Research Paper

Journal of Vascular Research

J Vasc Res 2015;52:279–288
DOI: 10.1159/000443402

Received: June 15, 2015
Accepted after revision: December 14, 2015
Published online: February 19, 2016

PKCα Deficiency in Mice Is Associated with Pulmonary Vascular Hyperresponsiveness to Thromboxane A2 and Increased Thromboxane Receptor Expression

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Key Words
Pulmonary vascular responsiveness · Hypoxic pulmonary vasoconstriction · Pulmonary arterial hypertension · Protein kinase C iota

Abstract
Pulmonary vascular hyperresponsiveness is a main characteristic of pulmonary arterial hypertension (PAH). In PAH patients, elevated levels of the vasoconstrictors thromboxane A2 (TXA2), endothelin (ET)-1 and serotonin further contribute to pulmonary hypertension. Protein kinase C (PKC) isozyme alpha (PKCα) is a known modulator of smooth muscle cell contraction. However, the effects of PKCα deficiency on pulmonary vasoconstriction have not yet been investigated. Thus, the role of PKCα in pulmonary vascular responsiveness to the TXA2 analog U46619, ET-1, serotonin and acute hypoxia was investigated in isolated lungs of PKCα$^{-/-}$ mice and corresponding wild-type mice, with or without prior administration of the PKC inhibitor bisindolylmaleimide I or Gö6976. mRNA was quantified from microdissected intrapulmonary arteries. We found that broad-spectrum PKC inhibition reduced pulmonary vascular responsiveness to ET-1 and acute hypoxia and, by trend, to U46619. Analogously, selective inhibition of conventional PKC isozymes or PKCα deficiency reduced ET-1-evoked pulmonary vasoconstriction. The pulmonary vasopressor response to serotonin was unaffected by either broad PKC inhibition or PKCα deficiency. Surprisingly, PKCα$^{-/-}$ mice showed pulmonary vascular hyperresponsiveness to U46619 and increased TXA2 receptor (TP receptor) expression in the intrapulmonary arteries. To conclude, PKCα regulates ET-1-induced pulmonary vasoconstriction. However, PKCα deficiency leads to pulmonary vascular hyperresponsiveness to TXA2, possibly via increased pulmonary arterial TP receptor expression.

Christoph Tabeling and Elena Noe contributed equally to this work. The study is part of the PhD thesis of Elena Noe.

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Introduction

Pulmonary arterial hypertension (PAH) is a fatal condition. Increased pulmonary vasoconstriction and progressive remodeling of the pulmonary arteries lead to elevated pulmonary arterial resistance and ultimately to right heart failure. Endothelial dysfunction, characterized by an imbalance between vasoconstrictive and vasodilative mediators, is an important feature of PAH pathobiology [1]. In PAH patients, increased levels of thromboxane A2 (TXA2) [2], endothelin (ET)-1 [3] and serotonin [4] as well as decreased levels of nitric oxide [5] and prostacyclin [2] have been observed. Moreover, the pulmonary vasopressor response to constrictive agents is enhanced in PAH patients [6], reflecting a hypercontractile state of pulmonary vascular smooth muscle cells. This hyperresponsiveness is believed to potentiate pulmonary vasoconstriction induced by the imbalance of the vasoactive agents.

In patients with systemic sclerosis-associated PAH, functional autoantibodies against the ET receptor A and/or the angiotensin II receptor type 1 may contribute to increased pulmonary vasoconstriction [7]. Pulmonary vascular hyperresponsiveness is also a key feature of commonly used experimental models of PAH including the monocrotaline [8], the T-helper (Th)2 inflammation [9, 10] and the hypoxia model [11], and it has been reported for clinically relevant vasoconstrictors like TXA2, ET-1, serotonin and alveolar hypoxia [8, 9]. Mechanistically, pulmonary vascular hyperresponsiveness has been proposed to be partially dependent on protein kinase C (PKC) signaling [9].

PKC is a family of serine/threonine kinases, which are classified into conventional (c)PKC, novel (n)PKC and atypical (a)PKC isozymes [12, 13]. Both conventional (α, β_I, β_{II} and γ) and novel PKC isozymes (δ, ε, η and θ) require phospholipids and 1,2-diacylglycerol for activation, whereas activation of the conventional isoforms additionally depends on Ca^{2+} [13]. Atypical isozymes (ζ, ι / λ) also require phospholipids but are activated independently of 1,2-diacylglycerol and Ca^{2+} [13].

Isozymes α, β, δ, ε and ζ have been implicated in smooth muscle cell contraction via myosin light-chain phosphorylation, Ca^{2+} channel activation and/or K^+ channel inhibition [13], and PKC has been proposed as a therapeutic target in pulmonary (arterial) hypertension [12, 14]. Nevertheless, PKC isozymes are differentially expressed and opposing effects of separate isozymes have been described [12, 15], highlighting the potential need for isozyme-specific targeting.

Hitherto, little is known about the functional role of specific PKC isozymes in the pulmonary vasculature [14]. Cogolludo et al. [16] reported the involvement of PKCζ in TXA2-evoked pulmonary arterial constriction, whereas PKCε has been shown to specifically modulate hypoxic pulmonary vasoconstriction (HPV) [17], which has previously been reported to be PKC-dependent [18]. Diminishing effects on HPV following broad-spectrum PKC inhibition have been repeatedly described [18–20], but cPKC isozymes do not seem to play a role in mediating HPV [20]. Snow et al. [21] found that intermittent hypoxia augments ET-1-induced pulmonary vasoreactivity in a PKCβ-dependent manner, and it has been suggested that PKCδ plays a role in modulating contraction in pulmonary arterial smooth muscle cells (PASMCs) due to angiotensin II [22]. Moreover, PKC is thought to promote ET-1-induced contraction of human pulmonary arteries [23], possibly via the mediation of ET-1-induced inhibition of K_v channels, which has been shown to be PKC-dependent [23]. However, the functional role of the specific isozyme PKCα in the pulmonary vasculature remains elusive.

TXA2, ET-1 and serotonin mediate pulmonary vasoconstriction via direct G protein-coupled receptor activation on vascular smooth muscle cells. Hypoxia-induced contraction of isolated PASMCs or pulmonary artery rings requires intrinsic mechanisms including hypoxia sensing and signal transduction [24]. Alternatively, HPV in the intact lung involves hypoxia sensing at the alveolocapillary level, from where the signal is conducted retrogradely to the upstream pulmonary arterioles where it is transferred to PASMCs which ultimately contract [25]. PKC-dependent vasoconstriction has been described for each of these vasoconstrictive stimuli [16, 18–21, 26–29]. Nevertheless, the role of PKC in TXA2- and serotonin-evoked pulmonary vascular responsiveness is controversial, since species- and compound-related differences have been observed [16, 18, 26, 27].

In this study, we dissected the role of PKCα in pulmonary vascular responsiveness to the PAH-associated vasoconstrictors TXA2, ET-1, serotonin and alveolar hypoxia, by investigating isolated perfused lungs of PKCα-deficient ($PKC\alpha^{-/-}$) mice. In order to compare the PKCα-specific effects to the overall PKC or cPKC signaling effects, broad-spectrum PKC inhibition and inhibition of the conventional PKC isozymes, PKCα and PKCβ, was studied in parallel.

Materials and Methods

Mice

All experiments were approved by the local Department of Health and Social Affairs (Landesamt für Gesundheit und Soziales, LAGeSo, Berlin, Germany). Female PKCα$^{-/-}$ and corresponding wild-type (WT) mice (8–11 weeks old) on a 129 SV background were used for all experiments. The generation of PKCα$^{-/-}$ mice has been described elsewhere [30]. The WT mice were purchased from Charles River Laboratories (Sulzfeld, Germany). All mice were housed in individually ventilated cages inside a climate- and light-cycle-controlled animal house, and were fed a diet of standard chow and tap water ad libitum.

Isolated Perfused and Ventilated Mouse Lungs

Mouse lungs were isolated as described previously [31, 32]. Briefly, mice were anesthetized i.p. with 160 mg ketamine and 75 mg xylazin per kilogram of body weight, tracheally intubated, ventilated and then heparinized by intracardial injection of 125 IU of heparin sodium and exsanguinated. The pulmonary artery and the left atrium were cannulated in order to perfuse the lung vasculature with sterile and carbonated (20 mmol/l $NaHCO_3$) 37°C Krebs-Henseleit hydroxyethylamylopectin buffer (1 ml/min; Serag-Wiessner, Naila, Germany). Left atrial pressure was set at +2.2 cm H_2O. Lungs were ventilated with negative pressure in a closed chamber (expiratory pressure of –4.5 cm H_2O; inspiratory pressure of –9.0 cm H_2O; 90 breaths/min) and hyperinflation (–24 cm H_2O) was performed at 4-min intervals. All hardware and software were purchased from HSE-Harvard Apparatus (March-Hugstetten, Germany). Mean pulmonary arterial pressure (Ppa mean) and airway resistance were continuously monitored. Data were discarded from further analyses if lungs had signs of hemostasis, atelectasis or edema.

Pulmonary Vascular Responsiveness

To study the effects of PKC inhibition, broad-spectrum PKC inhibitor bisindolylmaleimide I (BIM, 1 μmol/l; Santa Cruz Biotechnologies, Santa Cruz, Calif., USA), the selective inhibitor of the conventional PKC isozymes PKCα and PKCβ Gö6976 (1 μmol/l; Merck Millipore, Darmstadt, Germany) or solvent (DMSO <1‰) was added to the perfusate. After a steady-state period of approximately 12 min, increasing doses of ET-1 (10 and 100 nmol/l; Merck Millipore), serotonin (3, 10 and 30 μmol/l; Sigma-Aldrich, Steinheim, Germany) or TP receptor agonist U46619 (30, 100 and 300 nmol/l; Merck Millipore) were administered into the perfusion buffer for 10 (ET-1) [9], 0.5 (serotonin) [33] or 3 min (U46619) [34] each. To investigate the effects of PKCι inhibition on pulmonary vasoconstriction, the PKCι inhibitor sodium aurothiomalate hydrate (ATM, 20 μmol/l; Sigma-Aldrich) was dissolved in the perfusion buffer and the vasopressor response was assessed following a steady-state period of approximately 28 min [35, 36].

For the analysis of HPV, the murine lungs were isolated as described [37], and ventilated with negative pressure in a closed chamber with a tidal volume of approximately 10 ml/kg body weight and an end-expiratory pressure of –2 cm H_2O. HPV and the relationship between pressure (P) and volumetric flow-rate (Q) were analyzed as described before [38, 39]. Briefly, Nω-nitro-L-arginine methyl ester hydrochloride (1 mmol/l; Sigma-Aldrich) and indomethacin (30 μmol/l; Sigma-Aldrich) were added to the perfusate buffer to prevent nitric oxide and prostaglandin synthesis, respectively. After a steady-state period of 10 min, a 4-point pressure-flow (P-Q) curve was generated under normoxic conditions by perfusing the lungs with a flow of 0.5, 1, 1.5 or 2 ml/min for 30 s each. Perfusion flow was then set at 1 ml/min. After a second steady-state period of 4 min, the Ppa mean was recorded for 12 min in response to a change from normoxic to hypoxic (1% O_2) ventilation. Approximately 10 minutes after the initiation of HPV, a second P-Q curve was generated.

The difference in Ppa mean (ΔPpa mean) was expressed as a percentage and the pressure response to the highest stimulus concentration (ET-1, serotonin or U46619), hypoxic ventilation or highest perfusion rate (P-Q curve) of the respective control group was set at 100%.

Representative tracings of the Ppa mean in isolated perfused and ventilated mouse lungs in response to each vasoconstrictive stimulus are shown in online supplementary figure 1 (for all online suppl. material, see www.karger.com/doi/10.1159/000443402).

Microdissection of Intrapulmonary Arteries

Mouse lungs were isolated and flushed with NaCl 0.9% for 2 min, dissected and instilled with Tissue-Tek® O.C.T.™ compound (Sakura, Staufen, Germany)/PBS (1:4) through the trachea at a pressure of 20 cm H_2O for 15 min. Lungs were embedded in Tissue-Tek O.C.T. compound and quick-frozen. Serial 8-μm-thick cryosections were cut, prepared on membrane slides (membrane slides PEN 1.0, Carl Zeiss, Oberkochen, Germany) and fixated with –20°C ethanol 95% for 1.5 min. Hematoxylin and eosin staining was performed, followed by dehydration with increasing ethanol concentrations (75, 95 and 100%). The air-dried membrane slides were used for laser capture microdissection of intrapulmonary arteries with PALM MicroBeam (Carl Zeiss Microimaging GmbH, Bernried, Germany).

Real-Time Quantitative RT-PCR

Gene expression analysis of microdissected intrapulmonary arteries and whole tissue homogenates of dissected and quick-frozen PKCα$^{-/-}$ and WT lungs was performed. Total RNA was isolated with silica-membrane technology (NucleoSpin® RNA XS-Kit, Macherey & Nagel). Reverse transcription was performed using a high-capacity reverse-transcription kit (Applied Biosystems, Darmstadt, Germany). qRT-PCR was achieved with the 7300 real-time PCR system. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as a housekeeping control gene (HKG) while TP receptor and PKC isozymes were genes of interest (GOI). TaqMan® gene expression assays were obtained from Applied Biosystems. The assay IDs were Mm00436917_m1 (TP receptor), Mm00435749_m1 (PKCβ), Mm00440861_m1 (PKCγ), Mm00440891_m1 (PKCδ), Mm00440894_m1 (PKCε), Mm01340228_m1 (PKCθ), Mm00435769_m1 (PKCι), Mm00776345_g1 (PKCζ). Primer sequences for PKCη were CgAgggCTgggTggATCT (forward) and gTCTTCgCATAgCCCTTTgg (reverse). WT mice were considered as controls. Relative quantitative gene expression (RQ) was calculated according to the comparative Ct method: ΔCt: $Ct_{GOI} - Ct_{HKG}$; ΔΔCt: $\Delta Ct_{PKC\alpha-/-\text{ or WT}} - \Delta Ct_{\text{mean WT}}$; RQ: $2^{-\Delta\Delta CT}$.

Statistical Analysis

Two-way ANOVA was performed for the comparison of dose-response curves and the Mann-Whitney U test for the comparison of data between 2 groups. $p \leq 0.05$ was considered to be statistically significant and p values are presented as follows: * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$. All data are expressed as mean + SEM.

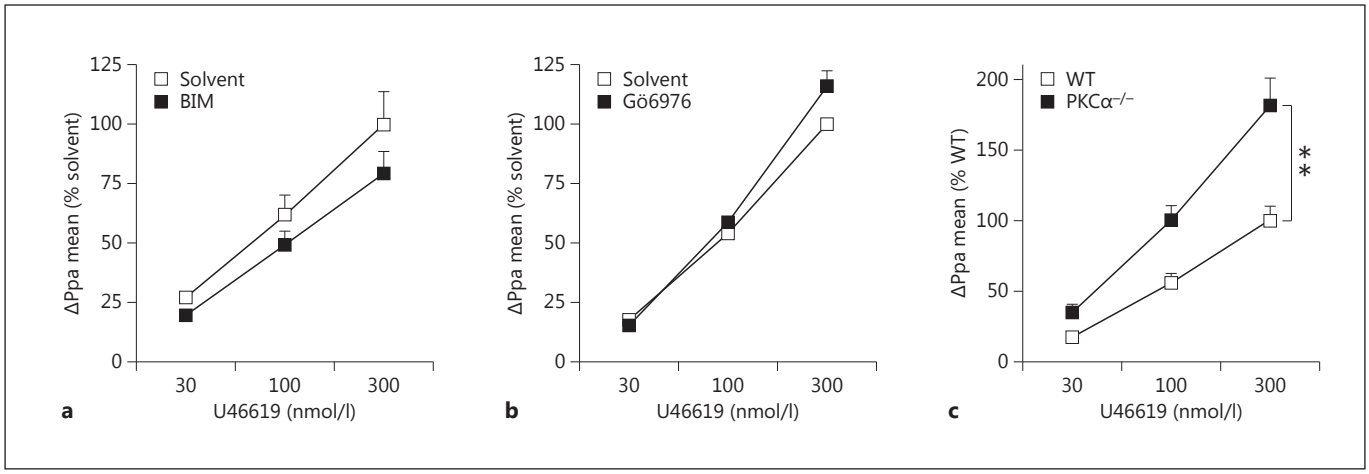

Fig. 1. PKCα deficiency induces pulmonary vascular hyperresponsiveness to TP receptor agonist U46619. **a** In isolated perfused and ventilated mouse lungs, U46619-evoked pulmonary vasoconstriction was reduced by trend following treatment with the PKC inhibitor BIM (1 μmol/l) compared to solvent treatment. **b** Selective inhibition of conventional PKC isozymes by Gö6976 (1 μmol/l) had no effect on pulmonary vascular responsiveness to U46619. **c** PKCα$^{-/-}$ mice showed a significantly greater increase in Ppa compared to the corresponding WT mice upon U46619 stimulation. Data represent the percentage of the pressure response observed in the respective control group at highest concentration. Values are shown as mean + SEM. **a** n = 11 per group; **b**, **c** n = 6–7 per group. ** $p \leq 0.01$ versus WT group.

Results

U46619 Caused Pulmonary Vascular Hyperresponsiveness in PKCα-Deficient Mice

To elucidate the role of PKCα in pulmonary vascular responsiveness, the effects of PKC inhibition and PKCα deficiency were analyzed in isolated perfused and ventilated mouse lungs. Under basal conditions, the Ppa mean was unaffected by pharmacological broad-spectrum PKC inhibition with BIM (mean ± SEM: 10.11 ± 0.09 cm H_2O) compared to solvent treatment (10.23 ± 0.12 cm H_2O), by selective inhibition of the conventional PKC isozymes PKCα and PKCβ with Gö6976 (10.51 ± 0.16 cm H_2O) compared to solvent treatment (10.30 ± 0.15 cm H_2O) or by PKCα deficiency (9.30 ± 0.18 cm H_2O) compared to the WT control (9.81 ± 0.15 cm H_2O).

Pulmonary vascular responsiveness to the TP receptor agonist U46619 was reduced by trend following broad-spectrum PKC inhibition when compared to solvent treatment (fig. 1a), suggesting a moderate role of PKC in thromboxane-evoked pulmonary vasoconstriction as shown before [26]. Selective cPKC inhibition had no effect on the U46619-evoked pressure response (fig. 1b), which is in line with previous findings [16]. Surprisingly, U46619-induced pulmonary vascular responsiveness was highly increased in PKCα$^{-/-}$ lungs compared to the corresponding WT lungs (fig. 1c). This pulmonary vascular hyperresponsiveness in PKCα$^{-/-}$ mice was, at first sight, contradictory, as PKCα is thought to promote vasoconstriction [40, 41]. To analyze whether this hyperresponsiveness is (1) thromboxane-specific or (2) specific for vasoconstriction initiated at the vascular site via direct G protein-coupled receptor activation, we studied pulmonary vascular responsiveness to ET-1 and serotonin as well as HPV.

ET-1-Induced Pulmonary Vascular Responsiveness Is PKCα-Dependent

Both broad-spectrum PKC inhibition (fig. 2a) and the selective inhibition of the cPKC isozymes PKCα and PKCβ (fig. 2b) reduced the pulmonary vasoconstriction induced by ET-1 application. Furthermore, pulmonary vascular responsiveness to ET-1 was markedly reduced by about 40% in PKCα$^{-/-}$ mice compared to in WT mice (fig. 2c), suggesting that PKCα is the main PKC isozyme promoting the pulmonary vasopressor response to ET-1.

In contrast, serotonin-induced pulmonary vascular responsiveness was not altered by broad-spectrum PKC inhibition (fig. 3a) or PKCα deficiency (fig. 3b).

HPV Is PKC-Dependent but Not PKCα-Dependent

Hypoxic ventilation led to the characteristic increase in Ppa mean. As shown before by Weissmann et al. [18], PKC inhibition distinctly reduced HPV (fig. 4a). Consistently, as revealed by the P-Q curves, PKC inhibition di-

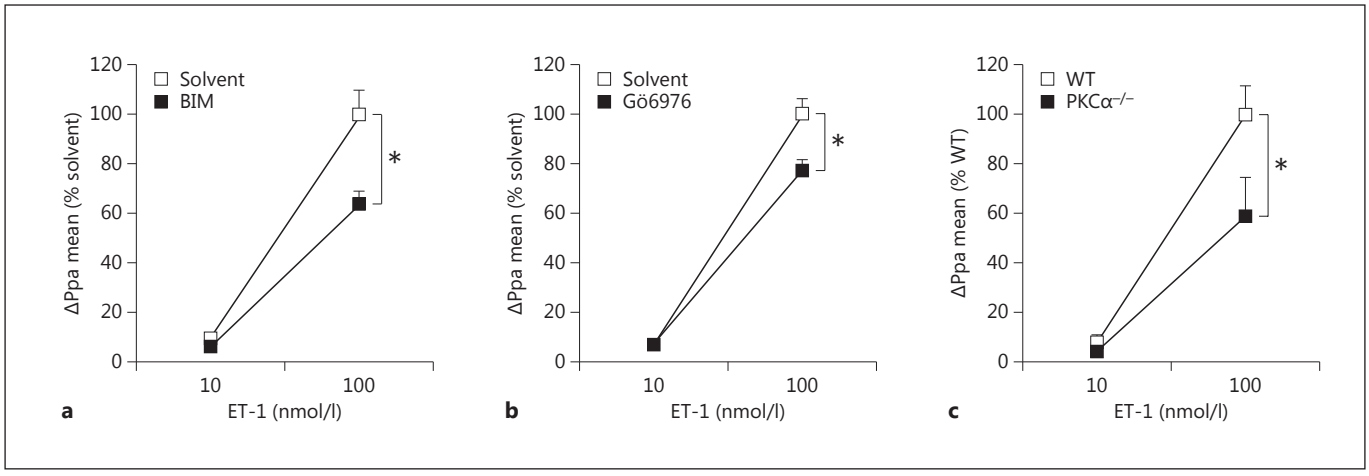

Fig. 2. PKCα is required for ET-1-induced pulmonary vasoconstriction. Broad-spectrum PKC inhibition (BIM; 1 μmol/l; **a**), selective inhibition of conventional PKC isozymes (Gö6976; 1 μmol/l; **b**) or PKCα deficiency (**c**) decreased the Ppa response towards ET-1 compared to the respective control group in isolated perfused mouse lungs. Data represent the percentage of the pressure response seen in the respective control group at highest concentration. Values are shown as mean + SEM. n = 4–7 per group. * $p \leq 0.05$ versus solvent or WT group.

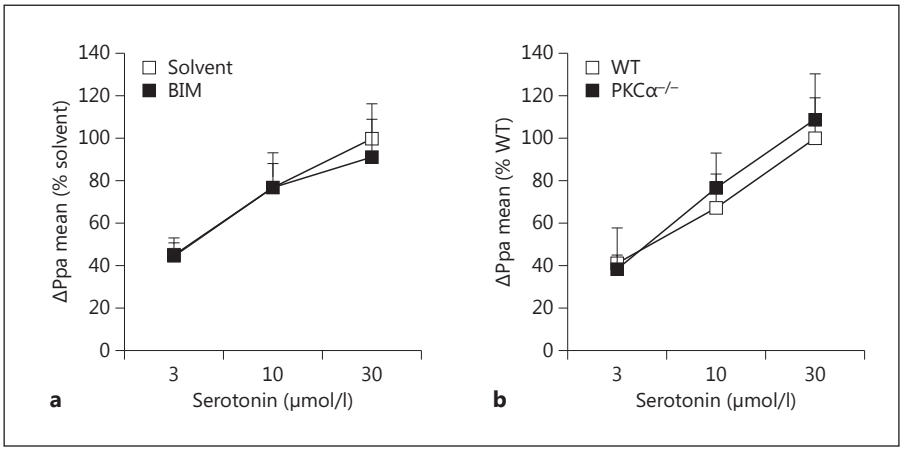

Fig. 3. Serotonin-evoked pulmonary vasoconstriction does not involve PKCα. **a** Following PKC inhibitor application (BIM; 1 μmol/l), pulmonary vascular responsiveness to serotonin was comparable to the vasopressor response observed in the solvent-treated control group. **b** Similarly, PKCα deficiency had no effect on serotonin-induced pulmonary vasoconstriction. Data represent the percentage of the pressure response observed in the respective control group at highest concentration. Values are shown as mean+ SEM. n = 7–8 per group.

minished Ppa mean over the course of all 4 flow rates under hypoxia, whereas the flow-induced increase in Ppa mean was unaltered by PKC inhibition under normoxic conditions (fig. 4b). PKCα deficiency, however, did not affect HPV (fig. 4c) or P-Q curves (fig. 4d) when compared to WT mice.

Thus, the initially observed pulmonary hyperresponsiveness to U46619 in PKCα$^{-/-}$ mice was most likely thromboxane-specific. To elucidate the underlying mechanism of the U46619-evoked hyperresponsiveness, we performed quantitative mRNA analyses of the TP receptor and PKC isozymes.

mRNA Expression of TP Receptor and Atypical PKCι Is Upregulated in Microdissected Intrapulmonary Arteries from PKCα$^{-/-}$ Mice

mRNA expression levels of the TP receptor and PKC isozymes were measured in laser-capture microdissected intrapulmonary arteries and whole-lung tissue homogenates from naïve PKCα$^{-/-}$ and WT mice. The rank order of PKC isozyme expression in the WT intrapulmonary arteries was as follows: PKCδ > PKCε > PKCζ > PKCι > PKCα > PKCη > PKCβ > PKCθ > PKCγ (online suppl. fig. 2). Notably, TP receptor mRNA expression in microdissected intrapulmonary arteries from PKCα$^{-/-}$ mice

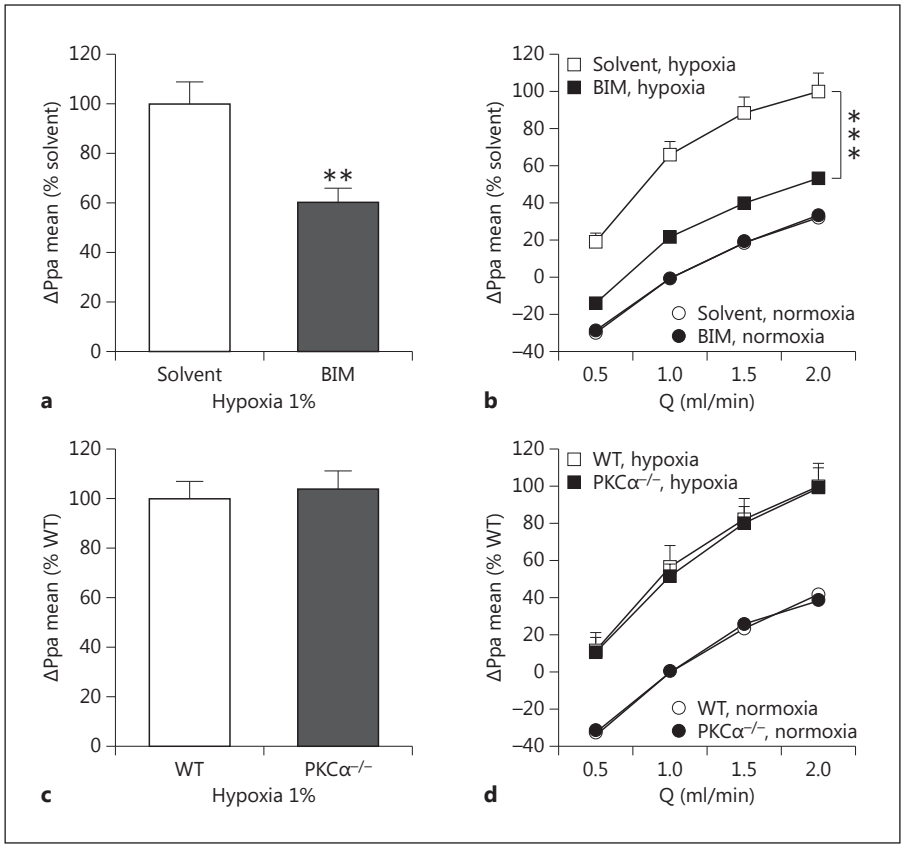

Fig. 4. HPV requires PKC, but not specifically PKCα. PKC inhibition (BIM; 1 μmol/l) reduced HPV (1% O_2) (**a**) and the flow (Q)-induced increase in Ppa under hypoxia (**b**) but not under normoxia. PKCα deficiency did not alter HPV (**c**) or pressure-flow curves (**d**). Data represent the percentage of the pressure response to hypoxic ventilation or the highest flow rate (Q) seen in the respective control group. Values are shown as mean + SEM. **a**, **b** n = 5 per group; **c**, **d** n = 8–9 per group. ** $p \leq 0.01$; *** $p \leq 0.001$ versus solvent group.

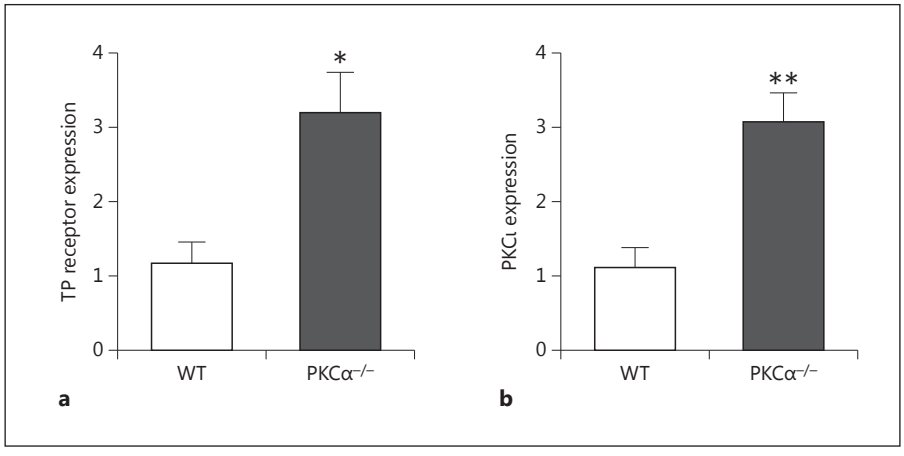

Fig. 5. PKCα deficiency leads to increased TP receptor and PKCι mRNA expression in intrapulmonary arteries. **a**, **b** mRNA expression analysis from microdissected intrapulmonary arteries showed a 2.7-fold increase in the expression of TP receptor and PKCι mRNA in PKCα$^{-/-}$ compared to in WT pulmonary arteries. Data represent the relative quantitative gene expression: mRNA expression levels were normalized to GAPDH levels and compared to expression levels in WT mice using the comparative Ct method. Values are shown as mean + SEM. n = 5–7 per group. * $p \leq 0.05$; ** $p \leq 0.01$ versus WT group.

was 2.7-fold greater compared to WT mice (fig. 5a). Similarly, PKCι mRNA levels were highly elevated in the intrapulmonary arteries from PKCα$^{-/-}$ mice (2.7-fold; fig. 5b).

In contrast, mRNA expression levels of conventional isozymes PKCβ and PKCγ, novel isozymes PKCδ, PKCε, PKCη, PKCθ and the atypical isozyme PKCζ were similar in the intrapulmonary arteries of PKCα$^{-/-}$ and WT mice (online suppl. table 1).

The elevated expression of TP receptor and PKCι in PKCα-deficient lungs seems to be specific to the pulmonary arteries, since no differential expression of TP receptor and PKC isozyme in whole-lung tissue homogenates was detected between PKCα$^{-/-}$ and WT mice (data not shown).

Elevated PKCι Expression in PKCα$^{-/-}$ Pulmonary Arteries Does Not Contribute to Vascular Hyperresponsiveness to U46619 in PKCα$^{-/-}$ Lungs

As revealed by mRNA analysis, increased TP receptor expression possibly leads to pulmonary vascular hyperresponsiveness to U46619 in PKCα$^{-/-}$ mice. However, since PKCι has been shown to mediate contraction of the rat corpus cavernosum [42], a contribution by PKCι to the vascular hyperresponsiveness that we observed also seems possible. To elucidate the functional role of upregulated PKCι in the vasculature of PKCα$^{-/-}$ lungs, the effect of PKCι inhibitor ATM on U46619-evoked pulmonary vasoconstriction was assessed. Basal Ppa mean was unaffected by PKCι inhibition (mean ± SEM: 9.64 ± 0.23 cm H_2O) compared to the solvent treatment (9.36 ± 0.33 cm H_2O). Importantly, PKCι inhibition did not reduce pulmonary vascular hyperresponsiveness to U46619 in PKCα$^{-/-}$ mice (fig. 6), allowing us to conclude that besides elevated TP receptor expression in intrapulmonary arteries, increased PKCι expression is most likely not causative for this increased vasopressor response.

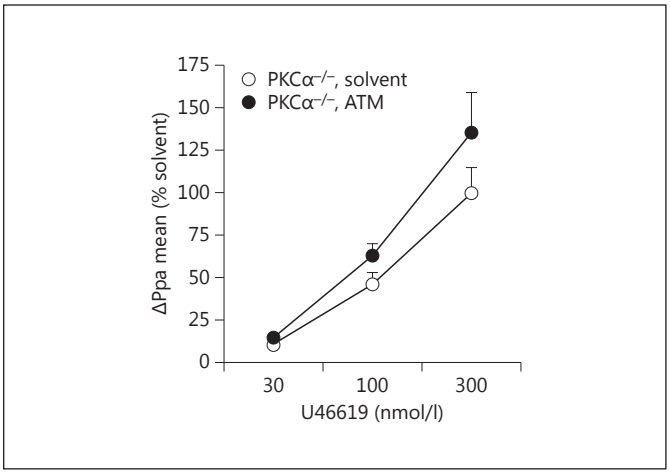

Fig. 6. PKCι inhibition does not reduce vascular hyperresponsiveness to U46619 in PKCα$^{-/-}$ lungs. Following application of PKCι inhibitor ATM (20 μmol/l), pulmonary vasoconstriction induced by TP receptor agonist U46619 was unaltered (p = 0.17) compared to solvent treatment. Data represent the percentage of the pressure response seen in the respective control group at highest concentration. Values are shown as mean + SEM; n = 7 per group.

Discussion

This study demonstrates PKC isozyme-specific functions in the pulmonary vasculature. PKCα contributed to pulmonary vasoconstriction induced by ET-1. Conversely, however, PKCα deficiency led to pulmonary vascular hyperresponsiveness to TXA2, possibly via the upregulation of the TP receptor in the intrapulmonary arteries. Furthermore, HPV, although diminished following PKC inhibition, did not depend on the isozyme, PKCα, whereas serotonin-induced pulmonary vasoconstriction was unaffected by both PKC inhibition or PKCα deficiency.

ET-1-evoked pulmonary vasoconstriction was reduced by approximately 40% in PKCα-deficient mice compared to WT mice, suggesting a prominent role for the PKC isozyme, PKCα, in the pulmonary vasopressor response to ET-1. These findings are consistent with a study by Feng et al. [41] demonstrating a significant role for PKCα signaling in ET-1-induced coronary microvascular vasoconstriction. Moreover, ET-1-induced contraction of isolated mesenteric arteries was reduced following treatment with a PKCα inhibitor peptide [43]. Interestingly, in a study by Weigand et al. [44], ET-1-induced contraction in pulmonary arteries from chronically hypoxic rats was shown to be unaffected by PKC inhibition, possibly pointing to altered mechanisms of ET-1-induced contraction under hypoxic conditions, which require further investigations. In contrast to the vasopressor response to ET-1, isozyme PKCα did not contribute to HPV, which has previously been shown to depend on PKC [18–20] and particularly isozyme PKCε [17], again indicating isozyme-specific functions in the pulmonary vasculature.

Surprisingly, PKCα deficiency led to pulmonary vascular hyperresponsiveness to the TXA2 analog U46619. As PKCα is proposed to be a promoter of smooth muscle cell contraction [40, 41, 43], this finding was unexpected. TXA2 is a prostanoid derived from arachidonic acid, which exerts its constrictive effects by binding TP receptors on vascular smooth muscle cells, leading to the inhibition of voltage-gated K^+ channels, membrane depolarization, the activation of L-type Ca^{2+} channels, and ultimately smooth muscle cell contraction [16]. The thromboxane system has been implicated in the pathogenesis of PAH [2, 45]. In this study, we observed that the pulmonary vascular hyperresponsiveness to U46619 in isolated PKCα$^{-/-}$ lungs coincided with pulmonary arterial TP receptor upregulation. Although it is tempting to speculate that compensatory TP receptor upregulation is a result of constitutive PKCα deficiency in PKCα$^{-/-}$ mice, the exact underlying mechanisms leading to this upregulation in PKCα deficiency remain elusive. Also, the pathophysiological impact of this observation is unclear. However, since neither broad-spectrum PKC inhibition nor selective cPKC inhibition led to an increased pulmonary vaso-

pressor response to U46619, the hyperresponsiveness in PKCα$^{-/-}$ lungs that we observed seems to rather be a result of TP receptor upregulation in the intrapulmonary arteries than a direct effect of the absence of PKCα.

To our knowledge, there are no published data on PKCα expression in PAH patients. A study by Shi et al. [46] demonstrated decreased PKCα expression in the pulmonary arteries of rats with hypoxia-induced pulmonary hypertension. Moreover, increased TP receptor density has been observed in the right ventricular tissue of PAH patients [47].

Besides TP receptor expression, PKCι expression was shown to be elevated in the intrapulmonary arteries of PKCα$^{-/-}$ mice. However, the role of PKCι in pulmonary vasoconstriction is currently unknown. A large body of literature has discussed PKCι as a tumor oncogene [48], but very little is known about its role in adult pulmonary vasculature. PKCι activation during embryonic vasculogenesis [49] and a potential role for PKCα and PKCι in phenylephrine-induced contraction of the rat corpus cavernosum have been proposed [42]. However, compensative modulation of pulmonary vascular function by PKCι in PKCα$^{-/-}$ mice is unlikely, since pulmonary vascular hyperresponsiveness to U46619 was not reduced in PKCα$^{-/-}$ lungs following PKCι inhibition, as shown in this study. In line with this notion, it has been reported that treatment with the PKCι inhibitor sodium aurothiomalate failed to inhibit adrenaline-induced constriction of rat anterior mesenteric artery and vein [50] as well as epidermal growth factor-urogastrone-evoked contraction in isolated longitudinal smooth muscle strips from guinea-pig stomach [51]. Nevertheless, to our knowledge, this is the first report on the effects of selective PKCι inhibition on pulmonary vasoconstriction focusing on the specific response to U46619 in PKCα$^{-/-}$ lungs, so a potential PKCι-mediated modulation of pulmonary vascular function needs to be further evaluated in future studies.

Remarkably, serotonin-induced pulmonary vasoconstriction was not altered by the PKC inhibitor BIM (1 μmol/l) or PKCα deficiency in isolated perfused naïve mouse lungs. This is consistent to some extent with the report by Kaye et al. [27], showing that the feline pulmonary vasoconstrictor response to serotonin was unaffected by pretreatment with the PKC inhibitor staurosporine or calphostin C. Nevertheless, in the same study, staurosporine was observed to diminish the pulmonary arterial pressor response to serotonin in isolated perfused rat lungs [27]. BIM (10 μmol/l) also reduced the hyperresponsive vasoconstriction towards serotonin in isolated perfused lungs of ovalbumin-sensitized and ovalbumin-challenged mice [9]. In addition, the PKC inhibitors Gö6976 and staurosporine have been shown to reduce serotonin-evoked reactivity of rat PASMCs [28] or the pulmonary vasculature in isolated canine lungs [29], respectively. However, staurosporine is the precursor of BIM and, with a relatively high affinity to other protein kinases, it is less PKC-specific than BIM [52]. Gö6976 is a selective inhibitor of conventional PKCα and PKCβ.

Our data suggest that serotonin-evoked pulmonary vasoconstriction is not mediated via PKC, and particularly not via PKCα in isolated naïve mouse lungs. Several explanations for the seemingly contradictory findings are possible, including species specificity, differences in the complexity of models or the varying isozyme selectivity profiles of different PKC inhibitors, particularly when applied at different concentrations. Since the BIM-evoked half maximal inhibition (IC_{50}) for relevant conventional and novel PKC isozymes ranges roughly from 8 to 210 nmol/l (according to the manufacturer), the currently employed concentration of 1 μmol/l BIM indicates an adequate cPKC/nPKC inhibition under physiological conditions, and is the preferred working concentration for vasoconstriction analysis in naïve isolated perfused lungs [53, 54]. On the other hand, IC_{50} for atypical PKCζ requires 5.8 μmol/l of BIM. Thus, the somewhat weak reduction of U46619-induced vasoconstriction that we observed can possibly be explained by only a partial inhibition of PKCζ, as PKCζ has been shown to promote U46619-evoked pulmonary vasoconstriction [16]. Moreover, as we could not entirely rule out PKCι-mediated pulmonary vasoconstriction, the weak or failed reduction of the vasopressor responses to U46619 and serotonin, respectively, by BIM, may also be in part explained by the fact that PKCι was probably not affected or was incompletely affected by 1 μmol/l of BIM.

In summary, we identified the PKC family as important agonist-specific modulators of pulmonary vascular function. HPV was affected by broad-spectrum PKC inhibition, but not by a deficiency of the PKCα isozyme. In contrast, the ET-1-evoked pulmonary vasoconstriction, which is assumed to be an important feature of PAH pathophysiology, was promoted by PKCα. However, PKCα deficiency led to pulmonary vascular hyperresponsiveness towards TXA2, which coincided with increased TP receptor expression. This finding may be of clinical relevance and should be kept in mind when discussing PKC isozymes and, in particular, PKCα, as potential targets for PAH therapy. Further studies are warranted to analyze whether the pharmacological, long-term inhibition of PKCα counteracts its possible beneficial effects on the pulmonary vasculature by TP receptor upregulation.

Acknowledgements

This project was supported in part by the German Research Foundation (SFB-TR84 C2 and C5 to S.H., B1 and Z2 to N.S., C3 and C6 to M.W.). The authors would like to thank Monika Schaerig and Denise Barthel for excellent technical assistance, Wolfgang M. Kuebler for thoughtful discussions, Michael Leitges for providing PKCα$^{-/-}$ mice and Jasmin Lienau for proof-reading of the manuscript.

Disclosure Statement

None of the authors have competing interests.

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