

the techniques of the [Ca²⁺]_i measurement using real-time confocal imaging has been described in detail previously (43). For the caffeine and thapsigargin stimulation, the cells were first perfused with zero Ca²⁺ Tyrodes for 2 min before the introduction of caffeine (5 mM) and thapsigargin (1 µM) in zero Ca²⁺ Tyrodes at 4 ml/min.

Statistical analysis

All values are presented as mean ± SEM as determined by Student's *t*-test. A *P*-value of <0.05 was considered statistically significant.

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