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Role of PKCζ and its adaptor protein p62 in K$_V$ channel modulation in pulmonary arteries.

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Nonstandard abbreviations: aPKC, atypical PKC; Ca_L, voltage-dependent L-type Ca^{2+} channels; DPO-1, ([1S-1α,2α,5β]-[5-Methyl-2-(1-methylethyl) cyclohexyl] diphenyl phosphine oxide); K_V, voltage-gated K^+ channels; PA, pulmonary artery; PASMC, pulmonary artery smooth muscle cells; PH, pulmonary hypertension; PKCζ, protein kinase C ζ; PKCζ-PI, PKCζ pseudosubstrate inhibitory peptide; TP, thromboxane-endoperoxide receptors; TXA_2, thromboxane A_2; U46619, 9,11-dideoxy-11α,9α-epoxymethano-prostaglandin $F_{2α}$.

Abstract: Voltage-gated potassium (K_V) channels play an essential role in regulating pulmonary artery function and underpin the phenomenon of hypoxic pulmonary vasoconstriction. Pulmonary hypertension is characterised by inappropriate vasoconstriction, vascular remodelling 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 analogue 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 PKCζ$^{-/-}$ and p62$^{-/-}$ mice, we demonstrate that these two proteins are involved in the K_V channel inhibition. PKCζ co-immunoprecipitated with K_V1.5 and this interaction was markedly reduced in p62$^{-/-}$ mice. Pulmonary arteries from PKCζ$^{-/-}$ mice also showed a diminished $[Ca^{2+}]_i$ and contractile response while genetic inactivation of p62$^{-/-}$ resulted in an absent $[Ca^{2+}]_i$ response but 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 (PASMC) and its inhibition causes membrane depolarisation, activation of L-type Ca^{2+} channels (Ca_L), increases in $[Ca^{2+}]_i$ and vasoconstriction (Archer et al., 1998; Yuan et al., 1998b; Barnes and Liu, 1995). These channels are common targets of pulmonary vasoconstrictor stimuli such as hypoxia, thromboxane A_2 (TXA_2) 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 PASMC 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 $K_V1.5$, since decreased expression or activity and mutations of $K_V1.5$ occurs 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 depolarization, activation of L-type Ca^{2+} channels, and vasoconstriction. Furthermore, using a protein kinase C ζ (PKCζ) pseudosubstrate inhibitory

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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, 2002). The aPKCs share with other members of their family a conserved catalytic domain but display a clearly distinct regulatory region since 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 (Cogolludo et al., 2003, 2005; Shizukuda and Buttrick, 2002), angiotensin II (Gayral et al., 2006; Godeny and Sayeski, 2006) or pro-inflammatory 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). Interestingly, nerve growth factor and catecholamines have been reported to increase the expression of p62, enabling the formation of the PKCζ-p62-K_Vβ 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

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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, 2006). However, recent evidences suggest that some considered isoform-specific PKC inhibitors, such as myristoylated PKCζ pseudosubstrate peptide, may exert other effects unrelated to inhibition of PKC and, thus, should be used with caution (Krotova et al., 2006).

Therefore, in the present study we aimed to further characterize the signalling pathway modulating K_V currents in PA. 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.

MATERIAL AND METHODS

All experiments were carried out in accordance with the European Animals Act 1986 (Scientific Procedures) and 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 (six to eight week old, either sex) were generously supplied by

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Drs. Moscat and Diaz-Meco which were generated as described (Leitges et al. 2001; Duran et al. 2004). PA 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 (Ca^{2+}-free PSS) of the following composition (in mmol/L): NaCl 130, KCl 5, $MgCl_2$ 1.2, glucose 10, and HEPES 10 (pH 7.3 with NaOH). Endothelium denuded PAs were cut into small segments (2x2 mm), and cells were isolated in Ca^{2+}-free PSS containing (in mg/mL) papain 1, dithiothreitol 0.8, and albumin 0.7. Cells were stored in Ca^{2+}-free PSS (4°C) and used within 8 hours 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 pA pF^{-1}. 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 section). Ltk$^-$ cells stably expressing $hK_V1.5$ channels (Valenzuela et al., 1995) were superfused with PSS containing 1 mmol/L $CaCl_2$. Currents were evoked following the application of 200 ms depolarizing pulses from -60 mV to test potentials from -60 mV to +40 mV in 10 mV increments. All experiments were performed at room temperature (22 to 24°C).

$[Ca^{2+}]_i$ recording.

PA rings were incubated for 80 minutes at room temperature in Krebs solution containing the fluorescent dye Fura-2 acetoximethylester (5 x 10^{-6} M) and cremophor EL (0.05%) and then mounted in a fluorimeter (CAF 110 model, Jasco, Tokyo). 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., 1998). The ratio of emitted fluorescence (F340/F380) obtained at the two excitation wavelengths was used as an indicator of $[Ca^{2+}]_i$. Arteries were stimulated with 30 nM and 300 nM U46619, added in a cumulative fashion. 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 ionomycin (15 μM) and EGTA (10 mM) at the end of the experiment.

Co-immunoprecipitation 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 U46619 (1 μM) 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 (mmol/L): HEPES 10 (pH 8), KCl 10, EDTA 1, EGTA 1, dithiothreitol 1, aprotinin 0.006, leupeptin 0.009, Nα-p-tosyl-l-lysine chloromethyl ketone 0.011, NaF 5, Na_2MoO_4 10, $NaVO_4$ 1, phenylmethanesulfonyl fluoride 0.5 and okadaic acid 0.00001. Homogenates were centrifuged at 13,000g for 5 min at 4ºC and the supernatant fraction was collected. For immunoprecipitation, sixty μg of protein were incubated for 2

hours 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-PAGE and transferred to a PVDF membrane for western blotting as described (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 (mM): NaCl 130, KCl 5, $MgCl_2$ 1.2, glucose 10, HEPES 10, buffered to pH 7.3 with NaOH. The Ca^{2+}-free pipette (internal) solution contained (mM): KCl 110, $MgCl_2$ 1.2, Na_2ATP 5, HEPES 10, EGTA 10, pH adjusted to 7.3 with KOH. The Krebs solution used for tissue bath experiments comprised (in mM): NaCl 118, KCl 4.75, $NaHCO_3$ 25, $MgSO_4$ 1.2, $CaCl_2$ 2.0, KH_2PO_4 1.2 and glucose 11. This solution was gassed with a 95% O_2-5% CO_2 gas mixture at 37° C. U46619 (9, 11-dideoxy-11α,9α-epoxymethano-prostaglandin $F_{2\alpha}$ methyl acetate solution) was obtained from Sigma Chemical Co., DPO-1 ([1S-1α,2α,5β]-[5-Methyl-2-(1-methylethyl) cyclohexyl] diphenyl phosphine oxide) from Tocris Cookson, secondary horseradish peroxidase conjugated antibodies and fura-2 AM from Calbiochem, rabbit anti-$K_V1.5$ from Alomone, goat anti-PKCζ from Santa Cruz Biotechnology and guinea-pig anti-p62 from Progen.

Statistical analysis.

Data are expressed as means ± s.e.mean; 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 PASMC when eliciting depolarizing

steps from -60 to +40 mV (Fig. 1A and 1B) from a holding potential of -60 mV. The

magnitude of the currents, the threshold voltage for activation and the current-voltage

relationship (Fig. 1C and 1D) was similar in PASMC 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^{-1}, 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$_2$ analogue U46619. U46619 (100 nM) caused a significant inhibition of K_V currents

in the whole range of channel activation in PASMC 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 PASMC (Fig. 1E). However, U46619 had no effect on either K_V currents or

membrane potentials in PASMC from PKCζ$^{-/-}$ mice (Figs. 1B, D, F).

Role of PKCζ in $[Ca^{2+}]_i$ increase and contraction induced by TXA$_2$

Changes in $[Ca^{2+}]_i$ and contraction induced by U46619 were simultaneously analyzed in fura-2 loaded PA 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 PA from wild-type and PKC$\zeta^{-/-}$ animals (Fig. 2A and 2B). However, the increase in $[Ca^{2+}]_i$ (Fig. 2C) and the contractile response (Fig. 2D) was significantly reduced in PKC$\zeta^{-/-}$ mice as compared to wild-type mice.

Role of p62 in K_V current inhibition, $[Ca^{2+}]_i$ increase and contraction

In order to analyze the functional role of p62 and the PKCζ-p62-K_V1.5 interaction, we analyzed the effects of U46619 (100 nM) 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. 3C and 3D) were similar in PASMC 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^{-1}, 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 PASMC from wild-type mice (Fig. 3A, 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$_2$ analogue had no effect on K_V currents in PASMC from p62$^{-/-}$ mice (Fig. 3B, D and E).

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Basal levels of $[Ca^{2+}]_i$ in p62$^{-/-}$ (184 ± 35 nM, n=5) were 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. 4B and 4C). 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$_V$1.5 channels in TXA$_2$-induced effects

K$_V$1.5 channels have been reported to be major contributors of K$_V$ currents in PASMCs in several animal species. Fig. 5A shows hK$_V$1.5 current traces recorded in Ltk$^-$ cells stably expressing hK$_V$1.5 channels. U46619 (100 nM) significantly inhibited hK$_V$1.5 currents. This inhibitory effect was only observed at the end of the depolarizing pulse; e.g. currents were almost unaffected at the peak (4.6 ± 2.4% decrease, not significant) but reduced by 17.8 ± 4.2% after 200 ms (n=4, p< 0.05). In rat PASMCs, U46619 also inhibited K$_V$ currents (Fig. 5B) as previously described (Cogolludo et al., 2003). The K$_V$1.5 channel blocker DPO-1 (Lagrutta et al., 2006) inhibited K$_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$_V$1.5 channels and p62. Rat pulmonary arteries were incubated for 30 s in the absence (control) or presence of U46619. Homogenates were immunoprecipitated with anti-PKCζ or anti-K$_V$1.5 antibodies and the content of K$_V$1.5, PKCζ or p62 in the immunoprecipitates was analyzed via Western blot. Fig. 5C shows that in immunoprecipitates of K$_V$1.5 both PKCζ and p62 were present. The K$_V$1.5-PKCζ and the K$_V$1.5-p62 association were 135 ± 13% (n= 8, not significant, p = 0.06) and 163 ± 31% (n= 7, p < 0.05), respectively, in U46619-treated vs untreated arteries. The K$_V$1.5-PKCζ interaction

was also observed in immunoprecipitates of PKCζ immunoblotted with the anti-K_V1.5 antibody (not shown).

Interaction of PKCζ with K_V channels: role of p62

In order to determine the potential role of the PKCζ scaffold protein p62, the PKCζ-K_V1.5 interaction was analyzed by co-immunoprecipitation 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 PASMC (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 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 co-immunoprecipitation (Fig. 6B).

DISCUSSION

By using non selective 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%

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and that the TXA_2 analogue 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 PA and mice 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 PASMC, 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 to

membrane depolarization. Herein, we demonstrated that, in mice, U46619 also inhibits K_V

currents in PASMC and induces a $[Ca^{2+}]_i$ response and vasoconstriction in isolated PA. The

degree of K_V channel inhibition in mice PASMC (~25% at 100 nM U46619) was similar to

that observed in porcine and in rat PA and was accompanied by a significant membrane

depolarization. In rat and porcine PA, 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 PASMC. In contrast, both

approaches only partially inhibited (~50-70%) the Ca^{2+} signal induced by U46619 in rat and

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mice PA, 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 two 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 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 contraction (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 PA, 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 3 of $K_V\beta$ subunits: $K_V\beta1$ to -3 have been identified by RT-PCR). However,

$K_V1.5$ subunits are believed to be major contributors of the native K_V currents in PA from

different species and their activity is regulated by vasoactive factors such as 5-HT (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 (Uelele et al., 1996). U46619 induced a weak

but significant inhibitory effect on this current, suggesting that $K_V1.5$ channels are involved

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in the effects of TP receptor activation in native PASMCs. The small inhibition in this cell type probably reflects a lower efficacy of the signalling pathway compared to rat or mice 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.

In the present paper we show that PKCζ co-immunoprecipitates 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-$K_V1.5$ co-immunoprecipitation was strongly reduced in p62$^{-/-}$ mice lung indicating that p62 physically associates PKCζ into the K_V channel complex.

$K_V\beta$ subunits function as molecular chaperones, and 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 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

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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 pharmacologic inactivation of PKCζ regarding K_V current modulation. Unexpectedly, we found that p62 gen deletion fully inhibited the Ca^{2+} response induced by U46619 in isolated PA as compared to a 50-70% 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 two week old piglet PA 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 downregulation 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 ($PKCζ^{-/-}$ mice) is compensated by upregulation of Ca^{2+} sensitization mechanisms.

In conclusion, PKCζ modulates K_V channel function and 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

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specific channel subtypes are functional targets for PKCζ. The adaptor protein p62 is
required for the PKCζ-K$_V$1.5 interaction and hence for the inhibition of K$_V$ currents
following TP receptor activation.

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Footnotes:

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Figure 1. **TP receptor activation leads to K_V current inhibition and depolarization in PASMCs from PKC$\zeta^{+/+}$ (A,C,E) but not from PKC$\zeta^{-/-}$ (B,D,F) mice**. Panels A and B show current traces for 200 ms depolarization pulses from -60 mV to +40 mV (in 10 mV increments) from a holding potential of -60 mV before (control) and after application of the TXA$_2$ analogue U46619 (100 nM). Panels C and D show the current-voltage relationship measured at the end of the 200 ms pulse (means $\pm$ SEM of 5 cells). Panels E and F show the effects of U46619 (100 nM) on membrane potential recorded under current clamp conditions. *, ** indicate $p < 0.05$ and $p < 0.01$, respectively, vs. control (paired Student's t test).

Figure 2. **PA from PKC$\zeta^{-/-}$ mice show reduced $[Ca^{2+}]_i$ and contractile responses induced by TP receptor activation.** Panels A and B show simultaneous recordings of $[Ca^{2+}]_i$ (upper trace) and force (lower trace) in PA from PKC$\zeta^{+/+}$ and PKC$\zeta^{-/-}$, respectively, stimulated by 30 and 300 nM U46619. The averaged values (means $\pm$ SEM of 5-7 PA) of U46619-induced increase in $[Ca^{2+}]_i$ and force are shown in panels C and D, respectively. * indicates $p < 0.05$ vs. PKC$\zeta^{+/+}$ (unpaired Student's t test).

Figure 3. **TP receptor activation leads to K_V current inhibition and depolarization in PASMCs from wild-type (A,C,E) but not from p62$^{-/-}$ (B,D,F) mice.** Panels A and B show current traces for 200 ms depolarization pulses from -60 mV to +40 mV (in 10 mV increments) from a holding potential of -60 mV before (control) and after application of the TXA$_2$ analogue U46619 (100 nM). Panels C and D show the current-voltage relationship measured at the end of the 200 ms pulse (means $\pm$ SEM of 5-6 cells). Panels E and F show

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the effects of U46619 on membrane potential recorded under current clamp conditions.*

indicates $p <0.05$ vs. control (paired Student's t test).

Figure 4. **PA from p62$^{-/-}$ mice show no [Ca^{2+}]$_i$ responses but preserved contractions induced by TP receptor activation.** Panels A and B show simultaneous recordings of [Ca^{2+}]$_i$ (upper trace) and force (lower trace) in PA from p62$^{+/+}$ and p62$^{-/-}$, respectively, stimulated by 30 and 300 nM U46619. The averaged values (means ± SEM of 5 PA) of U46619-induced increase in [Ca^{2+}]$_i$ and force are shown in panels C and D, respectively. * indicates $p <0.05$ vs. p62$^{+/+}$ (unpaired Student's t test).

Figure 5. Role of K$_V$1.5 channels: TP receptor activation inhibits K$_V$1.5 currents and increases co-immunoprecipitation of K$_V$1.5 with PKCζ. Panel A: Current traces recorded in Ltk$^-$ cells stably transfected with human K$_V$1.5 before (control) and after U46619 (100 nM) and current-voltage relationship (means ± SEM of 4 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. * indicates $p < 0.05$ vs. control (paired Student's t test). Panel B: Current traces recorded in rat PASMCs cells before (control) and after the U46619 (100 nM) (upper panel) or before (control), after DPO-1 (300 nM) and after DPO-1 plus U46619 (lower panel). Current-voltage relationships are shown at the right (means ± SEM of 3-4 cells). * indicates $p < 0.05$ vs. control (paired Student's t test). Panel C: Rat pulmonary arteries were incubated for 30 s in the absence (control) or presence of U46619 (100 nM), frozen and homogenated. Homogenates were immunoprecipitated with anti-K$_V$1.5 antibodies and immunoblotted with

anti-PKCζ or anti-p62. Results are representative of samples from 7-8 mice. Each pair of bands (control and U46619) is obtained from the same animal.

Figure 6. Similar expression of PKCζ and $K_V1.5$ but reduced interaction between PKCζ and $K_V1.5$ in p62$^{-/-}$ vs p62$^{+/+}$. Panel A: Representative western blots of lung homogenates using anti-$K_V1.5$ and anti-PKCζ antibodies. Panel B: Lung homogenates were immunoprecipitated with anti-PKCζ antibodies and immunobloted with anti-$K_V1.5$, membranes were re-blotted 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 percent of values in wild-type mice. . ** indicates $p < 0.01$ vs. wild-type.
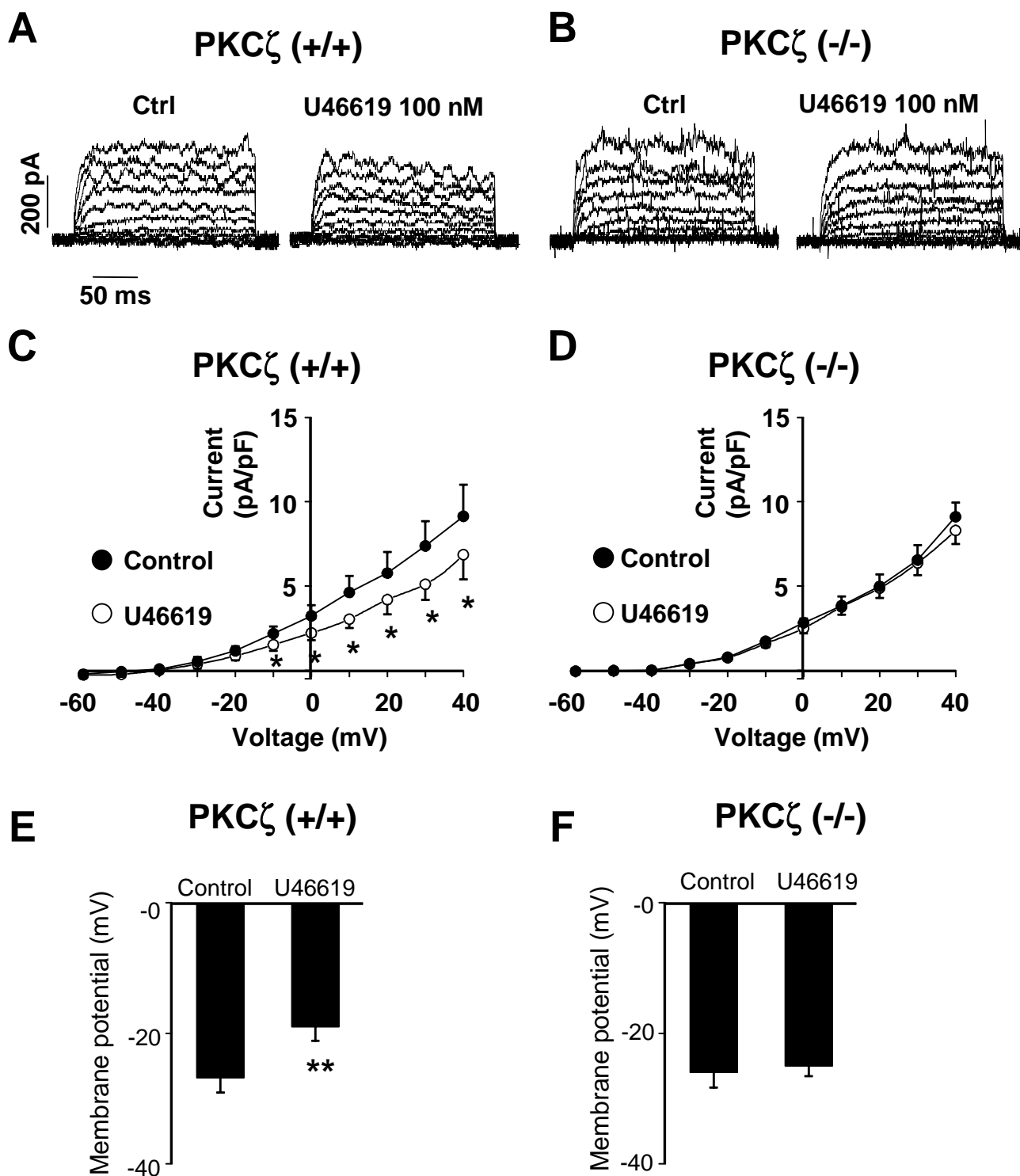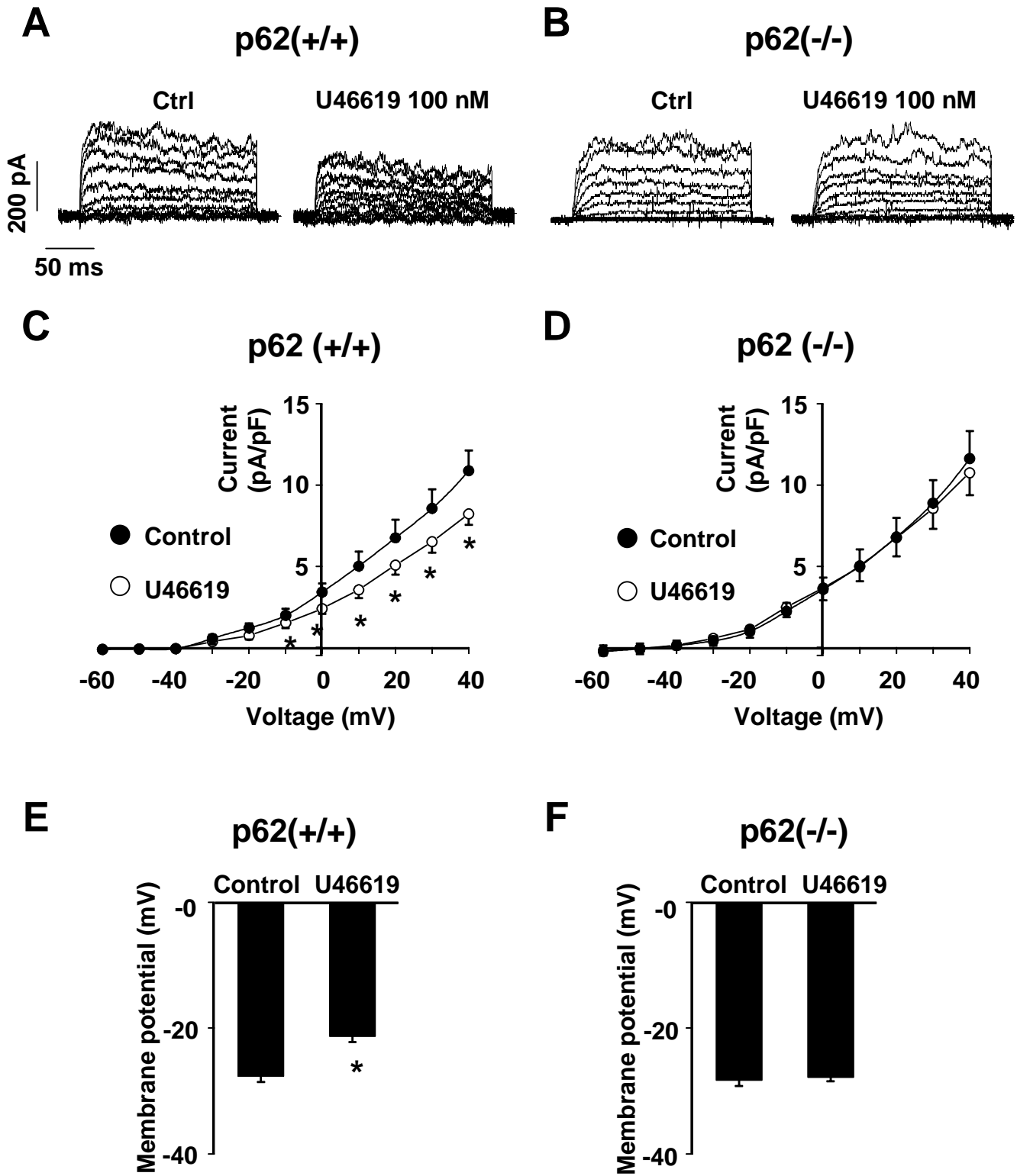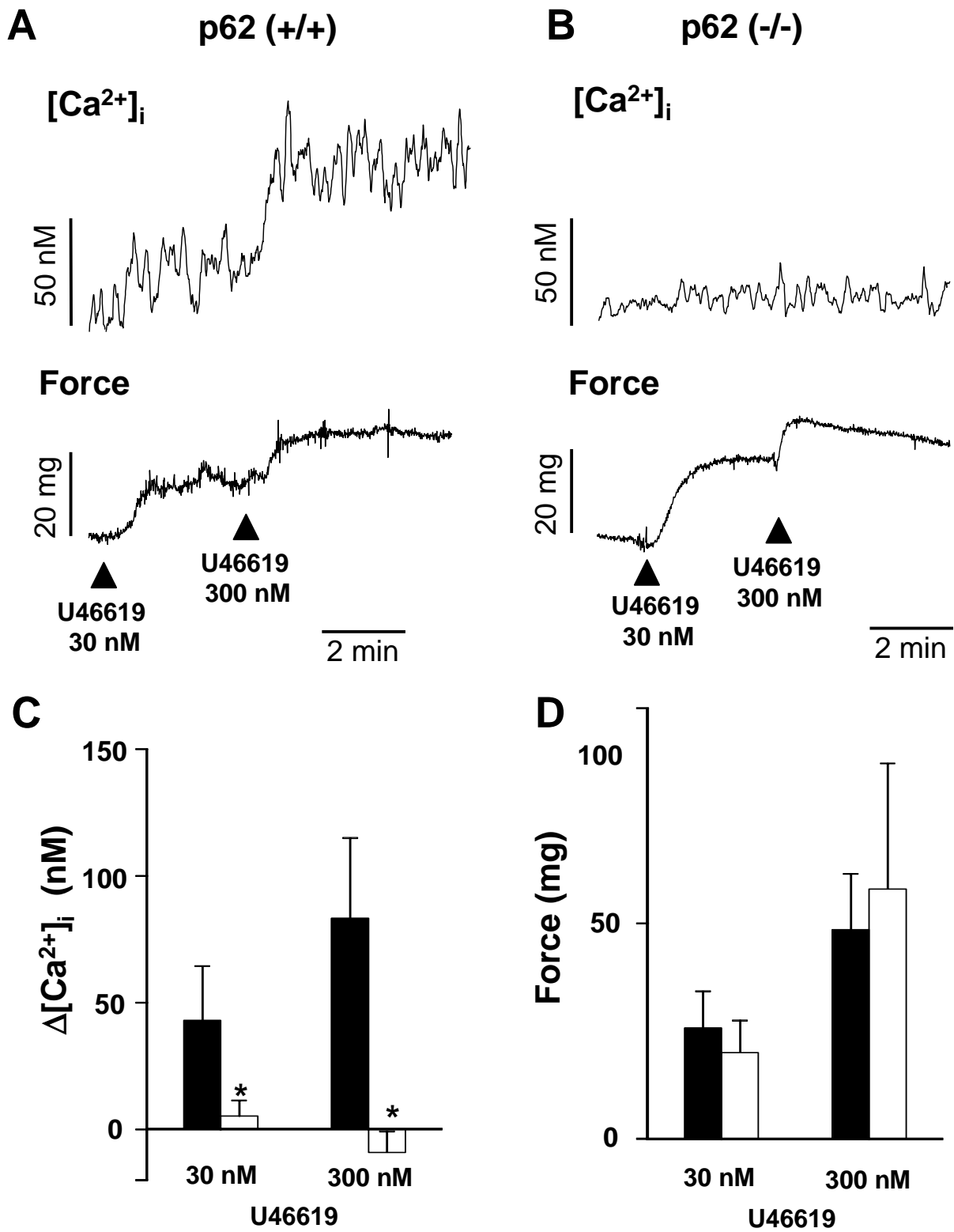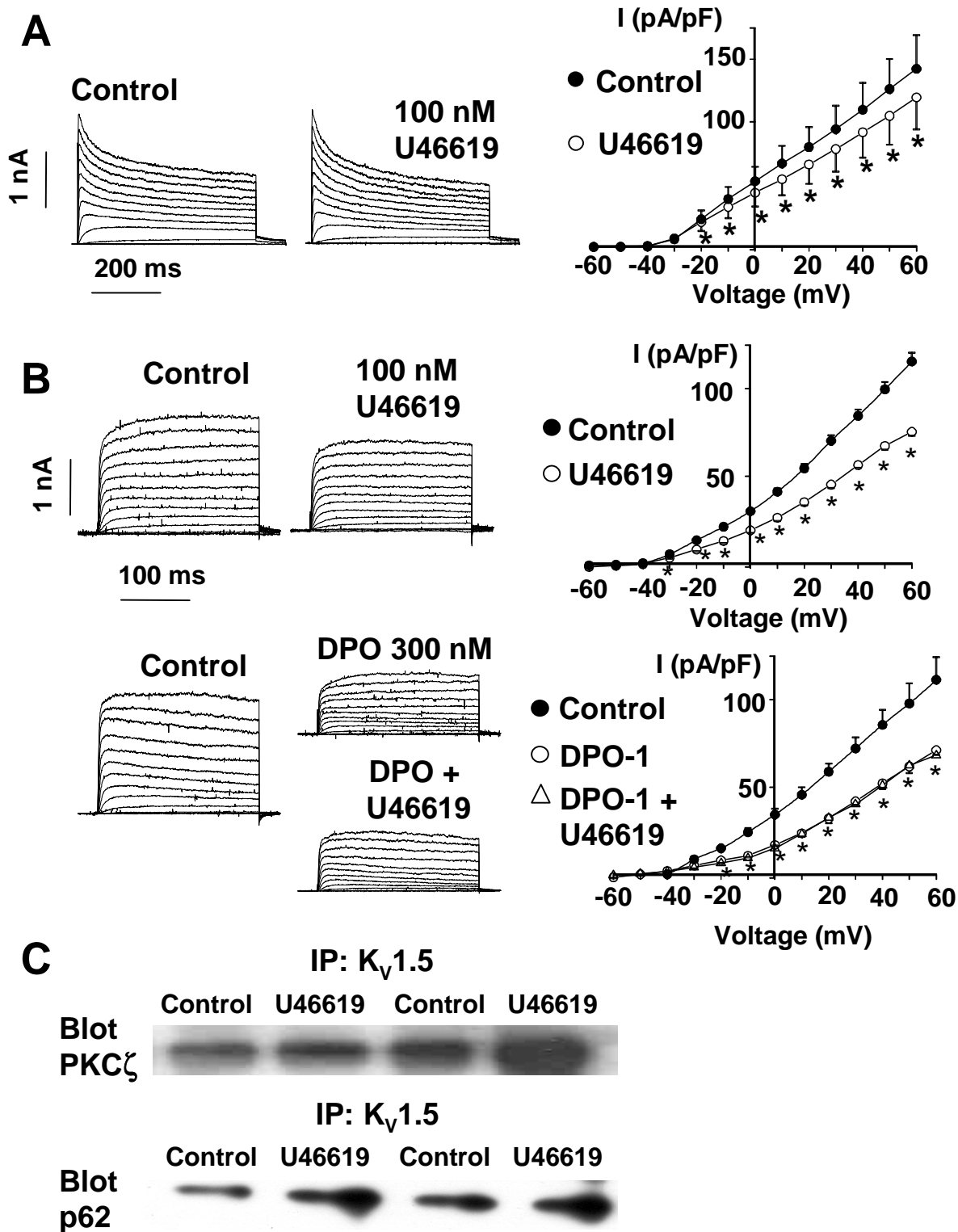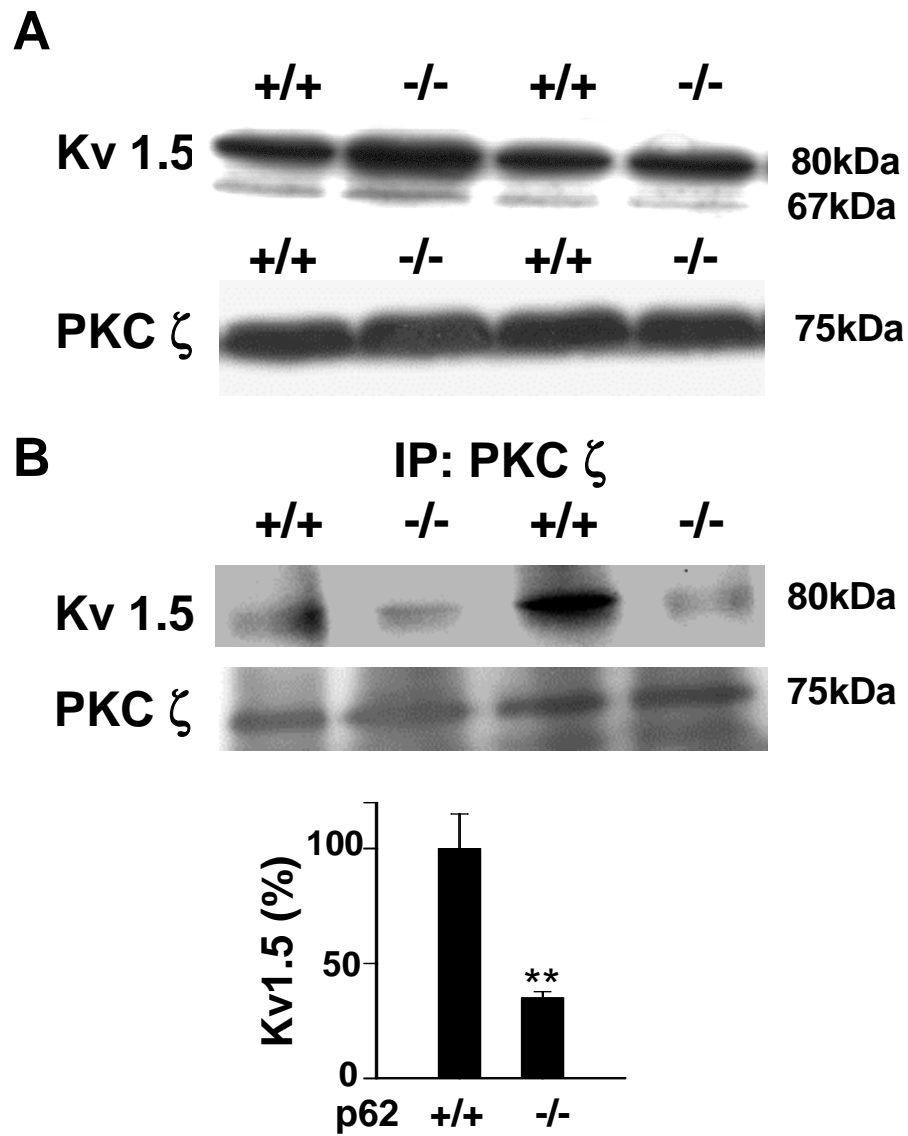
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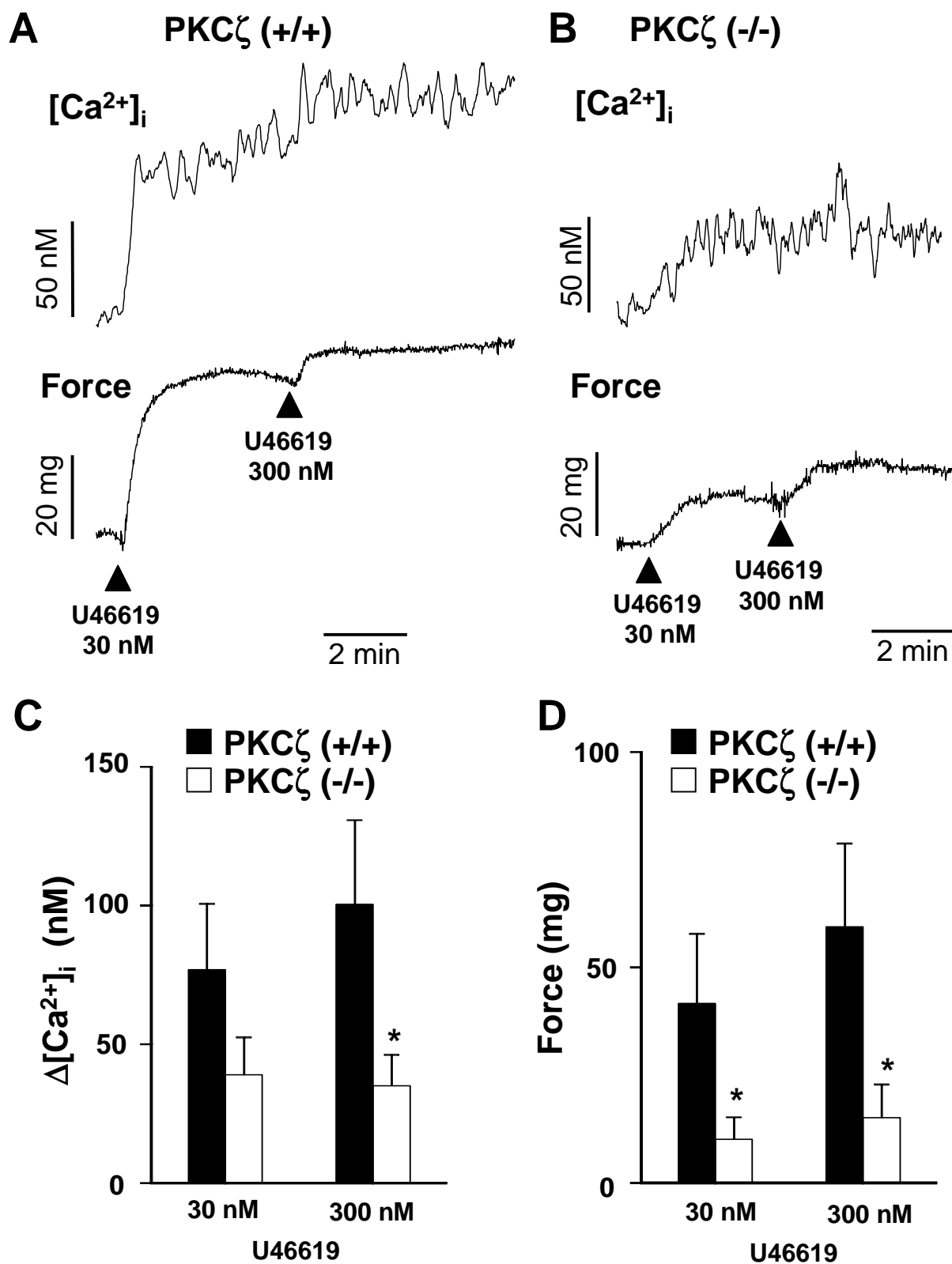
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