Thromboxane A_2-Induced Inhibition of Voltage-Gated K^+ Channels and Pulmonary Vasoconstriction

Role of Protein Kinase Cζ

Angel Cogolludo, Laura Moreno, Lisardo Bosca, Juan Tamargo, Francisco Perez-Vizcaino

Abstract—Voltage-gated K^+ channels (K_V) and thromboxane A_2 (TXA_2) play critical roles in controlling pulmonary arterial tone under physiological and pathological conditions. We hypothesized that TXA_2 might inhibit K_V channels, thereby establishing a link between these two major pathogenic pathways in pulmonary hypertension. The TXA_2 analogue U46619 inhibited $I_{K(V)}$ ($E_{max}=56.1\pm3.9\%$, $EC_{50}=0.054\pm0.019$ μmol/L) and depolarized pulmonary artery smooth muscle cells via activation of TP receptors. In isolated pulmonary arteries, U46619 simultaneously increased intracellular Ca^{2+} concentration and contractile force, and these effects were inhibited by nifedipine or KCl (60 mmol/L). U46619-induced contractions were not altered by the inhibitors of tyrosine kinase genistein or Rho kinase Y-27632 but were prevented by the nonselective protein kinase C (PKC) inhibitors staurosporine and calphostin C. Furthermore, these responses were sensitive to Gö-6983 but insensitive to bisindolylmaleimide I and Gö-6976. Based on the specificity of these drugs, we suggested a role for an atypical PKC in U46619-induced effects. Thus, treatment with a PKCζ pseudosubstrate inhibitor markedly prevented the vasoconstriction, the inhibition of $I_{K(V)}$, and the depolarization induced by U46619. Western blots showed a transient translocation of PKCζ from the cytosolic to the particulate fraction on stimulation with U46619. These results indicate that TXA_2 inhibits $I_{K(V)}$, leading to depolarization, activation of L-type Ca^{2+} channels, and vasoconstriction of rat pulmonary arteries. We propose PKCζ as a link between TP receptor activation and K_V channel inhibition. **(*Circ Res*. 2003;93:656-663.)**

Key Words: K^+ channels ■ pulmonary artery ■ protein kinase C ■ thromboxane A_2

Thromboxane A_2 (TXA_2) is a prostanoid synthesized by cyclooxygenase with potent vasoconstrictor, mitogenic, and platelet aggregant properties.[1] 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. In fact, TXA_2 has been involved in several forms of human and experimental pulmonary hypertension, including primary[2] and secondary pulmonary hypertension induced by sepsis, endotoxemia, heparin/protamine, leukotriene D_4, microembolism, and ischemia-reperfusion.[3–8]

TXA_2 contracts vascular smooth muscle by binding to specific $G_{q/11}$ protein–coupled receptors (TP receptors), which leads to an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and sensitization of the contractile proteins to Ca^{2+}.[9–12] Activation of TP receptors is also involved in the vasoconstrictor effects of several isoprostanes, a novel class of arachidonic acid metabolites generated by oxygen free radical–mediated peroxidation of membrane phospholipids, used as markers for many disease states, including pulmonary hypertension.[13] The signaling pathways mediating TP receptor–induced contraction remain controversial, because a variety of protein kinases, such as protein kinase C (PKC), Rho kinase, and tyrosine kinases, have been shown to be involved.[9,10,12]

K^+ channels play an essential role in regulating resting membrane potential, $[Ca^{2+}]_i$, and contraction of vascular smooth muscle.[14–18] Activation of K^+ channels leads to hyperpolarization, whereas their inhibition causes membrane depolarization, activation of voltage-gated L-type Ca^{2+} channels, increase in $[Ca^{2+}]_i$, and vasoconstriction. Different types of K^+ channels have been identified in pulmonary artery smooth muscle cells (PASMCs), including voltage-gated K^+ channels (K_V), large-conductance Ca^{2+}–activated channels (BK_{Ca}), and ATP-dependent channels (K_{ATP}).[14,17–19] There is increasing interest in K_V channels in the pulmonary circulation because of several different facts. First, they make a substantial contribution to whole-cell K^+ conductance and resting membrane potential in PASMCs.[14,15,18] Second, they are modulated by hypoxia and vasoactive factors such as nitric oxide, endothelin-1, and angiotensin II.[14,19–21] Finally, decreased expression or function of K_V channels in PASMCs has been involved in the pathogenesis of primary and anorexigen-induced pulmonary hypertension.[14,22–24]

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Very little is known about the effects of TXA_2 on vascular K^+ channels. It has been reported that TXA_2 analogues inhibit the activity of BK_{Ca} channels in bronchial and coronary arteries,[25,26] whereas their effects on vascular K_V channels are unknown. We hypothesized that TXA_2 might inhibit K_V channels, thereby establishing a link between these two pathogenic pathways in pulmonary hypertension. Therefore, in the present study we have analyzed the effects of the TXA_2 analogue U46619 on the current flowing through K_V channels ($I_{K(V)}$) recorded in rat PASMCs using the whole-cell configuration of the patch-clamp technique. The role of K_V channels in TXA_2-induced pulmonary vasoconstriction has also been studied in isolated pulmonary arteries (PAs). Our results indicate that U46619 inhibits K_V channels, leading to depolarization of PASMCs, an increase in $[Ca^{2+}]_i$, and vasoconstriction in PA. Furthermore, we provide evidence for the role of PKCζ as the link between TXA_2 receptor activation and inhibition of K_V channels.

Materials and Methods

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

Reagents

Drugs were obtained from Sigma, except nifedipine (Bayer España), Y-27632 (Tocris Cookson), fura-2 AM, calphostin C, Gö-6976, Gö-6983, PKCζ pseudosubstrate inhibitor, and secondary horseradish peroxidase–conjugated antibodies (Calbiochem). Polyclonal rabbit antibodies were from Santa Cruz Biotechnology.

Tissue Preparation and Cell Isolation

Second- to third-order branches of the PA (internal diameter, 0.5 to 1 mm) isolated from male Wistar rats (250 to 300 g; ANUC, Universidad Complutense, Madrid, Spain) 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 (2×2 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.

Contractile Tension Recording

Contractile responses in endothelium-denuded PA rings were recorded as previously reported.[9,27] Arteries were stimulated with U46619 (0.1 μmol/L) and, once a stable contraction was reached, were washed with Krebs solution for 30 minutes. A second stimulation with U46619 was elicited in the absence of treatment (controls) or after 30 minutes of treatment with different drugs. The values of the second contraction were expressed as a percentage of the initial response to the agonist and normalized to the values obtained in control experiments.

Simultaneous $[Ca^{2+}]_i$ and Tension Recording

PA rings were incubated for 2 to 3 hours in Krebs solution containing fura-2 acetoxymethylester (5 μmol/L) and Cremophor EL (0.05%) and then mounted in a bath that allows the estimation of changes in the fluorescence intensity of fura-2 and force development simultaneously.[9,28] The absolute values of $[Ca^{2+}]_i$ were estimated using the Grynkiewicz equation.[9,29]

Electrophysiological Studies

Membrane currents were measured using the whole-cell configuration of the patch-clamp technique,[30] normalized for cell capacitance and expressed in pA pF^{-1}. Membrane potential (E_m) was measured under current-clamp configuration.

K_V currents ($I_{K(V)}$) were recorded under essentially Ca^{2+}-free conditions using an external Ca^{2+}-free PSS (see above) and a Ca^{2+}-free pipette (internal) solution containing (in mmol/L) KCl 110, $MgCl_2$ 1.2, Na_2ATP 5, HEPES 10, and EGTA 10, pH adjusted to 7.3 with KOH. For recording L-type Ca^{2+} currents, KCl was replaced by CsCl in both the external and the pipette solutions and 10 mmol/L $BaCl_2$ was included in the external solution as charge carrier. All experiments were performed at room temperature (22 to 24°C).

Western Blot Analysis, Phosphorylation of T410, and Cell Fractionation

After dissection, PAs were placed in warm, oxygenated Krebs solution for 60 minutes and then in the absence or presence of U46619 (1 μmol/L) for 30 or 180 seconds. PAs were frozen in liquid nitrogen, homogenized in a glass potter in 200 μL of a buffer of the following composition: 10 mmol/L HEPES (pH 8), 10 mmol/L KCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L dithiothreitol, 40 μg/mL aprotinin, 4 μg/mL leupeptin, 4 μg/mL Nα-p-tosyl-L-lysine chloromethyl ketone, 5 mmol/L NaF, 10 mmol/L Na_2MoO_4, 1 mmol/L $NaVO_4$, 0.5 mmol/L phenylmethanesulfonyl fluoride, and 10 nmol/L okadaic acid. The homogenate was centrifuged at 100 000*g* for 30 minutes. The supernatant was collected (cytosolic fraction), and the pellet was resuspended in 200 μL of the same buffer containing nonidet P-40 1% and gently shaken for 30 minutes at 4°C and again centrifuged at 100 000*g* for 30 minutes. The pellet was discarded, and the supernatant was collected (particulate-enriched fraction). The enrichment of the subcellular fractions was evaluated by measuring the levels of cytosolic and membrane markers. Western blotting was performed with 10 μg of protein from the supernatant per lane. SDS-PAGE (7.5% acrylamide) electrophoresis was performed using the method of Laemmli in a mini-gel system (Bio-Rad). The proteins were transferred to PVDF membranes overnight at 4°C and incubated with rabbit anti-PKCζ, anti-PKCι, or anti-P-T410-PKCζ primary antibodies and secondary anti-rabbit horseradish peroxidase–conjugated antibodies. The bands were visualized by chemoluminescence (ECL, Amersham).

Statistical Analysis

Data are expressed as mean±SEM; n indicates the number of arteries or cells tested from different animals. Statistical analysis was performed using Student's *t* test for paired observations or one-way ANOVA followed by a Newman-Keuls test. Differences were considered statistically significant when $P<0.05$.

Results

Effects of the TXA_2 Analogue U46619 on $I_{K(V)}$ and Membrane Potential

The average cell capacitance of the freshly isolated rat PASMC was 17.4±0.6 pF (n=42). $I_{K(V)}$ was recorded under essentially Ca^{2+}-free conditions, and EGTA and ATP were included in the pipette solution to minimize the component of ATP-dependent ($I_{K(ATP)}$) and Ca^{2+}-activated ($I_{BK(Ca)}$) K^+ currents. Under these conditions, when cells were voltage-clamped at −60 mV, the application of 200-ms depolarizing pulses to test potentials from −60 to +50 mV in 10-mV increments induced a K^+ current (Figure 1A), which activated at potentials positive to −30 mV and was usually reproducible for at least 1 hour. This current was essentially abolished by the K_V channel inhibitor 4-aminopyridine (1 mmol/L, Figures 1F and 1G), indicating that it was evoked by the activation of K_V channels. U46619 inhibited $I_{K(V)}$ in a concentration-dependent manner (Figures 1A and 1B). The onset of the response to U46619 was fast (20 to 30 seconds) and reached a stable response within 2 to 3 minutes. The

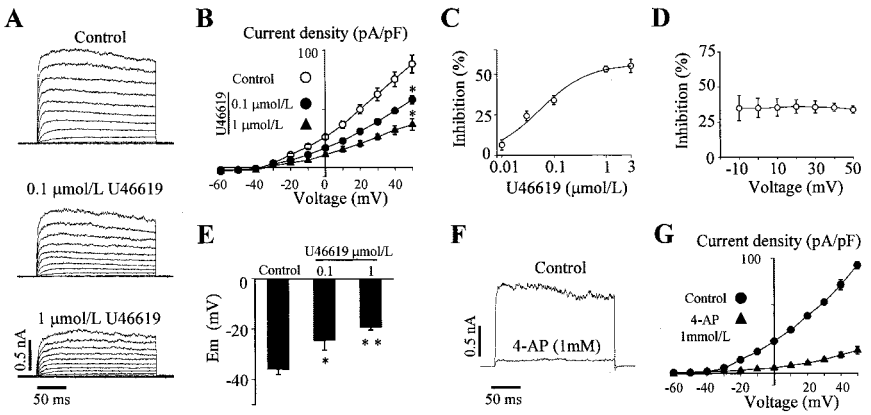

Figure 1. U46619 decreases $I_{K(V)}$ recorded in rat PASMCs. A, Current traces are shown for 200-ms depolarization pulses from −60 mV to +50 mV (in 10-mV increments) from a holding potential of −60 mV. U46619 (0.1 and 1 μmol/L) inhibited $I_{K(V)}$ in a concentration-dependent manner. B, Current-voltage relationships of $I_{K(V)}$ measured at the end of the pulse to +50 mV in the absence and the presence of U46619 (0.1 and 1 μmol/L). C, Concentration-dependent inhibition of $I_{K(V)}$ by U46619. D, Voltage dependency of the effects of U46619 (0.1 μmol/L) on $I_{K(V)}$ measured at 200 ms. The inhibitory effect resulted to be voltage independent. E, Effects of U46619 on membrane potential. The drug depolarized the membrane in a concentration-dependent manner. F, Current traces when applying depolarization pulses to +50 mV from a holding potential of −60 mV in the absence (control) and presence of 4-aminopyridine (4-AP, 1 mmol/L). G, Current-voltage relationships of $I_{K(V)}$ in the absence and the presence of 4-AP. Data show mean±SEM (n=4 to 7). *P<0.05 and **P<0.01 vs control, respectively.

concentration-response curve for the inhibition of $I_{K(V)}$ by U46619 at test potentials of +50 mV was fitted to a Hill equation, leading to E_{max} and EC_{50} values of 56.1±3.9% and 0.054±0.019 μmol/L, respectively (Figure 1C). The magnitude of this inhibition was similar at the potentials tested, indicating a voltage-independent blockade (Figure 1D). In addition, U46619 (0.1 and 1 μmol/L) significantly depolarized PASMCs in a concentration-dependent manner (Figure 1E).

In the presence of the TXA_2 receptor antagonist SQ-29548 (3 μmol/L), U46619 (0.1 μmol/L) did not modify the K_V currents or the membrane potential in isolated PASMCs (Figures 2A and 2B). These results indicated that the electrophysiological effects induced by U46619 were mediated through the activation of TP receptors.

Effects of U46619 on Contraction and $[Ca^{2+}]_i$: Role of L-Type Ca^{2+} Channels

Stimulation of endothelium-denuded PA rings with U46619 (0.1 μmol/L, which produced ≈50% of the maximal response) induced a sustained contractile response of 185±23 mg (n=10), which was suitably reproduced after a 30-minute washout (109±4% of the first contraction, P>0.05). Pretreatment with 3 μmol/L SQ-29548 before the second addition of U46619 completely abolished the vasoconstriction (Figure 2C).

Depolarization resulting from U46619-induced inhibition of K_V channels might increase vascular tone by promoting Ca^{2+} entry through voltage-gated L-type Ca^{2+} channels. This possibility was studied by measuring simultaneous changes in force and $[Ca^{2+}]_i$ in fura-2-loaded PA. Absolute values for $[Ca^{2+}]_i$ under basal conditions and after stimulation with U46619 (0.1 μmol/L) were 299±96 and 490±55 nmol/L, respectively, and the sustained contractile response averaged 163±34 mg (n=5).

Both the increase in $[Ca^{2+}]_i$ and the increase in force induced by U46619 (0.1 μmol/L) were reproducible (Figure 3A) and markedly inhibited in the presence of the L-type Ca^{2+} channel–blocker nifedipine (0.1 μmol/L, Figure 3B). Similarly, pretreatment with KCl (60 mmol/L) elicited a contraction of 145±21% of the response to U46619 and markedly inhibited the contraction and abolished the increase in $[Ca^{2+}]_i$ induced by U46619 (Figure 3C). These results suggested an important role of L-type Ca^{2+} channels in the U46619-induced increase in $[Ca^{2+}]_i$ and contraction. Figure 3E shows the concentration-response curves for the increase in $[Ca^{2+}]_i$ and force induced by U46619 (0.01 to 3 μmol/L), which yielded EC_{50} values of 0.13±0.01 and 0.14±0.01 μmol/L, respectively (n=6).

To test the possibility that U46619 could be modulating L-type Ca^{2+} channels not only via membrane depolarization but also directly, its effects were analyzed on L-type Ca^{2+} currents recorded in isolated PASMCs. Figure 4A shows traces of Ca^{2+} currents elicited when stepping from −60 to +10 mV. The current activated at ≈−27 mV reached a maximum at +10 mV (Figure 4B) and was not significantly affected by U46619 (0.1 μmol/L) but was completely abolished by nifedipine (0.1 μmol/L).

Role of Protein Kinases on U46619-Induced Effects

To additionally assess the mechanisms involved in U46619-induced contraction, PAs were incubated with different protein kinase inhibitors before the second addition of the agonist. As shown in Figure 5A, neither the tyrosine kinase inhibitor genistein (10 μmol/L) nor the Rho kinase inhibitor Y-27632 (1 μmol/L) modified U46619-induced contraction, whereas the PKC inhibitors staurosporine (0.01 μmol/L) and calphostin C (1 μmol/L) markedly attenuated the response to the TXA_2 analogue. Because staurosporine is a nonselective inhibitor of PKC and may also directly affect contractile proteins (eg, it inhibits myosin light chain kinase[31]), we analyzed its effects on 4-aminopyridine-induced contractions. At 10 mmol/L, 4-aminopyridine elicited a contractile response of 159±14 mg (n=8). Staurosporine (0.01 μmol/L) did not affect the contraction induced by 4-aminopyridine (162±15 mg; n=9; P>0.05), suggesting that at this concentration the drug had no direct effect on contractile proteins.

In PASMCs, Y-27632 (1 μmol/L) did not significantly modify the membrane potential or $I_{K(V)}$ and did not alter the depolarizing (not shown) or K_V channel inhibitory effects

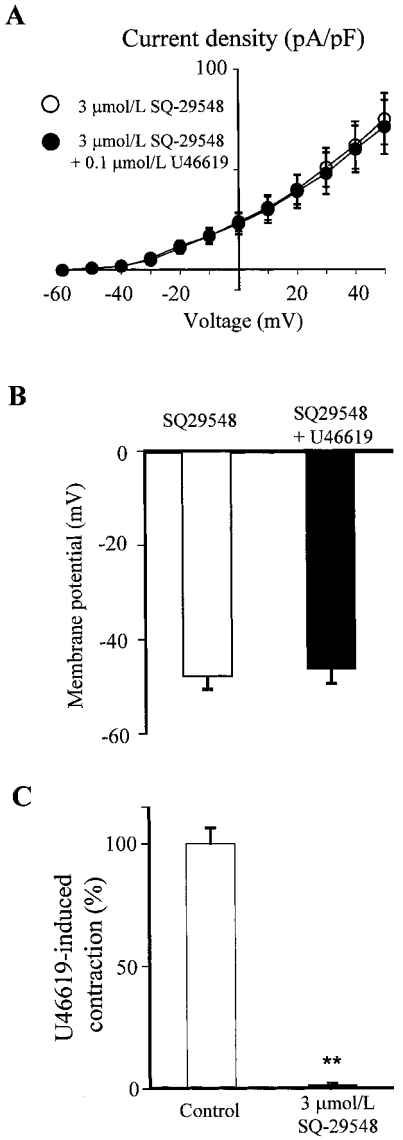

Figure 2. TP receptor activation mediates U46619-induced effects. Pretreatment with the TP receptor antagonist SQ-29548 (3 μmol/L) suppressed the effects of U46619 (0.1 μmol/L) on $I_{K(V)}$ (A) and membrane potential (B) in PASMCs and on contractile tension in PA (C). Data show mean±SEM (n=4 to 6). $^{**}P<0.01$ vs control.

induced by 0.1 μmol/L U46619 (Figure 5B). Perfusion with staurosporine (0.1 μmol/L) did not alter $I_{K(V)}$ but prevented the U46619-induced inhibition of this current (Figure 5C).

Role of PKCζ in U46619-Induced Effects

In view of the preceding data, we performed pharmacological studies with isotype-selective PKC inhibitors to evaluate the involvement of specific PKC isoforms in U46619-induced contractions. The inhibitors used were bisindolylmaleimide I, which at the concentration tested shows selectivity for the conventional PKC (cPKC) isoforms (α, βI, βII, and γ) and for some novel PKC (nPKC) isoforms (δ and ε); Gö-6976, which inhibits cPKC isoforms and PKCμ; and Gö-6983, which preferentially inhibits cPKC, some nPKC, and the atypical (aPKC) PKCζ but not PKCμ.[31,32] No changes were observed in U46619-induced contraction when PAs were pretreated with 1 μmol/L bisindolylmaleimide I or 0.01 μmol/L Gö-6976 (Figure 6A). However, exposure to Gö-6983 (0.01 μmol/L) markedly inhibited U46619-induced contraction. Because the only isoform known to be sensitive to Gö-6983 and insensitive to the other two drugs is PKCζ, we hypothesized that this aPKC might be involved in the responses induced by U46619. Therefore, we examined the effects of a PKCζ pseudosubstrate inhibitor (PKCζ-PI, 10 μmol/L). Figure 6A shows that PKCζ-PI produced an inhibition of U46619-induced pulmonary vasoconstriction similar to that induced by Gö-6983.

In PASMCs, U46619 did not alter $I_{K(V)}$ in the presence of 0.1 μmol/L Gö-6983 (which very slightly reduced $I_{K(V)}$ by 10.7±6.8%, $P>0.05$, Figure 6B). Similarly, in cells dialyzed with an internal solution containing 0.1 μmol/L PKCζ-PI, the addition of U46619 had no effect on $I_{K(V)}$ (Figure 6C) or membrane potential (Em=−47.2±1.5 and −47.9±1.3 mV before and after adding U46619, respectively). Altogether, these results indicated a key role of PKCζ in TXA_2-induced inhibition of $I_{K(V)}$, depolarization, and contraction of PA.

Subcellular Distribution of aPKC

Numerous reports in the literature using antibodies raised against the C-terminal domain of PKCζ found two bands of ≈75 to 80 kDa in fibroblasts, rabbit and ferret aorta, rat cardiac myocytes, PC12 cells, murine epidermis, basophilic RBL-2H3 cells, Jurkat T lymphoma cells, rat embryo fibroblasts, NIH 3T3 cells, the J774 macrophage cell line, the αT3-1 gonadotroph-derived cell line, rat brain, and bovine kidney cells (Reference 33 and references therein). The upper band is Ca^{2+}-dependent, can be downregulated by phorbol esters, and is actually considered a cPKC.[33] Western blots of homogenates from rat PA using polyclonal rabbit antibody directed toward the C-terminal peptide of PKCζ also recognized two bands of ≈81 and 75 kDa (Figure 6D). This antibody cross-reacts with the aPKCλ/ι. However, the expression of this aPKC was negligible using a specific anti-PKCλ/ι antibody toward the amino acids 168 to 243 of PKCι of human origin (equivalent to rodent PKCλ). Furthermore, an antibody directed toward the phosphorylated activation loop (T410) of PKCζ clearly identified the lower band even when the heavier one was also present in some blots. This indicates that an aPKC, most probably PKCζ, is strongly expressed and is at least partly phosphorylated at T410 in resting rat PA. Because the activity of PKC is mostly controlled by its intracellular compartmentalization, we analyzed its subcellular distribution and its possible translocation on stimulation with U46619. The 81-kDa band was located mostly in the particulate fraction (82±5%), and its cytosolic/particulate distribution did not change in PA stimulated with U46619. However, the 75-kDa band was less abundant in the particulate fraction (38±7%) and was rapidly but transiently translocated to the membrane fraction on U46619 stimulation (Figure 6D).

Discussion

In the present study, we have examined the effects of the TXA_2 analogue U46619 on K_V channels in freshly isolated

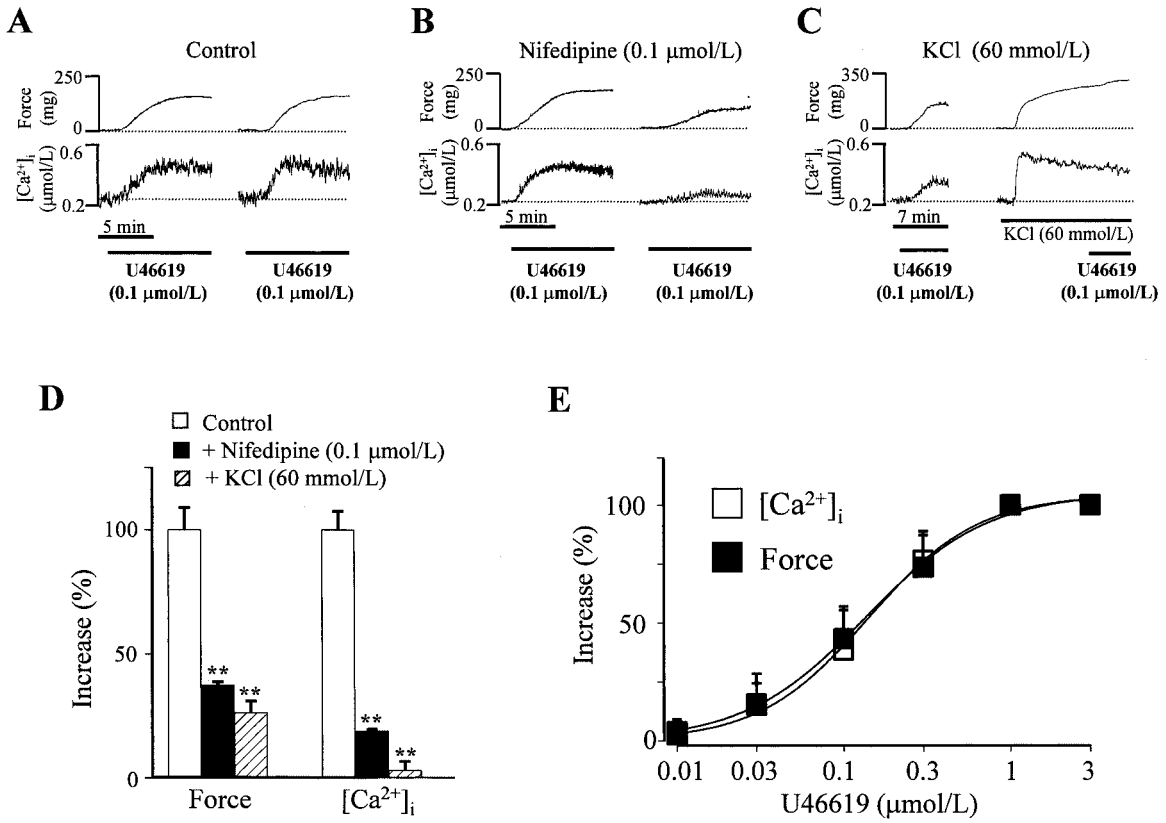

Figure 3. U46619 increases force and $[Ca^{2+}]_i$ via L-type Ca^{2+} channels. Recordings of the simultaneous changes on force (top) and $[Ca^{2+}]_i$ (bottom) elicited by U46619 (0.1 μmol/L). The second stimulation with U46619 was elicited in the absence (control, A) or after exposure to nifedipine (0.1 μmol/L, B) or KCl (60 mmol/L, C). D, Mean±SEM of 5 to 6 experiments, as those presented in panels A through C. Values were normalized to those obtained in control conditions (**$P<0.01$ vs control). E, Concentration response curves for the increase in $[Ca^{2+}]_i$ and contractile force induced by U46619 (0.01 to 3 μmol/L, mean±SEM, n=6).

PASMCs and its role in $[Ca^{2+}]_i$ and contraction in rat PA. We have demonstrated for the first time that U46619, through the activation of TP receptors, inhibited $I_{K(V)}$ and depolarized PASMCs in a concentration-dependent manner. These effects were totally abolished by nonselective PKC inhibitors and by selective inhibition of aPKCs. Additionally, the U46619-induced increases in $[Ca^{2+}]_i$ and contraction of the PA were markedly attenuated by L-type Ca^{2+} channel blockade, although this vasoconstrictor did not directly affect L-type Ca^{2+} currents. PKCζ was strongly expressed in PA and was translocated on stimulation with U46619, whereas expression of the other aPKC (PKCλ/ι) was negligible. All of these results indicate that U46619, via PKCζ-dependent pathway, inhibits K_V channel activity and causes membrane depolarization, leading to the activation of L-type Ca^{2+} channels, increase in $[Ca^{2+}]_i$, and contraction of PASMCs.

Membrane potential plays an essential role in regulating vascular diameter through the control of Ca^{2+} influx and, therefore, $[Ca^{2+}]_i$. In PASMCs, the resting membrane potential seems to be predominantly regulated by K_V channels.[14–16,21,23] Herein, we show that U46619 (via activation of TP receptors) inhibits K_V channels and depolarizes the membrane of PASMCs to values above the threshold of activation of L-type Ca^{2+} channels[15,18] and causes an increase in $[Ca^{2+}]_i$ and vasoconstriction. The fact that its effects on $[Ca^{2+}]_i$ and vasoconstriction are dihydropyridine-sensitive is

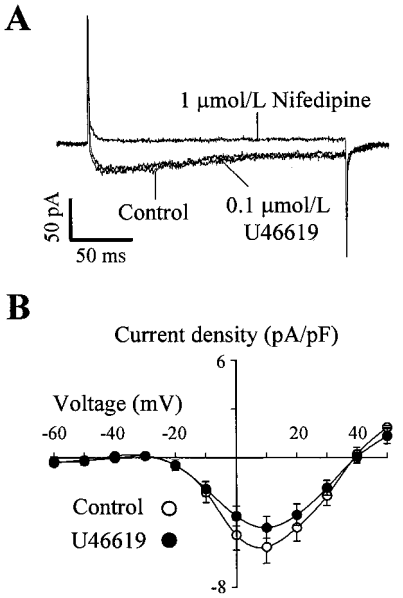

Figure 4. U46619 has no effect on L-type Ca^{2+} currents recorded in PASMCs. A, Current traces are shown when stepping from −60 mV to +10 mV in the absence (control) and presence of U46619 (0.1 μmol/L) or nifedipine (0.1 μmol/L). B, Current-voltage relationships of L-type Ca^{2+} currents measured at the peak in the absence and the presence of U46619. Data show mean±SEM (n=5).

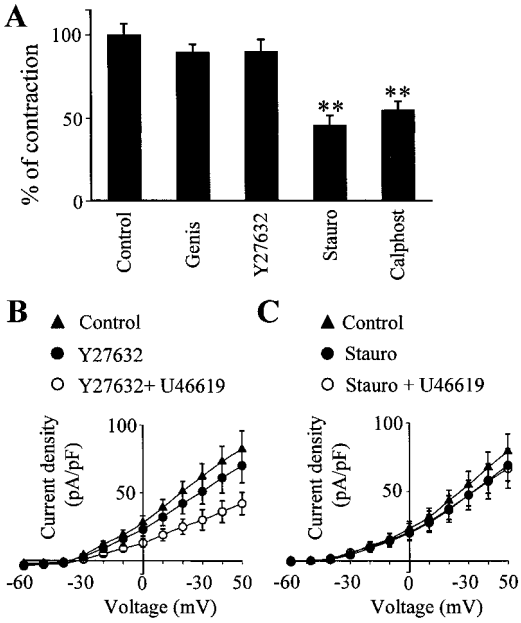

Figure 5. Inhibition of PKC decreases U46619-induced responses. A, Effects of genistein (Genis, 10 μmol/L), Y-27632 (1 μmol/L), staurosporine (Stauro, 0.01 μmol/L), and calphostin C (Calphost, 1 μmol/L) on U46619-induced contractions in rat PA. Results are normalized to values obtained in control experiments. Data represent mean±SEM (n=5 to 7). $^{**}P<0.01$ vs control. B and C, Effects of Y-27632 (1 μmol/L) and Y-27632 plus U46619 (0.1 μmol/L) (B) and staurosporine (0.1 μmol/L) and staurosporine plus U46619 (0.1 μmol/L) (C) on the current-voltage relationships of $I_{K(V)}$ measured at the end of the pulse. Data show mean±SEM (n=4).

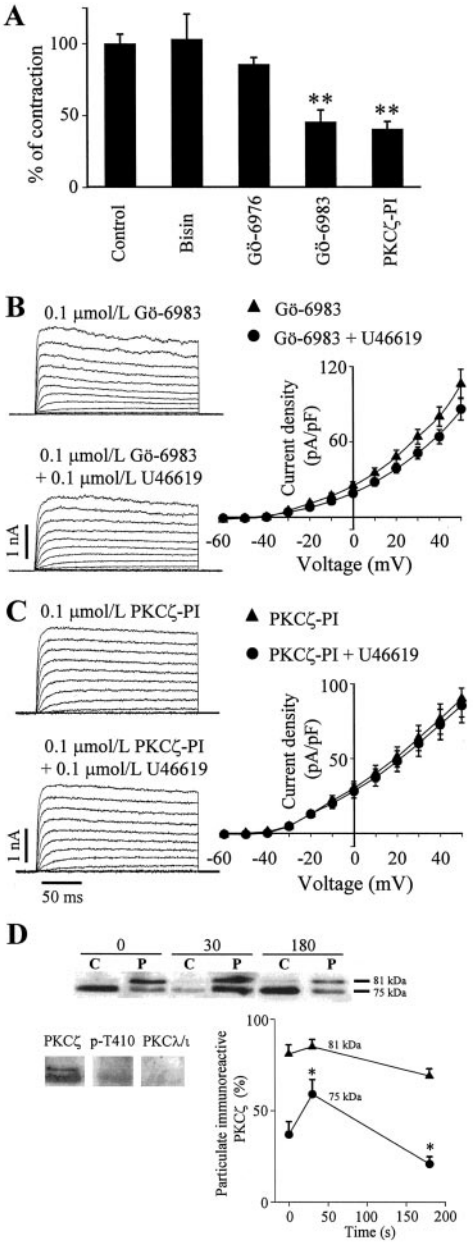

Figure 6. PKCζ mediates the contractile and electrophysiological effects induced by U46619. A, Effects of bisindolylmaleimide I (Bisin, 1 μmol/L), Gö-6976 (0.1 μmol/L), Gö-6983 (0.1 μmol/L), and a pseudosubstrate inhibitor of PKCζ (PKCζ-PI, 10 μmol/L) on U46619-induced contractions in rat PA. Results are normalized to values obtained in control experiments. Data represent mean±SEM (n=5 to 12). $^{**}P<0.01$ vs control. B and C, Effects of U46619 (0.1 μmol/L) on $I_{K(V)}$ in the presence of Gö-6983 (0.1 μmol/L) and PKCζ-PI (0.1 μmol/L, in the internal pipette solution), respectively. The current-voltage relationships of $I_{K(V)}$ in the presence of these drugs are also depicted. Data represent mean±SEM (n=5 to 6). D, Subcellular distribution of atypical PKCs. Top, Representative Western blots of cytosolic (C) and particulate (P) enriched fractions of homogenates of PA under resting conditions (0) or after exposure to U46619 (1 μmol/L) for 30 and 180 seconds using an antibody directed against the C-terminal domain of PKCζ. The graph shows the quantitative distribution of the PKCζ bands in the particulate fraction (percent of C+P) as a function of time of U46619 exposure. $^{*}P<0.05$ vs time 0 (mean±SEM, n=4 to 7). Bottom, Representative blots of total extracts of PA revealed with anti-PKCζ, anti-P-T410 PKCζ, and anti-PKC λ/ι antibodies.

widely assumed to involve a direct activation of TXA_2 on L-type Ca^{2+} channels.[11] However, such assumption has never been demonstrated, and, in fact, a blockade of these channels by TXA_2 agonists has been described in rat hippocampal CA1 neurons.[34] In the present study, L-type Ca^{2+} currents were not affected by U46619 but were abolished by nifedipine. Therefore, our results are consistent with earlier studies demonstrating the involvement of L-type Ca^{2+} channels in TXA_2-induced vasoconstriction[11] but highlight a relevant role for K_V channels as key modulators linking TP receptors to L-type Ca^{2+} channels in rat PA. However, our results cannot rule out that other mechanisms may also contribute to the contraction induced by the activation of TP receptors. Thus, there is a residual component of the contraction that is independent of changes in $[Ca^{2+}]_i$ (ie, implies Ca^{2+} sensitization[9,10]) and on the signaling events described herein.

TXA_2-induced pulmonary vasoconstriction has been shown to be mediated through different intracellular signaling cascades, such as PKC, tyrosine kinase, and Rho kinase.[9,10,12,27] In the present study, the analysis of the signaling pathways involved in TXA_2-induced effects revealed a lack of involvement of tyrosine and Rho kinases. However, the vasoconstrictor and electrophysiological effects of U46619 were attenuated by the nonselective PKC inhibitors staurosporine and calphostin C. Because staurosporine per se did not modify K_V currents or the contraction induced by 4-aminopyridine, the role of PKC in modulating K_V channels seems to be dependent on the activation of TP receptors. PKC

represents a family of several isoforms that can be divided into cPKC (α, βI, βII, and γ), nPKC (δ, ϵ, η, and θ), and aPKC (ζ and λ/ι) isoforms.[31,32] The former group includes Ca^{2+}-dependent isoforms, whereas nPKC and aPKC are Ca^{2+}-independent. Several isoforms (α, β, δ, ϵ, and ζ) seem to coexist in vascular smooth muscle cells,[36,37] and their modulation may account for the responses of vasoconstrictor agents such as angiotensin II, norepinephrine, and endothelin-1.[35,36,38] The contractile response to U46619 was sensitive to Gö-6983 (which preferentially inhibits cPKC, δ, and ζ isoforms) but insensitive to bisindolylmaleimide I or to Gö-6976 at concentrations at which cPKC, δ, ϵ, and μ isoforms should be substantially blocked.[31] These results suggest a role for an aPKC in TXA_2-induced effects. This proposal is additionally supported by the fact that TXA_2-induced inhibition of K_V channels was observed under Ca^{2+}-free conditions. A pseudosubstrate inhibitor peptide, highly specific for PKCζ, markedly inhibited the effects induced by U46619 on K_V channels and contractile force, which indicated a functional role for PKCζ in the signal transduction after TP receptor activation. In agreement with our results, PKCζ is also involved in TXA_2-induced apoptosis in ventricular myocytes.[39] The expression of PKCζ was confirmed by Western blot analysis in rat PA (present results) and in cultured canine pulmonary vascular smooth cells[36] using an antibody directed to the C-terminal peptide of PKCζ, which shows cross-reactivity with PKCλ/ι. However, the expression of PKC λ/ι was negligible using the specific anti-PKCλ/ι antibody, suggesting that the aPKC in this tissue is mainly PKCζ. Furthermore, the results obtained with an antibody directed toward the phosphorylated activation loop (T410) of PKCζ indicate that this kinase is at least partly phosphorylated at T410 in PA. Another piece of evidence in favor of the involvement of PKCζ comes from the results of the transient translocation of PKCζ from the cytosolic to the particulate-enriched fraction on stimulation with U46619.

K_V channels are composed by pore-forming $K_{V\alpha}$ and modulatory $K_{V\beta}$ subunits.[16] The β-subunits of K_V channels may play an important role in modulating the gating properties of α-subunits. Interestingly, PKCζ, via PKCζ-interacting proteins (ZIP1, ZIP2, and ZIP3) acting as scaffolds, has been shown to phosphorylate the auxiliary $K_{V\beta}2$-subunit,[40,41] whereas the consequences of this phosphorylation on K_V function were not analyzed. Therefore, we suggest that after TP receptor activation, the translocation of PKCζ to the membrane may facilitate its coupling with K_V channels. ZIPs, which dramatically enhance phosphorylation of K_V subunits, are attractive scaffold candidates in this interaction.

Increased activity of TXA_2 is associated with several forms of pulmonary hypertension.[1–8] It is interesting to note that calcium channel blockers are first-choice drugs in the treatment of pulmonary hypertension.[23,42] The present results demonstrate that PKCζ translocation, K_V channel inactivation, membrane depolarization, and L-type Ca^{2+} channel activation are key events mediating TXA_2-induced pulmonary vasoconstriction, establishing the rationale for the use of calcium channel blockers in pulmonary hypertension associated with increased vasoconstrictors such as TXA_2 and isoprostanes activating TP receptors.

In conclusion, we demonstrate 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, elevation of $[Ca^{2+}]_i$, and vasoconstriction. PKCζ seems to play a major role as a link between TP receptor activation and K_V channel inhibition.

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