Thromboxane A2-Induced Inhibition of Voltage-Gated K⁺ Channels and Pulmonary Vasoconstriction

Role of Protein Kinase Cζ

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Abstract—Voltage-gated K⁺ channels (Kᵥ) and thromboxane A₂ (TXA₂) play critical roles in controlling pulmonary arterial tone under physiological and pathological conditions. We hypothesized that TXA₂ might inhibit Kᵥ channels, thereby establishing a link between these two major pathogenic pathways in pulmonary hypertension. The TXA₂ analogue U46619 inhibited Iᵥ,K (E₉₀ = 56.1 ± 3.9%, EC₅₀ = 0.054 ± 0.019 μmol/L) and depolarized pulmonary artery smooth muscle cells via activation of TP receptors. In isolated pulmonary arteries, U46619 simultaneously increased intracellular Ca²⁺ 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 nonspecific 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 Iᵥ,K, 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 TXA₂ inhibits Iᵥ,K, leading to depolarization, activation of L-type Ca²⁺ channels, and vasoconstriction of rat pulmonary arteries. We propose PKCζ as a link between TP receptor activation and Kᵥ channel inhibition. (Circ Res. 2003;93:656-663.)

Key Words: K⁺ channels ■ pulmonary artery ■ protein kinase C ■ thromboxane A₂

Thromboxane A₂ (TXA₂) is a prostanoid synthesized by cyclooxygenase with potent vasocontractor, mitogenic, and platelet aggregant properties. The vasoconstrictor effects of TXA₂ are particularly pronounced in the pulmonary vascular bed, where it participates in the control of vessel tone under physiological and pathological situations. In fact, TXA₂ has been involved in several forms of human and experimental pulmonary hypertension, including primary and secondary pulmonary hypertension induced by sepsis, endotoxemia, heparin/protamine, leukotriene D₄, microembo- lism, and ischemia-reperfusion.

TXA₂ contracts vascular smooth muscle by binding to specific Gₛ₁₁ protein-coupled receptors (TP receptors), which leads to an increase in intracellular Ca²⁺ concentration ([Ca²⁺]ᵢ) and sensitization of the contractile proteins to Ca²⁺. 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. 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.

K⁺ channels play an essential role in regulating resting membrane potential, [Ca²⁺], and contraction of vascular smooth muscle. Activation of K⁺ channels leads to hyperpolarization, whereas their inhibition causes membrane depolarization, activation of voltage-gated L-type Ca²⁺ channels, increase in [Ca²⁺]ᵢ, and vasoconstriction. Different types of K⁺ channels have been identified in pulmonary artery smooth muscle cells (PASMCs), including voltage-gated K⁺ channels (Kᵥ), large-conductance Ca²⁺-activated channels (BKᵥ), and ATP-dependent channels (K₆.₅.₃). There is increasing interest in Kᵥ 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. Second, they are modulated by hypoxia and vasoactive factors such as nitric oxide, endothelin-1, and angiotensin II. Finally, decreased expression or function of Kᵥ channels in PASMCs has been involved in the pathogenesis of primary and anorexigen-induced pulmonary hypertension.
Very little is known about the effects of TXA₂ on vascular K⁺ channels. It has been reported that TXA₂ analogues inhibit the activity of BK channels in bronchial and coronary arteries, whereas their effects on vascular K⁺ channels are unknown. We hypothesized that TXA₂ might inhibit K⁺ 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 TXA₂ analogue U46619 on the current flowing through K⁺ channels (I_{K(V)}) recorded in rat PASMCs using the whole-cell configuration of the patch-clamp technique. The role of K⁺ channels in TXA₂-induced pulmonary vasoconstriction has also been studied in isolated pulmonary arteries (PAs). Our results indicate that U46619 inhibits K⁺ channels, leading to depolarization of PASMCs, an increase in [Ca²⁺], and vasoconstriction in PA. Furthermore, we provide evidence for the role of PKCα as the link between TXA₂ receptor activation and inhibition of K⁺ 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, PKCα 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 (Ca²⁺-free PSS) of the following composition (in mmol/L): NaCl 130, KCl 5, MgCl₂ 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 Ca²⁺-free PSS containing (in mg/mL) papain 1, detergents to test potentials from -50 to 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 (I_{K(MD)}) that activated at potentials positive to -30 mV and was usually reproducible for at least 1 hour. This current was essentially abolished by the Kₐ channel blocker 4-aminoypyrindine (1 mmol/L, Figure 1C), indicating that it was evoked by the activation of K⁺ 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

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 Na₃-p-tosyl-l-lysine chloromethyl ketone, 5 mmol/L NaF, 10 mmol/L Na₃MoO₄, 1 mmol/L NaVO₃, 0.5 mmol/L phenylmethylsulfonyl fluoride, and 10 mmol/L okadaic acid. The homogenate was centrifuged at 100 000g 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 4% and gently shaken for 30 minutes at 4°C and again centrifuged at 100 000g 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-PKCa, anti-PKCe, or anti-P-T410-PKCε 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.

Effects of the TXA₂ 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 Ca²⁺-free conditions, and EGTA and ATP were included in the pipette solution to minimize the component of ATP-dependent (I_{ATP}) and Ca²⁺-activated (I_{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 Kₐ channel inhibitor 4-aminoypyrindine (1 mmol/L, Figures 1F and 1G), indicating that it was evoked by the activation of K⁺ 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
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 TXA2 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 [Ca2+]: Role of L-Type Ca2+ 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 Ca2+ entry through voltage-gated L-type Ca2+ channels. This possibility was studied by measuring simultaneous changes in force and [Ca2+]i, in fura-2-loaded PA. Absolute values for [Ca2+]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 [Ca2+]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 Ca2+ 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 [Ca2+]i, induced by U46619 (Figure 3C). These results suggested an important role of L-type Ca2+ channels in the U46619-induced increase in [Ca2+]i, and contraction. Figure 3E shows the concentration-response curves for the increase in [Ca2+]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 Ca2+ channels not only via membrane depolarization but also directly, its effects were analyzed on L-type Ca2+ currents recorded in isolated PASMCs. Figure 4A shows traces of Ca2+ currents elicited when stepping from −60 to −10 mV. The current activated at $E_C$=−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 TXA2 analogue. Because staurosporine is a nonselective inhibitor of PKC and may also directly affect contractile proteins (eg, it inhibits myosin light chain kinase31), 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.
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 (\(E_m\)=−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 \(\approx75\) 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. Western blots of homogenates from rat PA using polyclonal rabbit antibody directed toward the C-terminal peptide of PKCζ also recognized two bands of \(\approx81\) and 75 kDa (Figure 6D). This antibody cross-reacts with the aPKCA/I. However, the expression of this aPKC was negligible using a specific anti-PKCA/I antibody toward the amino acids 168 to 243 of PKCζ. Western blots of homogenates from rat PA using polyclonal rabbit antibody directed toward the C-terminal peptide of PKCζ also recognized two bands of \(\approx81\) and 75 kDa (Figure 6D). This antibody cross-reacts with the aPKCA/I. However, the expression of this aPKC was negligible using a specific anti-PKCA/I antibody toward the amino acids 168 to 243 of PKCζ. Western blots of homogenates from rat PA using polyclonal rabbit antibody directed toward the C-terminal peptide of PKCζ also recognized two bands of \(\approx81\) and 75 kDa (Figure 6D). This antibody cross-reacts with the aPKCA/I. However, the expression of this aPKC was negligible using a specific anti-PKCA/I antibody toward the amino acids 168 to 243 of PKCζ. Western blots of homogenates from rat PA using polyclonal rabbit antibody directed toward the C-terminal peptide of PKCζ also recognized two bands of \(\approx81\) and 75 kDa (Figure 6D). This antibody cross-reacts with the aPKCA/I. However, the expression of this aPKC was negligible using a specific anti-PKCA/I antibody toward the amino acids 168 to 243 of PKCζ.
PASMCs and its role in \([\text{Ca}^{2+}]_i\) and contraction in rat PA. We have demonstrated for the first time that U46619, through the activation of TP receptors, inhibited \(I_{\text{K(V)}}\) 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 \([\text{Ca}^{2+}]_i\) 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. PKC\(\zeta\) was strongly expressed in PA and was translocated on stimulation with U46619, whereas expression of the other aPKC (PKC\(\alpha\)) was negligible. All of these results indicate that U46619, via PKC\(\zeta\)-dependent pathway, inhibits \(K_v\) channel activity and causes membrane depolarization, leading to the activation of L-type Ca \(^{2+}\) channels, increase in \([\text{Ca}^{2+}]_i\), and contraction of PASMCs.

Membrane potential plays an essential role in regulating vascular diameter through the control of Ca \(^{2+}\) influx and, therefore, \([\text{Ca}^{2+}]_i\). 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 \([\text{Ca}^{2+}]_i\), and vasoconstriction. The fact that its effects on \([\text{Ca}^{2+}]_i\), and vasoconstriction are dihydropyridine-sensitive is

**Figure 3.** U46619 increases force and \([\text{Ca}^{2+}]_i\) via L-type Ca \(^{2+}\) channels. Recordings of the simultaneous changes on force (top) and \([\text{Ca}^{2+}]_i\) (bottom) elicited by U46619 (0.1 \(\mu\)mol/L). The second stimulation with U46619 was elicited in the absence (control, A) or after exposure to nifedipine (0.1 \(\mu\)mol/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 \([\text{Ca}^{2+}]_i\) and contractile force induced by U46619 (0.01 to 3 \(\mu\)mol/L, mean±SEM, \(n=6\)).

**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 \(\mu\)mol/L) or nifedipine (0.1 \(\mu\)mol/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 (\(n=9\)).
widely assumed to involve a direct activation of TXA₂ on L-type Ca²⁺ channels. However, such assumption has never been demonstrated, and, in fact, a blockade of these channels by TXA₂ agonists has been described in rat hippocampal CA1 neurons. In the present study, L-type Ca²⁺ 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 Ca²⁺ channels in TXA₂-induced vasoconstriction but highlight a relevant role for Kv channels as key modulators linking TP receptors to L-type Ca²⁺ 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 [Ca²⁺], (ie, implies Ca²⁺ sensitization⁹,¹⁰) and on the signaling events described herein.

TXA₂-induced pulmonary vasoconstriction has been shown to be mediated through different intracellular signaling cascades, such as PKC, tyrosine kinase, and Rho kinase. In the present study, the analysis of the signaling pathways involved in TXA₂-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 Kv currents or the contraction induced by 4-aminopyridine, the role of PKC in modulating Kv channels seems to be dependent on the activation of TP receptors. PKC 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, measured at the end of the pulse. Data show mean±SEM (n=4).

Figure 5. PKCζ mediates the contractile and electrophysiologi- cal effects induced by U46619. A, Effects of bisindolylmaleimide I (Bisin, 1 μmol/L), Go6-6976 (0.1 μmol/L), Go6-6983 (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 in the presence of Go6-6983 (0.1 μmol/L) and PKCζ-PI (0.1 μmol/L). 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 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<0.01 vs control.
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 Ca²⁺-dependent isoforms, whereas nPKC and aPKC are Ca²⁺-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 TXA₂-induced effects. This proposal is additionally supported by the fact that TXA₂-induced inhibition of Kᵥ channels was observed under Ca²⁺-free conditions. A pseudosubstrate inhibitor peptide, highly specific for PKCζ, markedly inhibited the effects induced by U46619 on Kᵥ 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 TXA₂-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 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.

Kᵥ channels are composed by pore-forming Kᵥα and modulatory Kᵥβ subunits.16 The β-subunits of Kᵥ 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 Kᵥβ2-subunit, whereas the consequences of this phosphorylation on Kᵥ function were not analyzed. Therefore, we suggest that after TP receptor activation, the translocation of PKCζ to the membrane may facilitate its coupling with Kᵥ channels, ZIPs, which dramatically enhance phosphorylation of Kᵥ subunits, are attractive scaffold candidates in this interaction.

Increased activity of TXA₂ 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.2342 The present results demonstrate that PKCζ translocation, Kᵥ channel inactivation, membrane depolarization, and L-type Ca²⁺ channel activation are key events mediating TXA₂-induced pulmonary vasoconstriction, establishing the rationale for the use of calcium channel blockers in pulmonary hypertension associated with increased vasoconstrictors such as TXA₂ and isoprostanates activating TP receptors.

In conclusion, we demonstrate that in intact PAs and freshly isolated PASMCs, TXA₂, via activation of TP receptors, inhibits Kᵥ channels, leading to membrane depolarization, activation of L-type Ca²⁺ channels, elevation of [Ca²⁺], and vasoconstriction. PKCζ seems to play a major role as a link between TP receptor activation and Kᵥ channel inhibition.

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References


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