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Role of Protein Kinase Cζ and Its Adaptor Protein p62 in Voltage-Gated Potassium Channel Modulation in Pulmonary Arteries

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ABSTRACT

Voltage-gated potassium (K_V) channels play an essential role in regulating pulmonary artery function, and they underpin the phenomenon of hypoxic pulmonary vasoconstriction. Pulmonary hypertension is characterized by inappropriate vasoconstriction, vascular remodeling, and dysfunctional K_V channels. In the current study, we aimed to elucidate the role of PKCζ and its adaptor protein p62 in the modulation of K_V channels. We report that the thromboxane A_2 analog 9,11-dideoxy-11α,9α-epoxymethano-prostaglandin $F_{2\alpha}$ methyl acetate (U46619) inhibited K_V currents in isolated mice pulmonary artery myocytes and the K_V current carried by human cloned K_V1.5 channels expressed in Ltk$^-$ cells. Using protein kinase C (PKC)$\zeta^{-/-}$ and p62$^{-/-}$ mice, we demonstrate that these two proteins are involved in the K_V channel inhibition. PKCζ coimmunoprecipitated with K_V1.5, and this interaction was markedly reduced in p62$^{-/-}$ mice. Pulmonary arteries from PKC$\zeta^{-/-}$ mice also showed a diminished $[Ca^{2+}]_i$ and contractile response, whereas genetic inactivation of p62$^{-/-}$ resulted in an absent $[Ca^{2+}]_i$ response, but it preserved contractile response to U46619. These data demonstrate that PKCζ and its adaptor protein p62 play a key role in the modulation of K_V channel function in pulmonary arteries. These observations identify PKCζ and/or p62 as potential therapeutic targets for the treatment of pulmonary hypertension.

Voltage-gated potassium (K_V) channels play an essential role in regulating vascular smooth muscle function. They make a substantial contribution to whole-cell K^+ conductance and resting membrane potential in pulmonary artery smooth muscle cells (PASMCs), and its inhibition causes membrane depolarization, activation of L-type Ca^{2+} channels (Ca_L), increases in $[Ca^{2+}]_i$, and vasoconstriction (Barnes and Liu, 1995; Archer et al., 1998; Yuan et al., 1998b). These channels are common targets of pulmonary vasoconstrictor stimuli such as hypoxia, thromboxane A_2 (TXA_2), 5-hydroxytryptamine, endothelin-1 or angiotensin-II (Archer et al., 1998; Shimoda et al., 2001; Cogolludo et al., 2003, 2006). In addition, decreased expression or function of K_V channels in PASMCs has been involved in the pathogenesis of pulmonary arterial hypertension (PH) (Weir et al., 1996, Yuan et al., 1998a; Pozeg et al., 2003). From the variety of K_V channels expressed in PASMC (Platoshyn et al., 2006), special interest has been paid to K_V1.5, because decreased expression or activity and mutations of K_V1.5 occur in human (Yuan et al., 1998a; Remillard et al., 2007) and experimental (Archer et al., 1998; Pozeg et al., 2003) idiopathic and hypoxic PH, and in vivo gene transfer of K_V1.5 reduces PH (Pozeg et al., 2003).

TXA_2 is a prostanoid synthesized by cyclooxygenase with potent vasoconstrictor, mitogenic, and platelet aggregant properties via activation of thromboxane-endoperoxide (TP) receptors (Halushka et al., 1989). The vasoconstrictor effects of TXA_2 are particularly pronounced in the pulmonary vascular bed, where it participates in the control of vessel tone under physiological and pathological situations, including PH. We have previously reported that in intact PAs and freshly isolated PASMCs, TXA_2, via activation of TP receptors, inhibits K_V channels, leading to membrane depolariza-

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ABBREVIATIONS: K_V, voltage-gated K^+; PASMC, pulmonary artery smooth muscle cell; Ca_L, voltage-dependent L-type Ca^{2+} channel; TXA_2, thromboxane A_2; PH, pulmonary hypertension; TP, thromboxane-endoperoxide; PKC, protein kinase C; PA, pulmonary artery; IP, inhibitory peptide; aPKC, atypical PKC; PSS, physiological salt solution; h, human; U46619, 9,11-dideoxy-11α,9α-epoxymethano-prostaglandin $F_{2\alpha}$; DPO-1, [1S-1α,2α,5β]-[5-methyl-2-(1-methylethyl) cyclohexyl] diphenyl phosphine oxide.

tion, activation of L-type Ca^{2+} channels, and vasoconstriction. Furthermore, using a protein kinase C (PKC)ζ pseudosubstrate inhibitory peptide (PKCζ-PI), we provided evidence for the role of this kinase as a link between TP receptor activation and K_V channel inhibition (Cogolludo et al., 2003, 2005). PKCζ (together with PKCλ/ι) belongs to the atypical PKC (aPKC) subclass. Both aPKCs play key roles in different signaling pathways regulating cell growth, survival, and differentiation (Moscat and Díaz-Meco, 2000). The aPKCs share with other members of their family a conserved catalytic domain, but they display a clearly distinct regulatory region because they have been shown to be independent of Ca^{2+}, diacylglycerol, and phorbol esters, all of which are potent activators of other PKC isoforms. PKCζ is activated by phosphatidylinositols, arachidonic acid, and other lipids (Hirai and Chida, 2003) as well as by a variety of mediators, including insulin (Liu et al., 2006), thromboxane A_2 (Shizukuda and Buttrick, 2002; Cogolludo et al., 2003, 2005), angiotensin II (Gayral et al., 2006; Godeny and Sayeski, 2006), or proinflammatory cytokines (Frey et al., 2006).

The mechanism underlying the activation of aPKCs responsible for its diverse physiological functions remains unclear, but several groups have identified a number of aPKC-interacting proteins, including p62 (also called ZIP1 or sequestosome 1), Par-4, Par-6, and MEK5 (Moscat and Diaz-Meco, 2000). It is noteworthy that nerve growth factor and catecholamines have been reported to increase the expression of p62, enabling the formation of the PKCζ-p62-$K_V\beta$ complex, which results in a hyperpolarizing shift in the K_V current activation curve (Gong et al., 1999; Kim et al., 2004, 2005).

The role of PKC on pulmonary vasoconstriction has been widely reported (Ward et al., 2004); however, many of these studies have been conducted with PKC modulators of dubious selectivity, thereby limiting their conclusions. Molecular biology and genetic approaches and the currently available isoform-selective PKC inhibitors have made possible the elucidation of the involvement of specific PKC isoforms in cellular processes (such as vascular contractility) (Salamanca and Khalil, 2005). However, recent evidence suggests that some considered isoform-specific PKC inhibitors, such as myristoylated PKCζ pseudosubstrate peptide, may exert other effects unrelated to inhibition of PKC; thus, they should be used with caution (Krotova et al., 2006).

Therefore, in the present study, we aimed to further characterize the signaling pathway modulating K_V currents in PAs. Using PKC$\zeta^{-/-}$ and p62$^{-/-}$ mice, we provide evidence for the interaction of PKCζ with K_V channels, which further support the role of this interaction in TXA_2-induced effects. In addition, we hypothesized that the PKCζ-K_V-L-type Ca^{2+} channels pathway might involve other proteins such as p62. This possibility was tested by analyzing the modulation of K_V channels in wild-type and p62 homozygous null mice.

Materials and Methods

All experiments were carried out in accordance with the European Animals Act 1986 (Scientific Procedures), and they were approved by our institutional review board.

Animals. Lungs from PKC$\zeta^{-/-}$ (mixed C57BL/6 and SV129J background), p62$^{-/-}$ (C57BL/6), and corresponding wild-type mice (6–8 weeks old; either sex) were generously supplied by Drs. J. Moscat and M. T. Diaz-Meco (both from the Genome Research Institute, University of Cincinnati, Cincinnati, OH). These mice were generated as described previously (Leitges et al., 2001; Duran et al., 2004). PAs from male Wistar rats (250–300 g) were also used in these experiments.

Tissue Preparation and Cell Isolation. Second-order branches of the PA (internal diameter, ≤0.5 mm) isolated from mice were dissected into a nominally calcium-free physiological salt solution (PSS) of the following composition: 130 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES, pH 7.3, with NaOH. Endothelium denuded PAs were cut into small segments (2 × 2 mm), and cells were isolated in Ca^{2+}-free PSS containing 1 mg/ml papain, 0.8 mg/ml dithiothreitol, and 0.7 mg/ml albumin. Cells were stored in Ca^{2+}-free PSS (4°C) and used within 8 h of isolation.

Electrophysiological Studies. Membrane currents were measured using the whole-cell configuration of the patch-clamp technique (Cogolludo et al., 2003) normalized for cell capacitance and expressed in picoamperes per picofarad. Membrane potential (E_m) was measured under current-clamp configuration. K_V currents were recorded under essentially Ca^{2+}-free conditions using an external Ca^{2+}-free PSS and a Ca^{2+}-free pipette (internal) solution (see Solutions and Chemicals). Ltk^- cells stably expressing hK_V1.5 channels (Valenzuela et al., 1995) were superfused with PSS containing 1 mM $CaCl_2$. Currents were evoked after the application of 200-ms depolarizing pulses from −60 mV to test potentials from −60 to +40 mV in 10-mV increments. All experiments were performed at room temperature (22–24°C).

$[Ca^{2+}]_i$ Recording. PA rings were incubated for 80 min at room temperature in Krebs' solution containing the fluorescent dye fura-2 acetoxymethyl ester (5×10^{-6} M) and 0.05% cremophor EL, and then they were mounted in a fluorimeter (model CAF 110; Jasco, Tokyo, Japan). PA rings were alternatively illuminated (128 Hz) with two excitation wavelengths (340 and 380 nm), and the emitted fluorescence was filtered at 505 nm (Pérez-Vizcaíno et al., 1999). The ratio of emitted fluorescence (F_{340}/F_{380}) obtained at the two excitation wavelengths was used as an indicator of $[Ca^{2+}]_i$. Arteries were stimulated with 30 and 300 nM U46619, added in a cumulative manner. In preliminary experiments in wild-type mice, these concentrations produced ~60 and ~80% of the maximal response, respectively. The $[Ca^{2+}]_i$ signal in each vessel was calibrated according to the Grynkiewicz equation by sequential addition of 15 μM ionomycin and 10 mM EGTA at the end of the experiment.

Coimmunoprecipitation and Western Blot Analysis. Mice lungs were rapidly frozen in liquid nitrogen. In some experiments, rat PA were placed in warm Krebs' solution and then in the absence or presence of 1 μM U46619 for 30 s and then rapidly frozen. Frozen tissues were homogenized in a glass potter in 200 μl of a buffer of the following composition: 10 mM HEPES, pH 8, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 6 μM aprotinin, 9 μM leupeptin, 11 μM *N*α-*p*-tosyl-L-lysine chloromethyl ketone, 5 mM NaF, 10 mM Na_2MoO_4, 1 mM $NaVO_4$, 0.5 mM phenylmethanesulfonyl fluoride, and 10 nM okadaic acid. Homogenates were centrifuged at 13,000*g* for 5 min at 4°C, and the supernatant fraction was collected. For immunoprecipitation, 60 μg of protein was incubated for 2 h with anti-PKCζ or anti-K_V1.5 antibody at 4°C, followed by the addition of protein A/G beads and further incubation overnight. These immune complexes or 20 μg of the homogenates from mice lungs or rat PA were separated by SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane for Western blotting as described previously (Cogolludo et al., 2003). Membranes were probed for K_V1.5-, PKCζ-, and p62-like immunoreactivity.

Solutions and Chemicals. For the single cell electrophysiological studies, the composition of the Ca^{2+}-free bath solution (external Ca^{2+}-free PSS) was as follows: 130 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES, buffered to pH 7.3 with NaOH. The Ca^{2+}-free pipette (internal) solution contained 110 mM KCl, 1.2 mM $MgCl_2$, 5 mM Na_2ATP, 10 mM HEPES, and 10 mM EGTA, pH adjusted to 7.3 with KOH. The Krebs' solution used for

tissue bath experiments included 118 mM NaCl, 4.75 mM KCl, 25 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 2.0 mM $CaCl_2$, 1.2 mM KH_2PO_4, and 11 mM glucose. This solution was gassed with a 95% O_2, 5% CO_2 mixture at 37°C. U46619 was obtained from Sigma Chemical Co. (Tres Cantos, Spain). [1*S*-1α,2α,5β]-[5-Methyl-2-(1-methylethyl) cyclohexyl] diphenyl phosphine oxide (DPO-1) was from Tocris Cookson Inc. (Bristol, UK), secondary horseradish peroxidase-conjugated antibodies and fura-2 acetoxymethyl ester were from Calbiochem (Barcelona, Spain), rabbit anti-K_V1.5 was from Alomone Labs (Jerusalem, Israel), goat anti-PKCζ was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and guinea pig anti-p62 was from Progen (Heidelberg, Germany).

Statistical Analysis. Data are expressed as means ± S.E.M.; *n* indicates the number of arteries or cells tested. All experiments were conducted in arteries or cells from at least four different animals. Statistical analysis was performed using Student's *t* test for paired or unpaired observations. Differences were considered statistically significant when *p* was less than 0.05.

Results

Role of PKCζ in K_V Current Inhibition Induced by TXA_2. A family of K_V currents [$I_{K(V)}$] were obtained in mice PASMCs when eliciting depolarizing steps from −60 to +40 mV (Fig. 1, A and B) from a holding potential of −60 mV. The magnitude of the currents, the threshold voltage for activation, and the current-voltage relationship (Fig. 1, C and D) was similar in PASMCs from wild-type and PKCζ$^{-/-}$ mice (e.g., current density at +40 mV was 9.1 ± 1.9 and 8.7 ± 0.8 pA/pF, respectively). Current inactivation was also similar in both strains (i.e., at 200 ms, the current decayed by 11.5 ± 3 and 12.1 ± 3.8%, respectively). Currents were recorded before (control) and after addition of the TXA_2 analog U46619. U46619 (100 nM) caused a significant inhibition of K_V currents in the whole range of channel activation in PASMCs from wild-type mice (Fig. 1A). The degree of current inactivation at +40 mV was increased by U46619 (i.e., at 200 ms, the current decayed by 25.6 ± 4.8%; $p < 0.05$). In addition, U46619 induced membrane depolarization in wild-type PASMCs (Fig. 1E). However, U46619 had no effect on either K_V currents or membrane potentials in PASMC from PKCζ$^{-/-}$ mice (Fig. 1, B, D, and F).

Role of PKCζ in $[Ca^{2+}]_i$ Increase and Contraction Induced by TXA_2. Changes in $[Ca^{2+}]_i$ and contraction in-

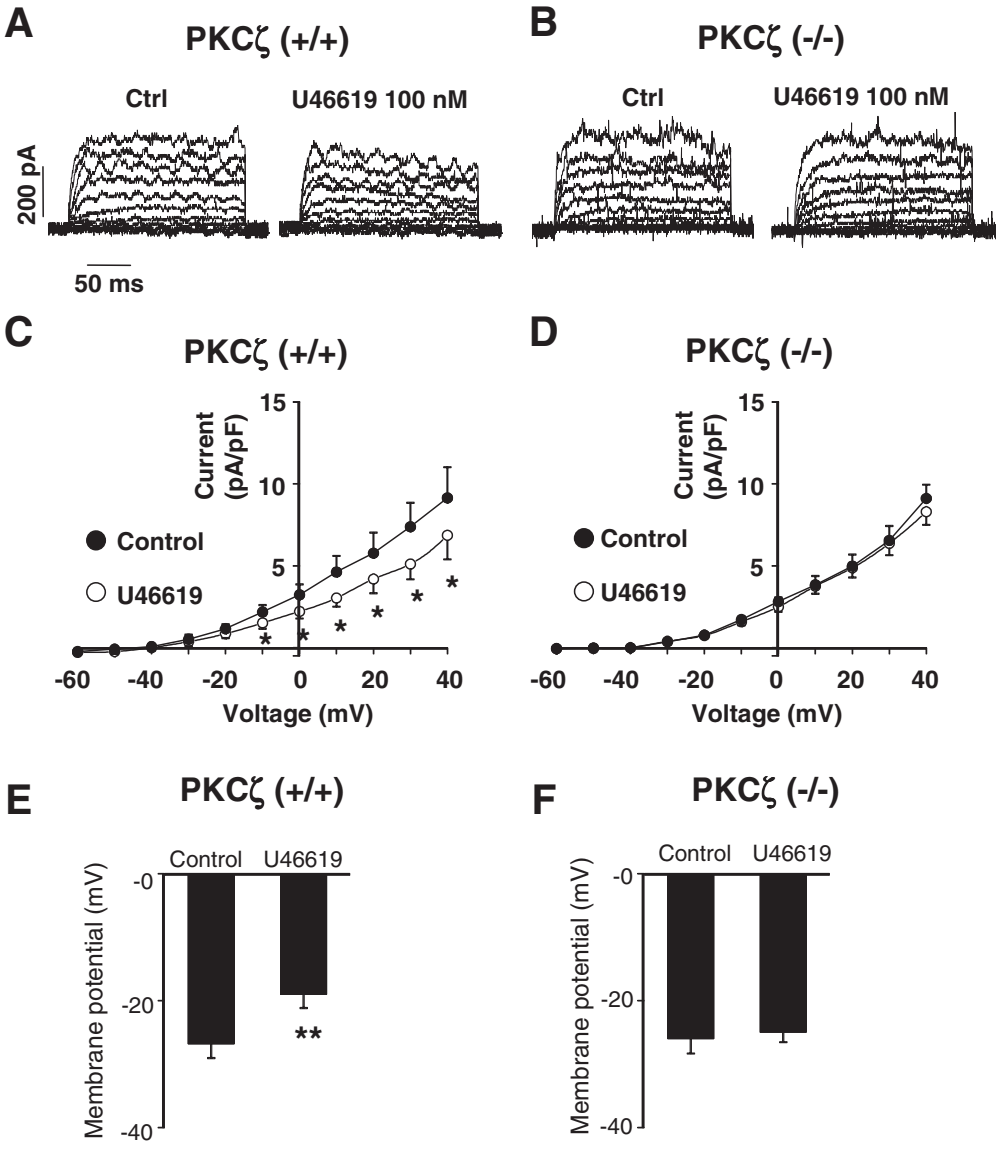

Fig. 1. TP receptor activation leads to K_V current inhibition and depolarization in PASMCs from PKCζ$^{+/+}$ (A, C, and E) but not from PKCζ$^{-/-}$ (B, D, and F) mice. A and B, current traces for 200-ms depolarization pulses from −60 to +40 mV (in 10-mV increments) from a holding potential of −60 mV before (control) and after application of the TXA_2 analog U46619 (100 nM). C and D, current-voltage relationship measured at the end of the 200-ms pulse (means ± S.E.M. of five cells). E and F, effects of 100 nM U46619 on membrane potential recorded under current clamp conditions. *, $p < 0.05$ and **, $p < 0.01$, respectively, versus control (paired Student's *t* test).

duced by U46619 were simultaneously analyzed in fura-2-loaded PAs from wild-type and from PKC$\zeta^{-/-}$ mice. Basal levels of $[Ca^{2+}]_i$ in PKC$\zeta^{-/-}$ (203 ± 40 nM; $n = 6$) were not significantly different from those in wild-type mice (160 ± 40 nM; $n = 6$). Stimulation of endothelium-denuded PA rings with 30 and 300 nM U46619 induced a sustained elevation in $[Ca^{2+}]_i$ and a contractile response in PAs from wild-type and PKC$\zeta^{-/-}$ animals (Fig. 2, A and B). However, the increase in $[Ca^{2+}]_i$ (Fig. 2C) and the contractile response (Fig. 2D) was significantly reduced in PKC$\zeta^{-/-}$ mice compared with wild-type mice.

Role of p62 in K_V Current Inhibition, $[Ca^{2+}]_i$ Increase, and Contraction. To analyze the functional role of p62 and the PKCζ-p62-K_V1.5 interaction, we analyzed the effects of 100 nM U46619 on K_V currents in p62$^{-/-}$ and the corresponding wild-type mice. The magnitude of the currents, the threshold voltage for activation, the current-voltage relationship, and the current inactivation (Fig. 3, C and D) were similar in PASMCs from wild-type and p62$^{-/-}$ mice (e.g., current density at +40 mV was 10.9 ± 1.3 and 11.6 ± 1.7 pA/pF, respectively; and at 200 ms, current decayed by 12.7 ± 2.8 and 14.3 ± 2.9%, respectively). As expected, U46619 caused a significant inhibition in the whole range of channel activation and depolarized the membrane in PASMCs from wild-type mice (Fig. 3, A, C, and E). Current inactivation at +40 mV was also increased by U46619 (i.e., at 200 ms, the current decayed by 21.7 ± 2.9%; $p < 0.05$). However, the TXA_2 analog had no effect on K_V currents in PASMCs from p62$^{-/-}$ mice (Fig. 3, B, D, and E).

Basal levels of $[Ca^{2+}]_i$ in p62$^{-/-}$ (184 ± 35 nM; $n = 5$) were

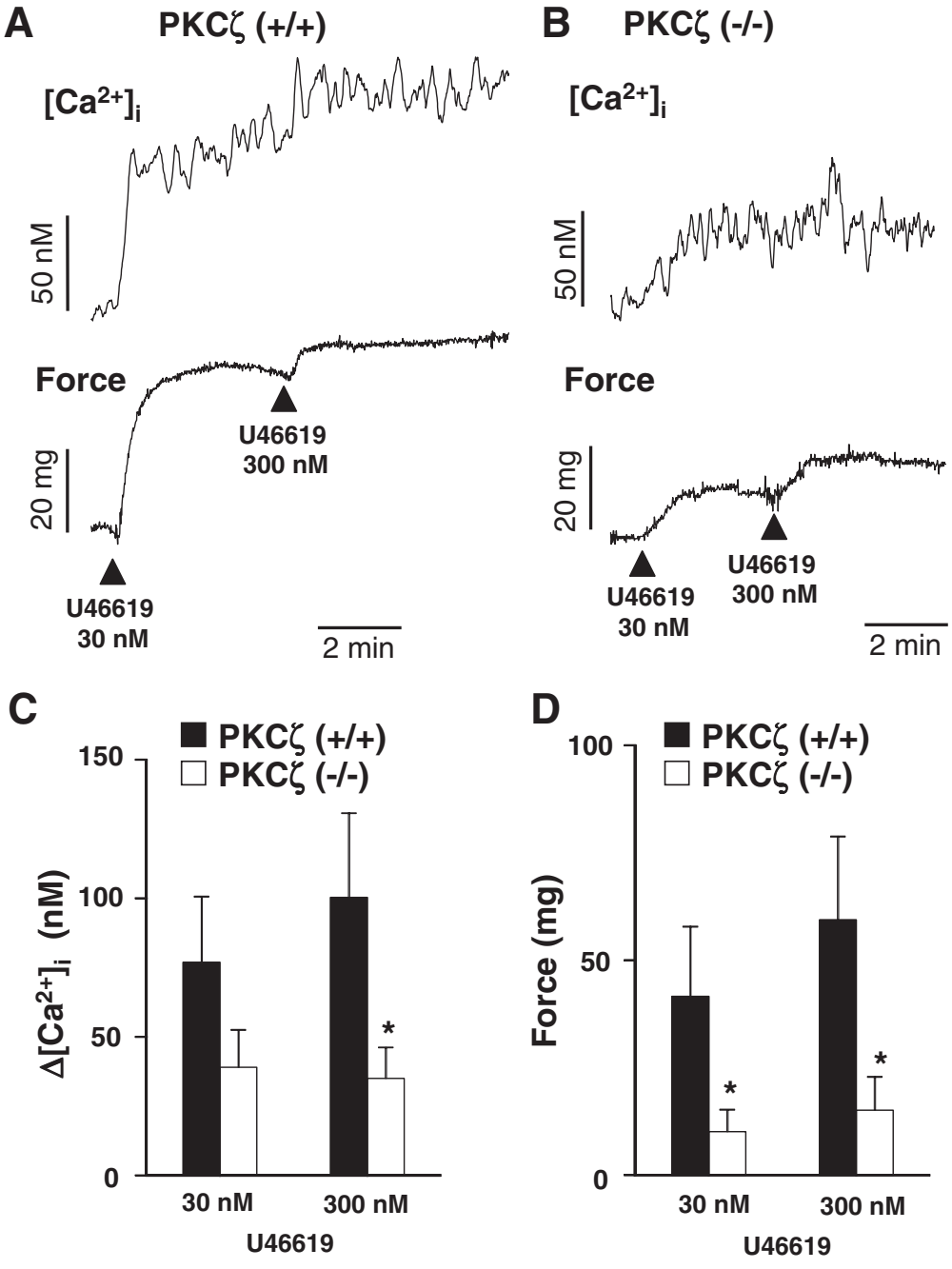

Fig. 2. PA from PKC$\zeta^{-/-}$ mice show reduced $[Ca^{2+}]_i$ and contractile responses induced by TP receptor activation. A and B, simultaneous recordings of $[Ca^{2+}]_i$ (top trace) and force (bottom trace) in PAs from PKC$\zeta^{+/+}$ and PKC$\zeta^{-/-}$, respectively, stimulated by 30 and 300 nM U46619. The averaged values (means ± S.E.M. of five to seven PAs) of U46619-induced increase in $[Ca^{2+}]_i$ and force are shown in C and D, respectively. *, $p < 0.05$ versus PKC$\zeta^{+/+}$ (unpaired Student's t test).

not significantly different from those in wild-type mice (170 ± 45 nM; $n = 6$). We found that genetic inactivation of p62 abolished the increase in $[Ca^{2+}]_i$ induced by U46619 (Fig. 4, B and C). However, the contractile response induced by the two concentrations of U46619 tested was remarkably similar in $p62^{-/-}$ and wild-type mice (Fig. 4D).

Role of K_V1.5 Channels in TXA_2-Induced Effects. K_V1.5 channels have been reported to be major contributors of K_V currents in PASMCs in several animal species. Figure 5A shows $hK_V1.5$ current traces recorded in Ltk^- cells stably expressing $hK_V1.5$ channels. U46619 (100 nM) significantly inhibited $hK_V1.5$ currents. This inhibitory effect was only observed at the end of the depolarizing pulse; e.g., currents were almost unaffected at the peak (4.6 ± 2.4% decrease; not significant), but they were reduced by 17.8 ± 4.2% after 200 ms ($n = 4$; $p < 0.05$). In rat PASMCs, U46619 also inhibited K_V currents (Fig. 5B) as described previously (Cogolludo et al., 2003). The K_V1.5 channel blocker DPO-1 (Lagrutta et al., 2006) inhibited K_V currents in rat PASMCs. In the presence of DPO-1, U46619 produced no further inhibitory effects (Fig. 5B). Therefore, we analyzed a possible interaction between PKCζ, K_V1.5 channels and p62. Rat pulmonary arteries were incubated for 30 s in the absence (control) or presence of U46619. Homogenates were immunoprecipitated with anti-PKCζ or anti-K_V1.5 antibodies, and the content of K_V1.5, PKCζ, or p62 in the immunoprecipitates was analyzed via Western blot. Figure 5C shows that in immunoprecipitates of K_V1.5 both PKCζ and p62 were present. The K_V1.5-PKCζ and the K_V1.5-p62 association were 135 ± 13% ($n = 8$; $p = 0.06$, not significant) and 163 ± 31% ($n = 7$; $p < 0.05$), respectively, in U46619-treated versus untreated arteries. The K_V1.5-PKCζ interaction was also observed in immunoprecipitates of PKCζ immunoblotted with the anti-K_V1.5 antibody (data not shown).

Interaction of PKCζ with K_V Channels: Role of p62. To determine the potential role of the PKCζ scaffold protein p62, the PKCζ-K_V1.5 interaction was analyzed by coimmunoprecipitation in lungs from wild-type and $p62^{-/-}$ mice. Genetic inactivation of p62 in mice did not modify the expression levels of either PKCζ or K_V1.5 channels in PASMCs (Fig. 6A). In immunoprecipitates of PKCζ from wild-type mice immunoblotted with the anti-K_V1.5 antibody, a band of approx. 80 kDa was

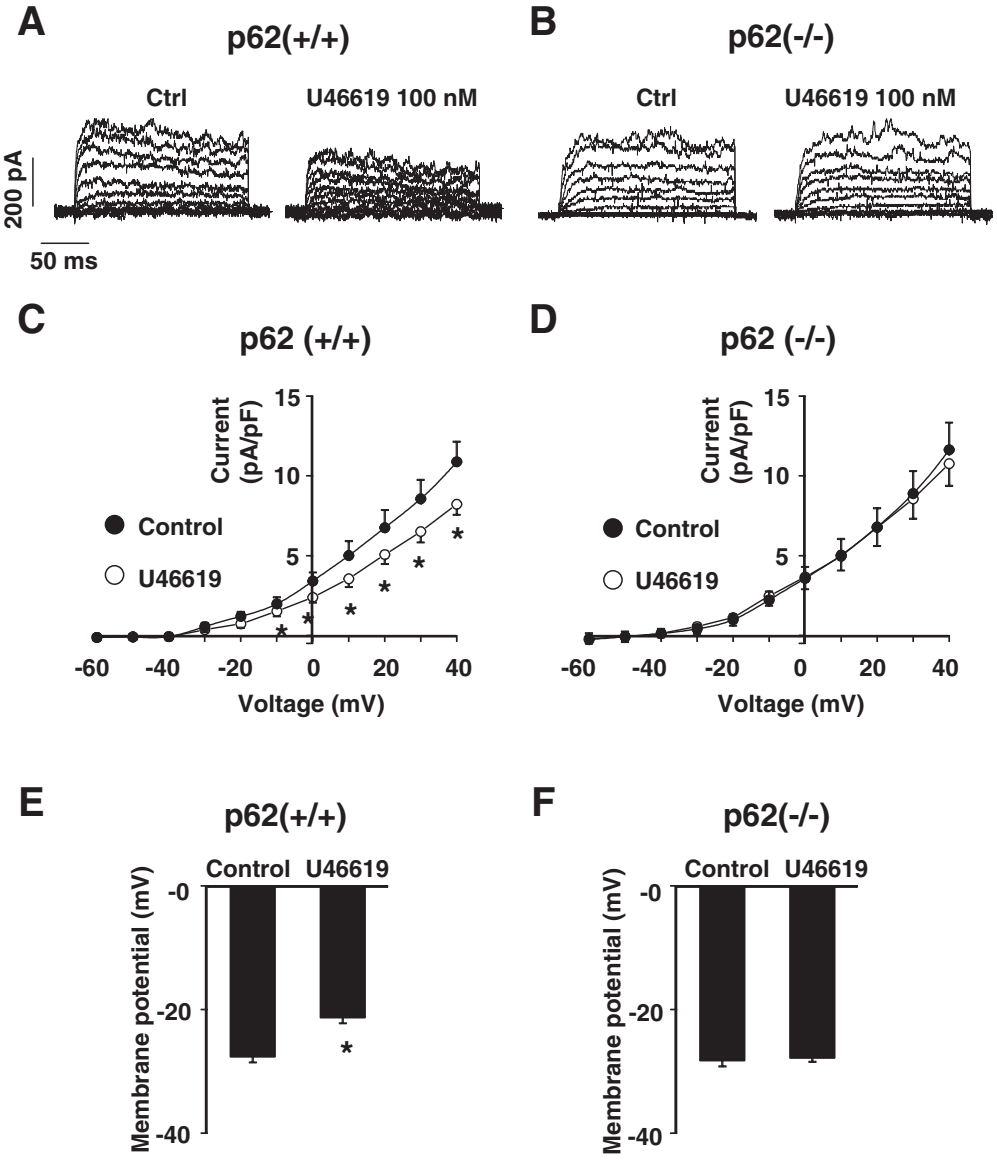

Fig. 3. TP receptor activation leads to K_V current inhibition and depolarization in PASMCs from wild-type (A, C, and E) but not from $p62^{-/-}$ (B, D, and F) mice. A and B, current traces for 200-ms depolarization pulses from −60 to +40 mV (in 10-mV increments) from a holding potential of −60 mV before (control) and after application of the TXA_2 analog U46619 (100 nM). C and D, current-voltage relationship measured at the end of the 200-ms pulse (means ± S.E.M. of five to six cells). E and F, effects of U46619 on membrane potential recorded under current-clamp conditions.*, $p < 0.05$ versus control (paired Student's t test).

observed, which presumably reflects the mature (glycosylated) form of the channel expressed in the membrane (Li et al., 2000). However, p62-deficient mice showed a weak PKCζ-K_V1.5 coimmunoprecipitation (Fig. 6B).

Discussion

By using nonselective PKC inhibitors and the PKCζ-selective inhibitor PKCζ-PI, we suggested that PKCζ was involved in the K_V channel inhibition and the contractile response induced by TXA_2 in rat pulmonary artery myocytes (Cogolludo et al., 2003, 2005). Herein, we confirmed the role of PKCζ in native K_V currents by using PASMCs from PKCζ$^{-/-}$ mice. Consistent with the essential role of K_V1.5 channels in the pulmonary vasculature, we show that the K_V1.5 inhibitor DPO-1 inhibited K_V currents in native rat PASMCs by approx. 50% and that the TXA_2 analog U46619 had no further inhibitory effects. In addition, cloned human K_V1.5 channels expressed in Ltk$^-$ cells were also inhibited by U46619. Moreover, our results demonstrate the interaction between PKCζ and K_V1.5 in both rat PAs and mouse lungs, which was minimal in p62$^{-/-}$ mice. Deletion of p62 abolished K_V channel inhibition and Ca^{2+} responses induced by TXA_2, further supporting the role of p62 as a key mediator between PKCζ and K_V1.5. However, our study also showed that the contractile response induced by U46619 in PA was similar in wild-type and p62$^{-/-}$ mice.

In both rat and newborn porcine PASMCs, U46619 inhibited K_V currents, depolarized cell membrane, increased $[Ca^{2+}]_i$ through Ca_L channels, and induced a contractile response (Cogolludo et al., 2003, 2005). U46619 had no direct effect on Ca_L channels in voltage-clamped cells, indicating that increased Ca^{2+} entry through Ca_L channels is secondary

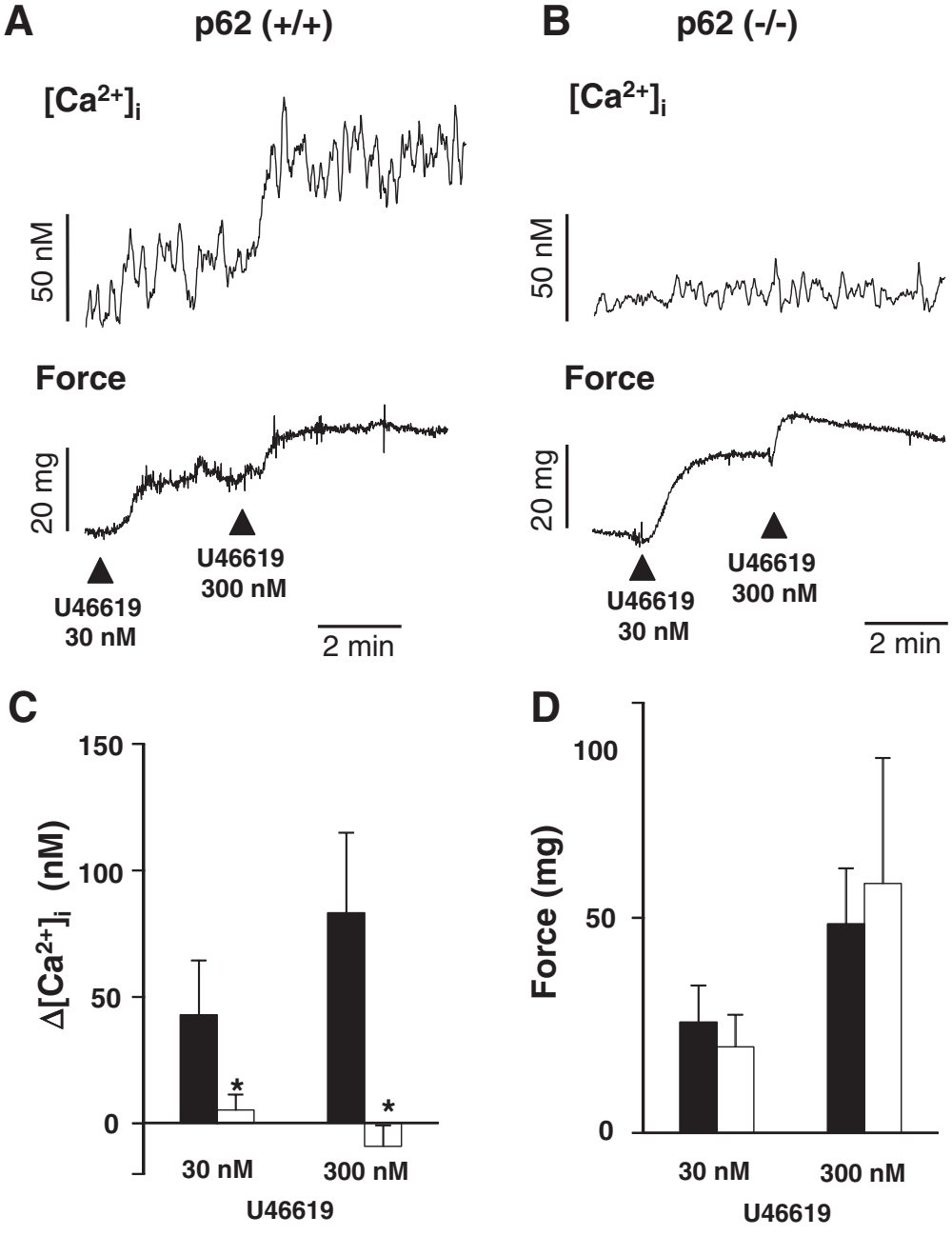

Fig. 4. PAs from p62$^{-/-}$ mice show no $[Ca^{2+}]_i$ responses but preserved contractions induced by TP receptor activation. A and B, simultaneous recordings of $[Ca^{2+}]_i$ (top trace) and force (bottom trace) in PA from p62$^{+/+}$ and p62$^{-/-}$, respectively, stimulated by 30 and 300 nM U46619. The averaged values (means ± S.E.M. of five PAs) of U46619-induced increase in $[Ca^{2+}]_i$ and force are shown in C and D, respectively. *, $p < 0.05$ versus p62$^{+/+}$ (unpaired Student's t test).

to membrane depolarization. Herein, we demonstrated that, in mice, U46619 also inhibits K_V currents in PASMCs and induces a $[Ca^{2+}]_i$ response and vasoconstriction in isolated PA. The degree of K_V channel inhibition in mice PASMCs (~25% at 100 nM U46619) was similar to that observed in porcine and in rat PA, and it was accompanied by a significant membrane depolarization. In rat and porcine PAs, all these effects were inhibited by calphostin C and PKCζ-PI (Cogolludo et al., 2003, 2005). These experiments suggested a role for PKCζ as a link between TP receptors and K_V channels, which was confirmed in the present study using PKCζ$^{-/-}$ mice. The magnitude and current-voltage relationship of K_V currents were similar in the wild-type and knockout animals, suggesting no changes in the channel proteins underlying K_V currents. Thus, genetic inactivation or pharmacological inhibition of PKCζ abolished the effects of U46619 on K_V currents or membrane potential in PASMCs.

In contrast, both approaches only partially inhibited (~50–70%) the Ca^{2+} signal induced by U46619 in rat and mice PAs, indicating that, in addition to the PKCζ-K_V-Ca_L pathway, mechanisms increasing $[Ca^{2+}]_i$ (e.g., Ca^{2+} release from intracellular stores) are also activated in response to U46619 (Snetkov et al., 2006).

The present experiments also indicate that in mice, PKCζ contributes to the vasoconstriction induced by TP receptor activation. These results are in agreement with those obtained in rats and newborn piglets using PKCζ-PI (Cogolludo et al., 2003, 2005). However, in 2-week-old piglets (Cogolludo et al., 2005), PKCζ-PI and the Ca^{2+} channel blocker nifedipine almost fully inhibited U46619-induced increases in $[Ca^{2+}]_i$, but they had no effect on U46619-induced contractile responses; i.e., there was a contractile response in the absence of changes in $[Ca^{2+}]_i$. Therefore, in these animals, the up-regulation of Ca^{2+}-independent mechanisms for contrac-

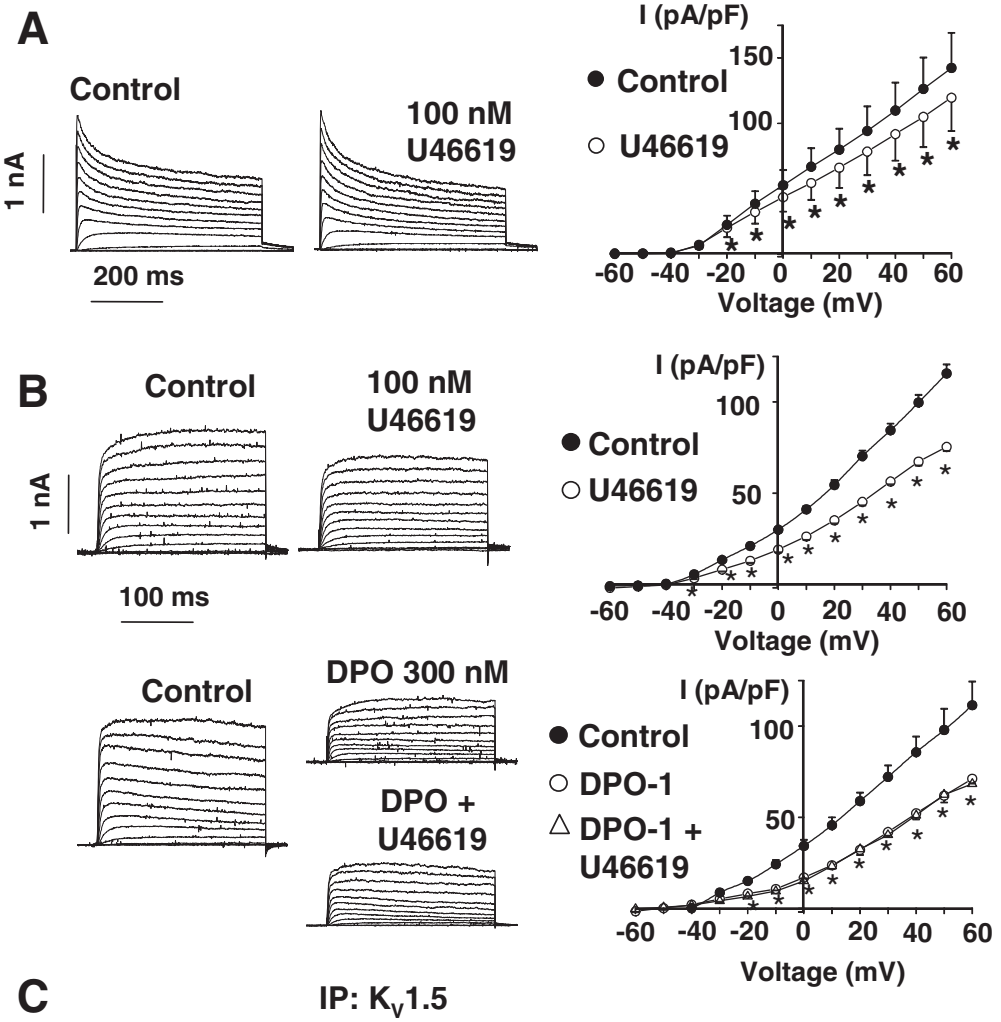

Fig. 5. Role of K_V1.5 channels: TP receptor activation inhibits K_V1.5 currents and increases coimmunoprecipitation of K_V1.5 with PKCζ. A, current traces recorded in Ltk$^-$ cells stably transfected with human K_V1.5 before (control) and after 100 nM U46619 and current-voltage relationship (means ± S.E.M. of four cells) measured at the end of the 200-ms pulse. Depolarizing steps from −60 to +60 mV were applied from a holding potential of −60 mV. *, $p < 0.05$ versus control (paired Student's t test). B, current traces recorded in rat PASMCs cells before (control) and after the 100 nM U46619 (top) or before (control), after DPO-1 (300 nM), and after DPO-1 plus U46619 (bottom). Current-voltage relationships are shown at the right (means ± S.E.M. of three to four cells). *, $p < 0.05$ versus control (paired Student's t test). C, rat pulmonary arteries were incubated for 30 s in the absence (control) or presence of 100 nM U46619, frozen, and homogenated. Homogenates were immunoprecipitated with anti-K_V1.5 antibodies and immunoblotted with anti-PKCζ or anti-p62. Results are representative of samples from seven to eight mice. Each pair of bands (control and U46619) is obtained from the same animal.

tion (Somlyo and Somlyo, 2000) makes PKCζ and the $[Ca^{2+}]_i$ signal redundant.

K_V currents recorded in native PASMCs reflect the contribution of multiple K_V channel proteins [e.g., in human PAs, 22 transcripts of $K_V\alpha$ subunits: $K_V1.1$ to $K_V1.7$, $K_V1.10$, $K_V2.1$, $K_V3.1$, $K_V3.3$, $K_V3.4$, $K_V4.1$, $K_V4.2$, $K_V5.1$, $K_V6.1$ to -6.3, $K_V9.1$, $K_V9.3$, $K_V10.1$, and $K_V11.1$, and three of $K_V\beta$ subunits $K_V\beta1$ to -3 have been identified by reverse transcription-polymerase chain reaction]. However, $K_V1.5$ subunits are thought to be major contributors of the native K_V currents in PAs from different species, and their activity is regulated by vasoactive factors such as 5-hydroxytryptamine (Cogolludo et al., 2006) and hypoxia (Platoshyn et al., 2006). Therefore, we analyzed the effects of U46619 on the K_V current carried by human cloned $K_V1.5$ channels expressed in mouse fibroblast (Ltk^-) cells. This cell line expresses endogenously the $K_V\beta2.1$ subunit, which assembles with the transfected hKv1.5 protein (Uebele et al., 1996). U46619 induced a weak but significant inhibitory effect on this current, suggesting that $K_V1.5$ channels are involved in the effects of TP receptor activation in native PASMCs. The small inhibition in this cell type probably reflects a lower efficacy of the signaling pathway compared with rat or mouse PASMCs. Furthermore, after pharmacological inhibition of $K_V1.5$ channels with DPO-1, U46619 had no further inhibitory effects on K_V currents in rat PASMCs.

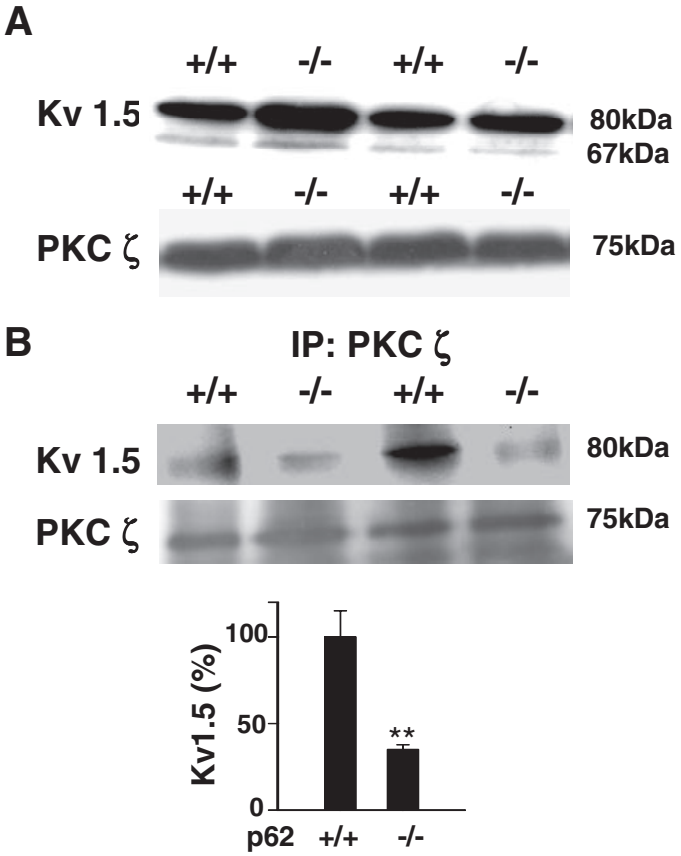

Fig. 6. Similar expression of PKCζ and $K_V1.5$ but reduced interaction between PKCζ and $K_V1.5$ in $p62^{-/-}$ versus $p62^{+/+}$. A, representative Western blots of lung homogenates using anti-$K_V1.5$ and anti-PKCζ antibodies. B, lung homogenates were immunoprecipitated with anti-PKCζ antibodies and immunoblotted with anti-$K_V1.5$; membranes were reblotted with the anti-PKCζ antibody as a loading control. The graph shows the densitometric analysis of the $K_V1.5$ protein relative to PKCζ and expressed as a percentage of values in wild-type mice. **, $p < 0.01$ versus wild type.

In the present article, we show that PKCζ coimmunoprecipitates with $K_V1.5$ channels. In a previous study (Cogolludo et al., 2003), we reported that U46619 induced the translocation of PKCζ from the cytosolic to the membrane fraction. Therefore, TP receptor-induced K_V channel inhibition is associated with the translocation of PKCζ to the plasma membrane where it interacts with $K_V1.5$ channels. This PKCζ-$K_V1.5$ interaction is not necessarily a direct protein-protein interaction; it seems more likely that it is mediated by adaptor proteins. In this regard, it has been described that PKCζ can interact with the β subunit $K_V\beta2$ of the K_V channel via the p62 adaptor protein (Gong et al., 1999). In immunoprecipitation experiments, we found that p62 was present in the $K_V1.5$-PKCζ complex. Even when the complex was constitutive, the association of p62 with $K_V1.5$ increased significantly by U46619. Furthermore, the PKCζ-$K_V1.5$ coimmunoprecipitation was strongly reduced in $p62^{-/-}$ mouse lung, indicating that p62 physically associates PKCζ into the K_V channel complex.

$K_V\beta$ subunits function as molecular chaperones, and they can directly regulate channel inactivation, voltage dependence, and current amplitude (Martens et al., 1999). p62 overexpression stimulates PKCζ-dependent phosphorylation of $K_V\beta2$ (Gong et al., 1999), and it induces a hyperpolarizing shift of K_V current activation in pheochromocytoma cells (Kim et al., 2004). Thus, we analyzed the effect of genetic inactivation of p62 on K_V currents and its modulation by TP receptor activation. K_V currents in PASMCs from $p62^{-/-}$ were similar to wild type. As expected, U46619 had no effect on K_V currents in $p62^{-/-}$ PASMCs, indicating that the p62-dependent PKCζ-$K_V1.5$ interaction is required for the inhibitory effect of TP receptor activation on K_V current.

Thus, genetic inactivation of p62 had a similar effect to genetic or pharmacological inactivation of PKCζ regarding K_V current modulation. We were surprised to find that p62 gene deletion fully inhibited the Ca^{2+} response induced by U46619 in isolated PAs compared with a 50 to 70% inhibition by PKCζ inactivation. More intriguingly, the contractile response to U46619 was not affected in PA from $p62^{-/-}$ mice. This contractile response in the absence of changes in $[Ca^{2+}]_i$ must then be attributed to Ca^{2+}-independent mechanisms (i.e., Ca^{2+} sensitization; Somlyo and Somlyo, 2000). This response to U46619 in $p62^{-/-}$ mice PA is similar to that observed in 2-week-old piglet PAs after inhibition of PKCζ (i.e., contraction without $[Ca^{2+}]_i$ signal) (Cogolludo et al., 2005). In these animals, there is an up-regulation of Rho kinase (Bailly et al., 2004), a key enzyme in Ca^{2+}-sensitizing mechanisms. In addition, Rho kinase inhibitors were more effective inhibiting U46619 contractions in these piglets than in newborn piglets or adult rats (Cogolludo et al., 2005). Thus, we speculate that the chronic down-regulation of the PKCζ-p62-K_V-Ca_L-dependent pathway, either at the level of K_V channel activity (as occurs in older piglets) or p62 ($p62^{-/-}$ mice), but not PKCζ ($PKC\zeta^{-/-}$ mice), is compensated by up-regulation of Ca^{2+} sensitization mechanisms.

In conclusion, PKCζ modulates K_V channel function, and it is involved in pulmonary vasoconstriction induced by TP receptor activation. The interaction between PKCζ and $K_V1.5$ and the inhibitory effect of U46619 in cloned human $K_V1.5$ channels suggest that these specific channel subtypes are

functional targets for PKCζ. The adaptor protein p62 is required for the PKCζ-K_V1.5 interaction and hence for the inhibition of K_V currents after TP receptor activation.

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