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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 $F_{2\alpha}$.

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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.

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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

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 PKCζ$^{-/-}$ 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 PKCζ$^{-/-}$(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

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).

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

PA rings were incubated for 80 minutes at room temperature in Krebs solution containing the fluorescent dye Fura-2 acetoximethylester (5×10^{-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 37º 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

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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

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Changes in $[Ca^{2+}]_i$ and contraction induced by U46619 were simultaneously analyzed in fura-2 loaded PA from wild-type and from PKCζ$^{-/-}$ mice. Basal levels of $[Ca^{2+}]_i$ in PKCζ$^{-/-}$ (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 PKCζ$^{-/-}$ animals (Fig. 2A and 2B). However, the increase in $[Ca^{2+}]_i$ (Fig. 2C) and the contractile response (Fig. 2D) was significantly reduced in PKCζ$^{-/-}$ 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_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. Fig. 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 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_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. Fig. 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, not significant, $p = 0.06$) and 163 ± 31% (n= 7, $p < 0.05$), respectively, in U46619-treated vs untreated arteries. The K_V1.5-PKCζ interaction

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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 $PKC\zeta^{-/-}$ 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 $PKC\zeta^{-/-}$ 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 ($PKC\zeta^{-/-}$ 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_V1.5 interaction and hence for the inhibition of K_V currents following TP receptor activation.

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References

Archer S, Souil E, Dinh-Xuan AT, Schremmer B, Mercier JC, El Yaagoubi A, Nguyen-Huu L, Reeve HL, Hampl V (1998) Molecular identification of the role of voltage-gated K^+ channels, Kv1.5 and Kv1.2, in hypoxic pulmonary vasoconstriction and control of resting membrane potential in rat pulmonary artery myocytes. *J Clin Invest* **101**:2319-2330.

Bailly K, Ridley AJ, Hall SM, Haworth SG (2004) RhoA activation by hypoxia in pulmonary arterial smooth muscle cells is age and site specific. *Circ Res* **94**:1383-1391.

Barnes PJ, Liu SF (1995) Regulation of pulmonary vascular tone. *Pharmacol Rev* **47**:87-131.

Cogolludo A, Moreno L, Lodi F, Frazziano G, Cobeno L, Tamargo J, Perez-Vizcaino F (2006) Serotonin inhibits voltage-gated K^+ currents in pulmonary artery smooth muscle cells: role of 5-HT2A receptors, caveolin-1, and $K_V1.5$ channel internalization. *Circ Res* **98**:931-938.

Cogolludo A, Moreno L, Lodi F, Tamargo J, Perez-Vizcaino F (2005) Postnatal maturational shift from PKCzeta and voltage-gated K^+ channels to RhoA/Rho kinase in pulmonary vasoconstriction. *Cardiovasc Res* **66**:84-93.

Cogolludo A, Moreno L, Bosca L, Tamargo J, Perez-Vizcaino F (2003) Thromboxane A_2-induced inhibition of voltage-gated K^+ channels and pulmonary vasoconstriction: role of protein kinase Czeta. *Circ Res* **93**:656-663.

Duran A, Serrano M, Leitges M, Flores JM, Picard S, Brown JP, Moscat J, Diaz-Meco MT (2004) The atypical PKC-interacting protein p62 is an important mediator of RANK-activated osteoclastogenesis. *Dev Cell* **6**:303-309.

Frey RS, Gao X, Javaid K, Siddiqui SS, Rahman A, Malik AB (2006) Phosphatidylinositol 3-kinase gamma signaling through protein kinase Czeta induces NADPH oxidase-mediated oxidant generation and NF-kappaB activation in endothelial cells. *J Biol Chem* **281**:16128-16138.

Gayral S, Deleris P, Laulagnier K, Laffargue M, Salles JP, Perret B, Record M, Breton-Douillon M (2006) Selective activation of nuclear phospholipase D-1 by g protein-coupled receptor agonists in vascular smooth muscle cells. *Circ Res* **99**:132-139.

Godeny MD, Sayeski PP (2006) ANG II-induced cell proliferation is dually mediated by c-Src/Yes/Fyn-regulated ERK1/2 activation in the cytoplasm and PKCzeta-controlled ERK1/2 activity within the nucleus. *Am J Physiol Cell Physiol* **291**:C1297-307.

Gong J, Xu J, Bezanilla M, van Huizen R, Derin R, Li M (1999) Differential stimulation of PKC phosphorylation of potassium channels by ZIP1 and ZIP2. *Science* **285**:1565-9.

Halushka PV, Mais DE, Mayeux PR, Morinelli TA (1989) Thromboxane, prostaglandin and leukotriene receptors. *Annu Rev Pharmacol Toxicol* **29**: 213-239.

Hirai T, Chida K (2003) Protein kinase C ζ (PKCζ): activation and cellular functions, *J Biochem (Tokyo)* **133**:1-7.

Kim Y, Uhm DY, Shin J, Chung S (2004) Modulation of delayed rectifier potassium channel by protein kinase C zeta-containing signaling complex in pheochromocytoma cells. *Neuroscience* **125**:359-368.

Kim Y, Park MK, Uhm DY, Shin J, Chung S (2005) Modulation of delayed rectifier potassium channels by alpha1-adrenergic activation via protein kinase C zeta and p62 in PC12 cells. *Neurosci Lett* **387**:43-48.

Krotova K, Hu H, Xia SL, Belayev L, Patel JM, Block ER, Zharikov S (2006) Peptides modified by myristoylation activate eNOS in endothelial cells through Akt phosphorylation. *Br J Pharmacol* **148**:732-740.

Lagrutta A, Wang J, Fermini B, Salata JJ (2006) Novel, potent inhibitors of human Kv1.5 K^+ channels and ultrarapidly activating delayed rectifier potassium current. *J Pharmacol Exp Ther* **317**:1054-1063.

Leitges M, Sanz L, Martin P, Duran A, Braun U, Garcia JF, Camacho F, Diaz-Meco MT, Rennert PD, Moscat J (2001) Targeted disruption of the zetaPKC gene results in the impairment of the NF-kappaB pathway. *Mol Cell* **8**:771-780.

Li D, Takimoto K, Levitan ES (2000) Surface expression of Kv1 channels is governed by a C-terminal motif. *J Biol Chem* **275**: 11597-11602.

Liu LZ, Zhao HL, Zuo J, Ho SK, Chan JC, Meng Y, Fang FD, Tong PC (2006) Protein kinase Czeta mediates insulin-induced glucose transport through actin remodelling in L6 muscle cells. *Mol Biol Cell* **17**:2322-2330.

Martens JR, Kwak Y-G, Tamkun MM (1999) Modulation of Kv channel α/β subunit interactions. *Trends Cardiovasc Med* **9**:253-258.

Moscat J, Diaz-Meco MT (2000) The atypical protein kinase Cs. Functional specificity mediated by specific protein adapters. *EMBO Rep* **1**:399-403.

Perez-Vizcaino F, Cogolludo A, Tamargo J (1999) Modulation of arterial Na^+-K^+-ATPase-induced $[Ca^{2+}]_i$ reduction and relaxation by norepinephrine, ET-1, and PMA. *Am J Physiol* **276**:H651-H657.

Platoshyn O, Brevnova EE, Burg ED, Yu Y, Remillard CV, Yuan JX (2006) Acute hypoxia selectively inhibits KCNA5 channels in pulmonary artery smooth muscle cells. *Am J Physiol Cell Physiol* **290**:C907-916.

Pozeg ZI, Michelakis ED, McMurtry MS, Thebaud B, Wu XC, Dyck JR, Hashimoto K, Wang S, Moudgil R, Harry G, Sultanian R, Koshal A, Archer SL (2003) In vivo gene transfer

of the O_2-sensitive potassium channel Kv1.5 reduces pulmonary hypertension and restores hypoxic pulmonary vasoconstriction in chronically hypoxic rats. *Circulation* **107**:2037-2044.

Remillard CV, Tigno DD, Platoshyn O, Burg ED, Brevnova EE, Conger D, Nicholson A, Rana BK, Channick RN, Rubin LJ, O'connor DT, Yuan JX (2007) Function of Kv1.5 Channels and Genetic Variations in KCNA5 in Patients with Idiopathic Pulmonary Arterial Hypertension. *Am J Physiol Cell Physiol* **292**: C1837-C1853.

Salamanca DA, Khalil RA (2005) Protein kinase C isoforms as specific targets for modulation of vascular smooth muscle function in hypertension. *Biochem Pharmacol* **70**:1537-1547.

Shimoda LA, Sylvester JT, Booth GM, Shimoda TH, Meeker S, Undem BJ, Sham JS (2001) Inhibition of voltage-gated K+ currents by endothelin-1 in human pulmonary arterial myocytes. *Am J Physiol Lung Cell Mol Physiol* **281**:L1115-L1122.

Shizukuda Y, Buttrick PM (2002) Protein kinase C-zeta modulates thromboxane A2-mediated apoptosis in adult ventricular myocytes via Akt. *Am J Physiol Heart Circ Physiol* **282**:H320-H327.

Snetkov VA, Knock GA, Baxter L, Thomas GD, Ward JP, Aaronson PI (2006) Mechanisms of the prostaglandin F2alpha-induced rise in $[Ca^{2+}]_i$ in rat intrapulmonary arteries. *J Physiol* **571**:147-163.

Somlyo AP, Somlyo AV (2000) Signal transduction by G-proteins, Rho kinase and protein phosphatase to smooth muscle and non-muscle myosin II. *J Physiol* **522**:177-185.

Valenzuela C, Delpon E, Tamkun MM, Tamargo J, Snyders DJ (1995) Stereoselective block of a human cardiac potassium channel (Kv1.5) by bupivacaine enantiomers. *Biophys J* **69**:418-427.

Uebele VN, England SK, Chaudhary A, Tamkun MM, Snyders DJ (1996) Functional differences in Kv1.5 currents expressed in mammalian cell lines are due to the presence of endogenous Kv beta 2.1 subunits. *J Biol Chem* **271**:2406-2412.

Ward JP, Knock GA, Snetkov VA, Aaronson PI (2004) Protein kinases in vascular smooth muscle tone--role in the pulmonary vasculature and hypoxic pulmonary vasoconstriction. *Pharmacol Ther* **104**:207-231.

Weir EK, Reeve HL, Huang JM, Michelakis E, Nelson DP, Hampl V, Archer SL (1996) Anorexic agents aminorex, fenfluramine, and dexfenfluramine inhibit potassium current in rat pulmonary vascular smooth muscle and cause pulmonary vasoconstriction. *Circulation* **94**:2216-2220.

Yuan XJ, Wang J, Juhaszova M, Gaine SP, Rubin LJ (1998a) Attenuated K+ channel gene transcription in primary pulmonary hypertension. *Lancet* **351**:726-727.

Yuan X-J, Wang J, Juhaszova M, Golovina VA, Rubin LJ (1998b) Molecular basis and function of voltage-gated K+ channels in pulmonary arterial smooth muscle. *Am J Physiol* **274**: L621–L635.

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 ± 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 ± 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 ± SEM of 5-6 cells). Panels E and F show

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_V1.5 channels: TP receptor activation inhibits K_V1.5 currents and increases co-immunoprecipitation of K_V1.5 with PKCζ. Panel A: Current traces recorded in Ltk$^-$ cells stably transfected with human K_V1.5 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_V1.5 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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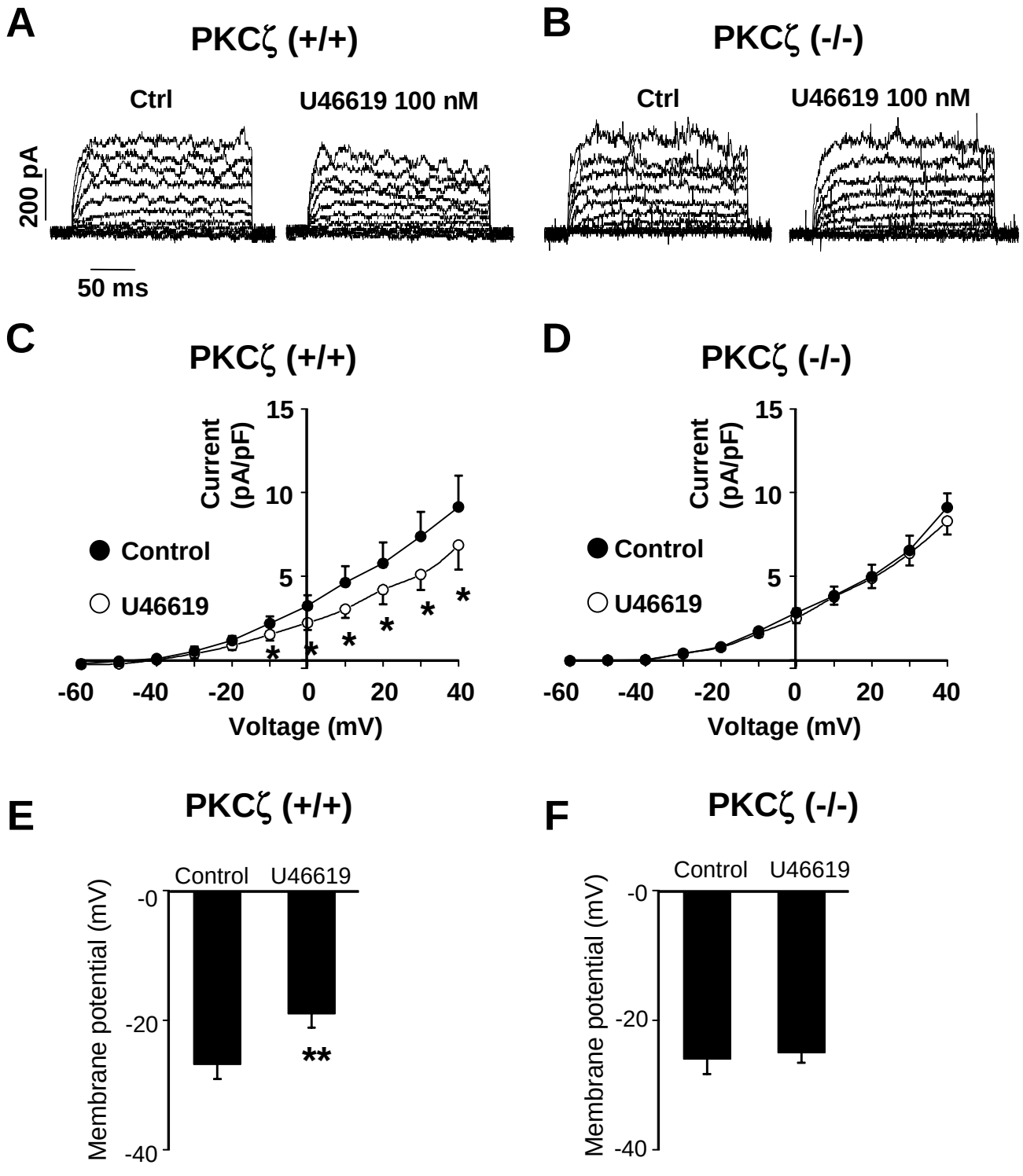

Figure 1

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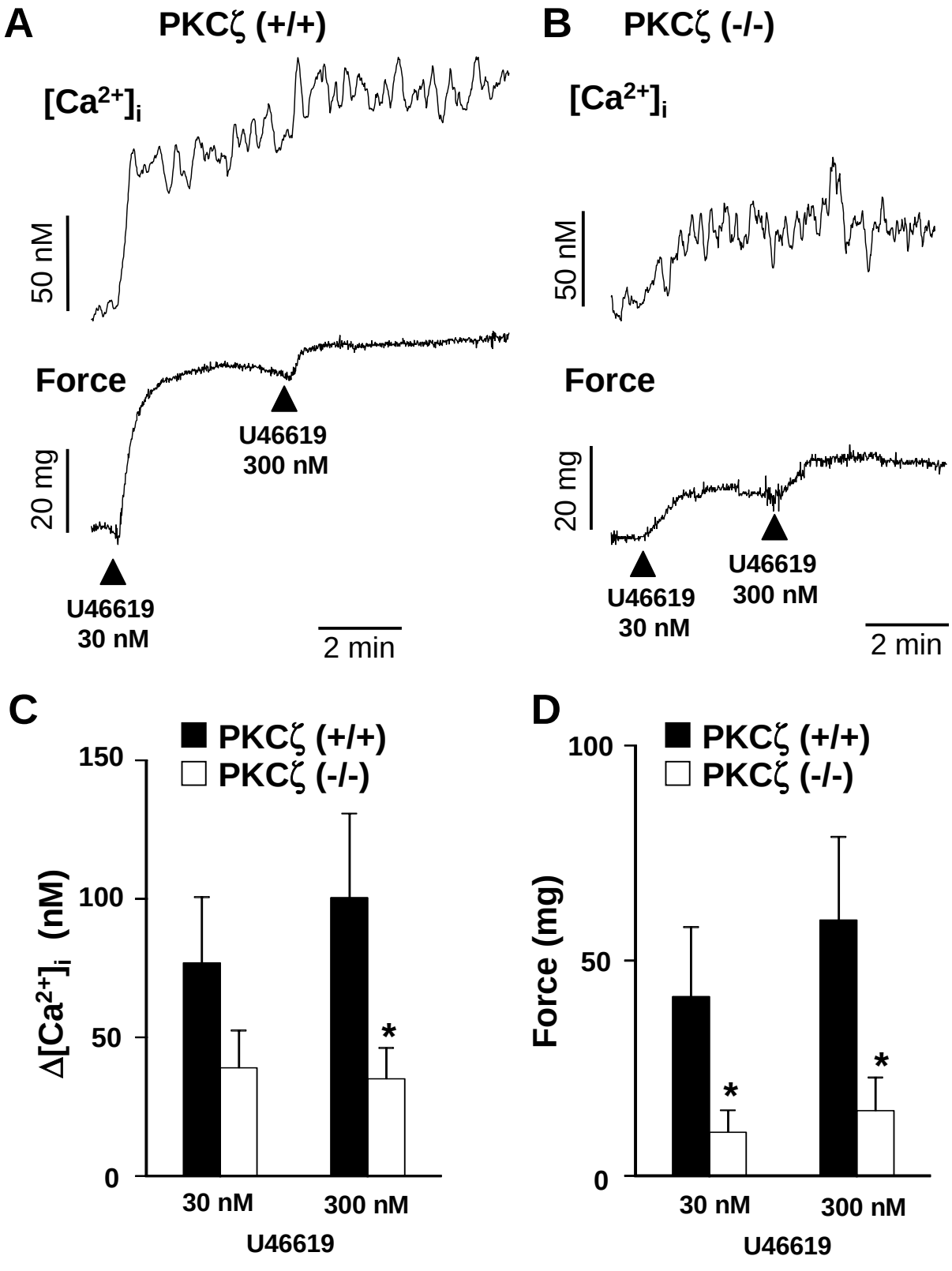

Figure 2

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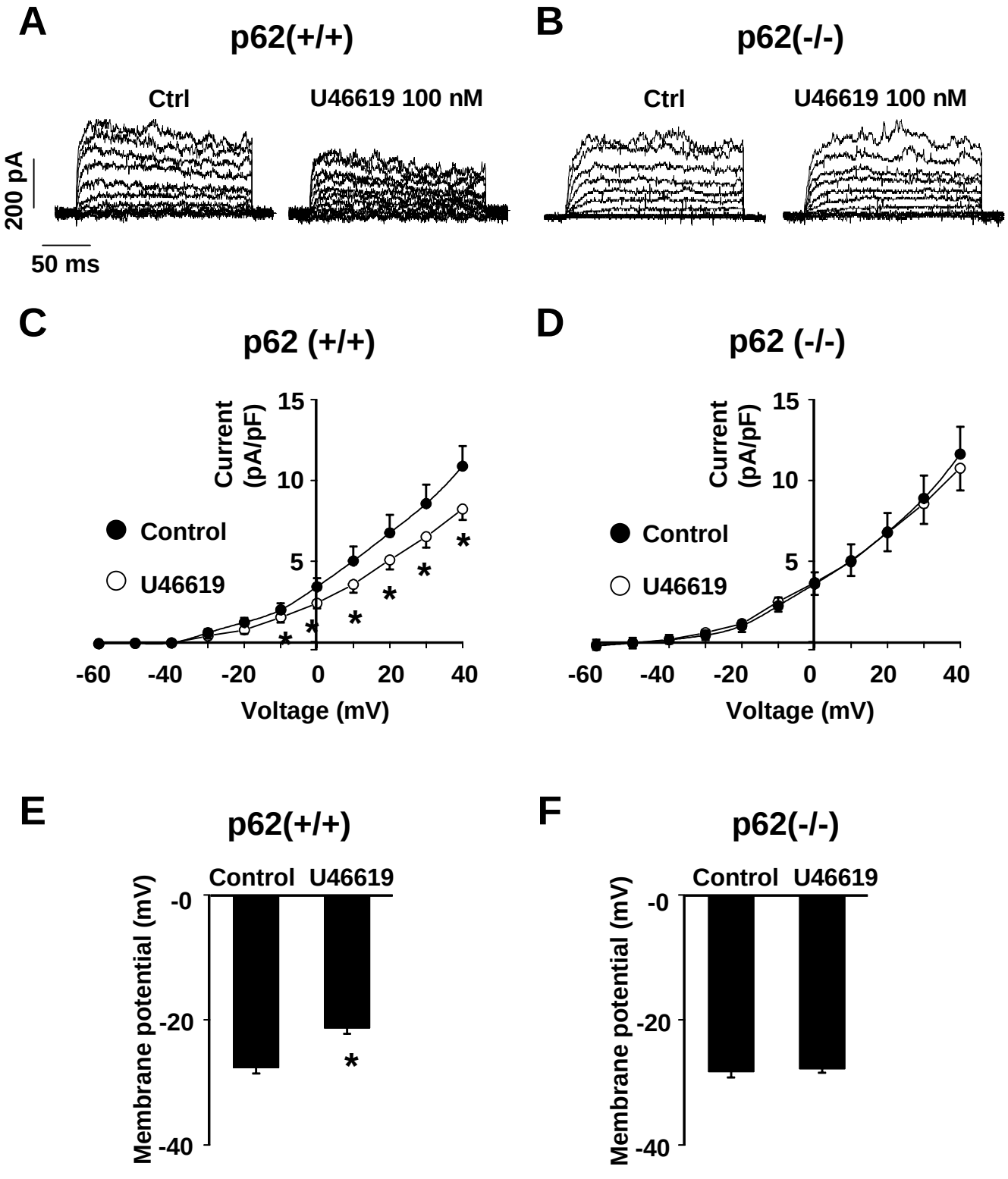

Figure 3

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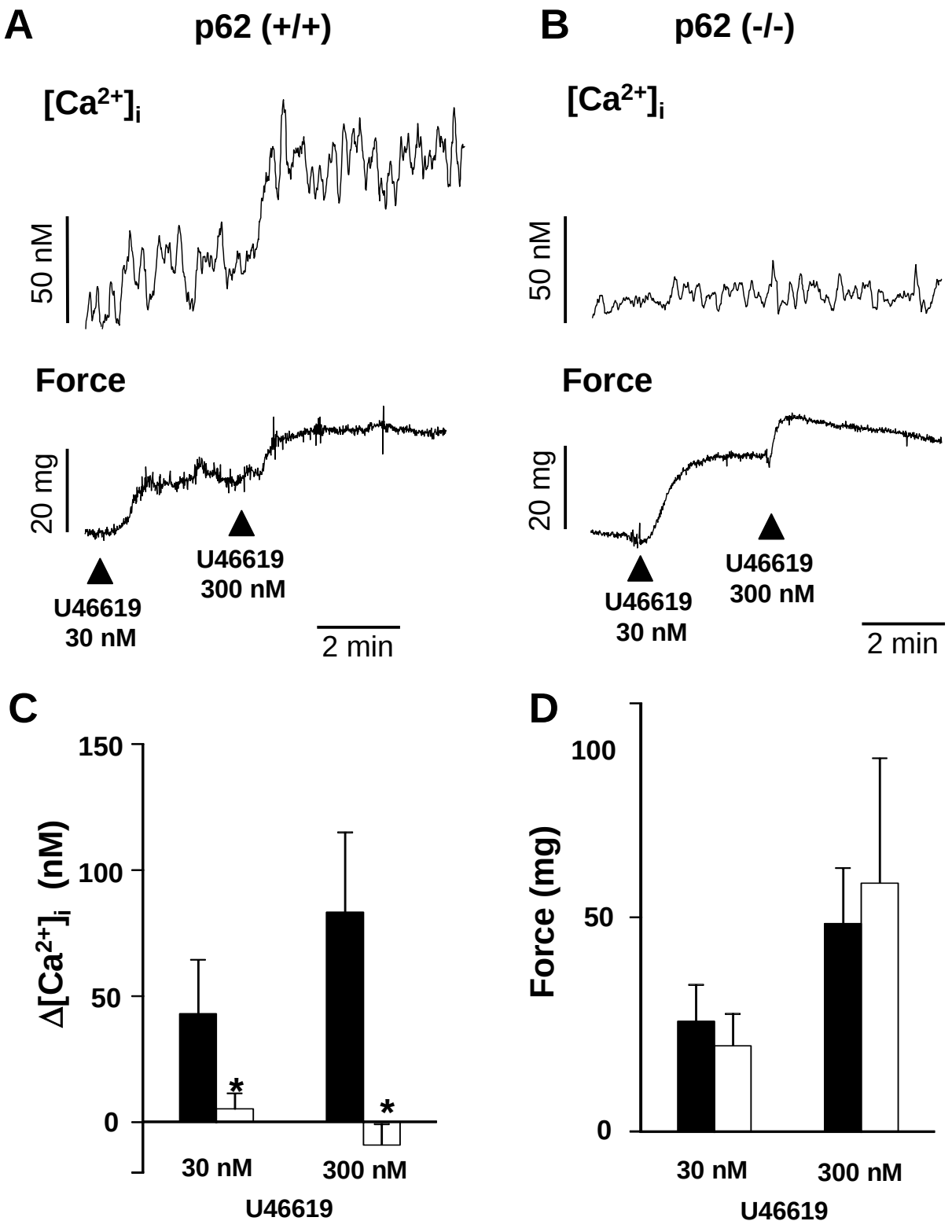

Figure 4

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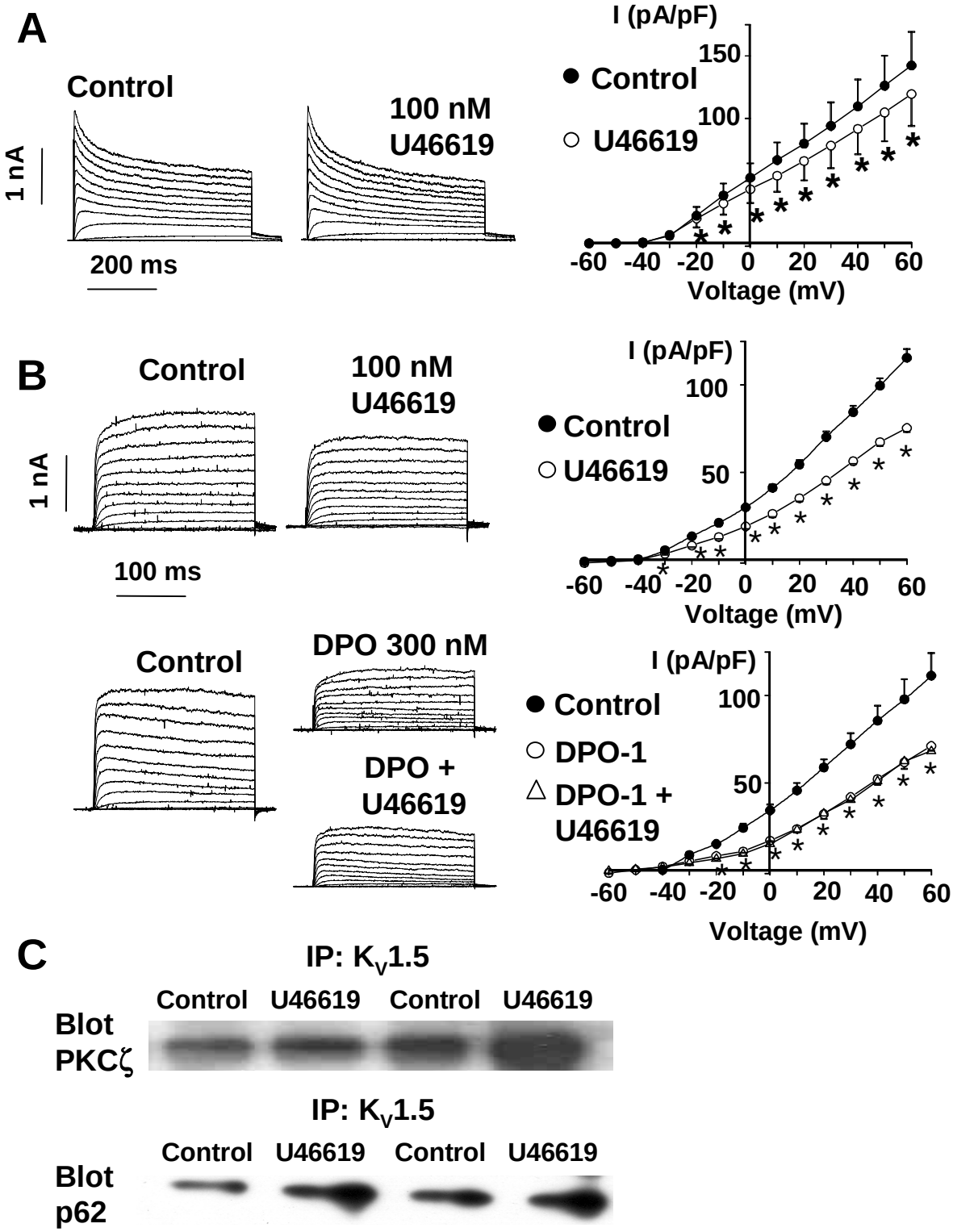

Figure 5

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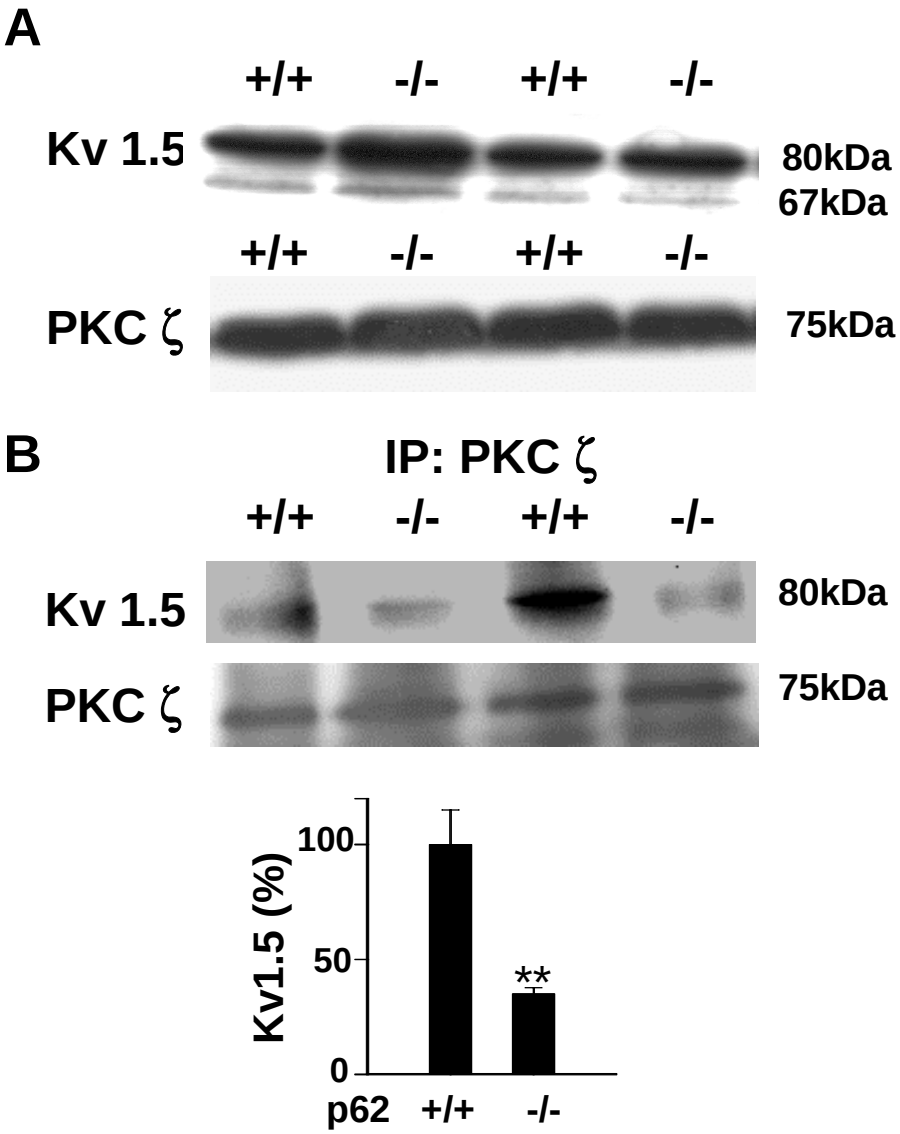

Figure 6