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Pulmonary Artery Vasoconstriction but not $[Ca^{2+}]_i$ Signal Stimulated by Thromboxane A_2 Is Partially Resistant to NO

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ABSTRACT

To characterize the thromboxane A_2 (TXA_2) -induced resistance to the vasodilator effects of the nitric oxide (NO)/cGMP pathway in pulmonary arteries, we have studied the effects of the NO donor sodium nitroprusside on intracellular calcium concentration ($[Ca^{2+}]_i$) and contractile force recorded simultaneously in isolated piglet pulmonary arteries loaded with fura-2 and contracted with norepinephrine or the TXA_2 mimetic U46619 and by activation of protein kinase C (PKC) with phorbol 12-myristate 13-acetate. In the TXA_2 mimetic- and phorbol 12-myristate 13-acetate plus norepinephrine-stimulated arteries, nitroprusside exhibited lower vasodilator efficacy (and lower potency in the TXA_2 mimetic-stimulated arteries) but similar reductions in $[Ca^{2+}]_i$ compared with arteries activated by norepinephrine. The nonselective serine/threonine kinase inhibitor staurosporine, but not the selective inhibitor of PKC bisindolylmaleimide, potentiated the relaxation of nitroprusside in the TXA_2 mimetic-stimulated arteries. In conclusion, the resistance to NO/cGMP-induced vasodilation in arteries stimulated by TXA_2 and PKC involves a reduced ability of the Ca^{2+}-independent mechanisms for smooth muscle vasodilation. The resistance to NO in arteries stimulated by TXA_2 is sensitive to staurosporine but not to bisindolylmaleimide, suggesting the involvement of an activation of a serine/threonine kinase distinct from PKC. **(*Pediatr Res* 50: 508–514, 2001)**

Abbreviations

$[Ca^{2+}]_i$, intracellular calcium concentration
E_{max}, maximal effect
F340/F380, ratio of fluorescence at 340 and 380 nm (index of cytosolic Ca^{2+})
NO, nitric oxide
pD_2, negative log molar of the drug concentration exhibiting 50% of the Emax
PMA, phorbol 12-myristate 13-acetate
PPHN, persistent pulmonary hypertension of the newborn
TXA_2, thromboxane A_2
U46619, 9,11-dideoxy-11α,9α-epoxymethano-prostaglandin $F_{2\alpha}$ (thromboxane mimetic)

NO plays a key role in the regulation of vascular smooth muscle tone through the activation of the soluble guanylate cyclase and the subsequent increase in cGMP (1, 2). Alterations of the NO/cGMP pathway have been associated with a number of vascular diseases. In the pulmonary circulation, NO is crucial for maintaining low vascular resistances and arterial pressure (3, 4). Reduced NO or cGMP activities have been associated with the high pulmonary pressure during fetal life (5) and PPHN (6–8), as well as primary and secondary pulmonary hypertension (9, 10). In addition, inhaled NO is widely used in the treatment of pulmonary hypertension in both adults and neonates (11–13).

Increased activity of the potent pulmonary vasoconstrictor TXA_2 has also been implicated in several forms of human and experimental pulmonary hypertension including those induced by sepsis (14), heparin/protamine (15), leukotriene D_4 (16), microembolism (17), and ischemia-reperfusion (18). Elevated levels of thromboxane B_2, the metabolite of TXA_2, have been found in patients with PPHN (19). Moreover, TXA_2 may play a key role in pulmonary hypertension not only because of its potent vasoconstrictor activity but also because TXA_2-induced vasoconstriction is highly resistant to the vasodilator effects of NO (20–24). Therefore, resistance of TXA_2-induced pulmonary vasoconstriction to NO might be implicated in the therapeutic failure of inhaled NO in some patients with PPHN (11, 12).

The reduced vasodilator activity of NO in TXA_2-induced pulmonary vasoconstriction was not related to a reduced cGMP synthesis, the initial step in the signal transduction of NO, but rather to changes downstream in the signaling cascade of

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cGMP (24), which involve both cytosolic $[Ca^{2+}]_i$-dependent and $[Ca^{2+}]_i$-independent pathways (25–35). At present, it is unknown whether the resistance to the NO/cGMP pathway in TXA_2-stimulated arteries is dependent on alterations of $[Ca^{2+}]_i$ or is caused by $[Ca^{2+}]_i$-independent effects. Inasmuch as the nonspecific protein kinase inhibitor staurosporine blunted the resistance to NO in TXA_2-stimulated arteries and the protein kinase C activator PMA mimicked the TXA_2-induced resistance to NO-induced vasodilation, it was suggested that protein kinase C was involved in this process (24).

Therefore, the aims of the present study were *1*) to analyze whether the resistance to the vasodilator effect of the NO/cGMP pathway in TXA_2-induced pulmonary vasoconstriction is associated with alterations in $[Ca^{2+}]_i$ and *2*) to further characterize the possible involvement of protein kinase C. For these purposes, we have studied the effects of the NO donor sodium nitroprusside on $[Ca^{2+}]_i$ and contractile force recorded simultaneously in isolated piglet pulmonary arteries stimulated with the TXA_2-mimetic U46619 and with the activator of protein kinase C, PMA. Norepinephrine-induced vasoconstriction was used as control, and staurosporine and bisindolylmaleimide, as nonspecific and specific inhibitors of protein kinase C, respectively.

METHODS

Tissue preparation. Twenty-four male piglets (10–17 d, 3–5 kg) were used in this study. The procedures were approved by the Complutense University Animal Care and Use Committee. Piglets were killed in the local abattoir, and the lungs were rapidly immersed in cold (4°C) Krebs solution (composition in mM: NaCl 118, KCl 4.75, $NaHCO_3$ 25, $MgSO_4$ 1.2, $CaCl_2$ 2.0, KH_2PO_4 1.2, and glucose 11) and transported to the laboratory. The intrapulmonary arteries (third branch, internal diameter 1–2 mm) were carefully dissected free of surrounding tissue and cut into rings of 2–3 mm length (20, 21). The endothelium was removed by gently rubbing the intimal surface of the rings with a metal rod. The procedure of endothelium removal was verified by the absence of a vasodilator response to acetylcholine (10^{-5} M) in arteries stimulated by norepinephrine.

Simultaneous measurements of $[Ca^{2+}]_i$ and tension. Endothelium-denuded rings were inverted and incubated for 4–6 h at room temperature in Krebs solution containing fura-2 acetoxymethylester (5×10^{-6} M) and Cremophor EL (0.05%). Thereafter, rings were mounted between two hooks under 0.5 g of tension in a 5-mL organ bath filled with Krebs solution at 37°C and gassed with a 95% O_2/5%CO_2 gas mixture in the absence of fura-2. The bath was part of a fluorimeter (CAF 110, Jasco, Tokyo, Japan), which allows us to estimate changes in the fluorescence intensity of fura-2 simultaneously with contractile force by an isometric force transducer as previously described (36, 37). The luminal face of the rings was alternately illuminated (128 Hz) with two excitation wavelengths (340 and 380 nm) from a xenon lamp coupled with two monochromators. The emitted fluorescent light at the two excitation wavelengths (F340 and F380) was measured by a photomultiplier through a 500-nm filter and recorded together with the force data by using data acquisition hardware (Mac Lab, model 8, AD Instruments Pty Ltd., Castle Hill, Australia) and data recording software (Chart v3.2, AD Instruments Pty Ltd.). The absolute values of $[Ca^{2+}]_i$ were estimated from the ratio of emitted fluorescence obtained at the two excitation wavelengths (F340/F380) using the Grynkiewicz equation as reported by Kanaide (38). The maximal and minimal F340 and F380 values for this equation were obtained by treatment with ionomycin (1.4×10^{-6} M) and then with EGTA (8 mM) as previously described (38).

After equilibration for 30–45 min, vessels were initially stimulated with 80 mM KCl for 10–15 min, which induced a sustained increase in $[Ca^{2+}]_i$ and force. After washing in normal Krebs solution, the rings were stimulated with norepinephrine (10^{-5} M) or the TXA_2 mimetic U46619 (10^{-7} M), and a concentration-response curve to nitroprusside (10^{-8} M to 3×10^{-5} M) was constructed by cumulative addition of the drug. The concentrations of norepinephrine and U46619 were chosen because in previous studies they produced a similar increase in contractile force (approximately 60–80% of the maximal response to U46619) (20, 24). In some experiments, PMA (10^{-7} M) was added 25 min before stimulation with norepinephrine, and in another set of experiments, staurosporine (10^{-7} M) or bisindolylmaleimide (10^{-6} M) was added 25 min before stimulation with U46619. Because staurosporine decreased the contractile response to U46619, in these experiments the concentration of U46619 was raised to 2×10^{-7} M to reach a contractile force similar to that induced by U46619 in the absence of the drug.

Drugs. The following drugs were used: (−)-norepinephrine bitartrate, sodium nitroprusside, PMA, thapsigargin, staurosporine, and bisindolylmaleimide I (Sigma Chemical Co., Alcobendas, Madrid, Spain), U46619 (Alexis Corporation, Läufelfingen, Switzerland) and fura-2 acetoxymethylester (1 mM solution in DMSO, Calbiochem, La Jolla, CA, U.S.A.). Drugs were dissolved initially in distilled deionized water (except for staurosporine, thapsigargin, bisindolylmaleimide, and PMA, which were dissolved in DMSO) to prepare a 10^{-2} M or 10^{-3} M stock solution, and further dilutions were made in Krebs solution. The concentrations are expressed as final molar concentration in the tissue chamber.

Statistical analysis. Results are expressed as mean ± SEM where n equals the number of animals. The results of both $[Ca^{2+}]_i$ and force are expressed as a percentage, considering the values at rest in normal Krebs solution and after 80 mM KCl-induced stimulation to be 0% and 100%, respectively (28). Individual cumulative concentration-response curves were fitted to a logistic equation. The drug concentration exhibiting 50% of the E_{max} was calculated from the fitted concentration-response curves for each ring and expressed as pD_2. Statistically significant differences among groups were calculated by one-way ANOVA followed by a Newman-Keuls' t test, with $p < 0.05$ considered statistically significant.

RESULTS

Responses to norepinephrine, the TXA_2 mimetic U46619, and PMA. In fura-2 unloaded arteries, 80 mM KCl induced a contractile response and weakly increased both the F340 and

F380 signals, but no change was observed in the ratio F340/F380. Stimulation of fura-2-loaded pulmonary arteries with 80 mM KCl induced a sustained increase in the F340/F380 signal of 16.7 ± 1.9% above resting values. Absolute values of $[Ca^{2+}]_i$ under basal conditions and after stimulation with KCl were 105 ± 19 and 196 ± 36 nM, respectively, and the sustained contractile response averaged 418 ± 37 mg in 38 arteries (not significantly different from that in fura-2 unloaded arteries). As shown in Figure 1, both norepinephrine (10^{-5} M) and the TXA_2 mimetic U46619 (10^{-7} M) induced a fast increase in $[Ca^{2+}]_i$ followed by a decay to a lower sustained level (Fig. 1, *A* and *B*). In contrast, PMA (10^{-7} M) had no effect on $[Ca^{2+}]_i$ (−3.7 ± 1.1% of the response to KCl, $p > 0.05$) but induced a slowly developing contractile response (16 ± 3% of the response to KCl at 30 min, $p < 0.05$, Fig. 1*C*). In the presence of phorbol esters, the increase in $[Ca^{2+}]_i$ induced by norepinephrine was strongly inhibited, which is consistent with data previously described in sheep cerebral arteries, possibly because of a reduction in phospholipase C activity (39). Nevertheless, application of thapsigargin at the end of the experiment increased $[Ca^{2+}]_i$ to values similar to those induced by KCl (Fig. 1*C*). Figure 2 shows that the Ca^{2+}–force relationship constructed with increasing concentrations of U46619 (10^{-9} M to 10^{-7} M) was very similar to that for norepinephrine (10^{-8} M to 10^{-5} M). At 10^{-7} M U46619 and 10^{-5} M norepinephrine, the force relative to $[Ca^{2+}]_i$ (% force/% $[Ca^{2+}]_i$) was 2.1 and 2 times greater, respectively, than that induced by 80 mM KCl ($p < 0.05$). In the presence of PMA, the norepinephrine-induced increase in $[Ca^{2+}]_i$ was strongly reduced, but the contractile force was not significantly different from that in the absence of PMA and thus a 4.5-fold increase in the force relative to $[Ca^{2+}]_i$ compared with PMA-untreated arteries was observed.

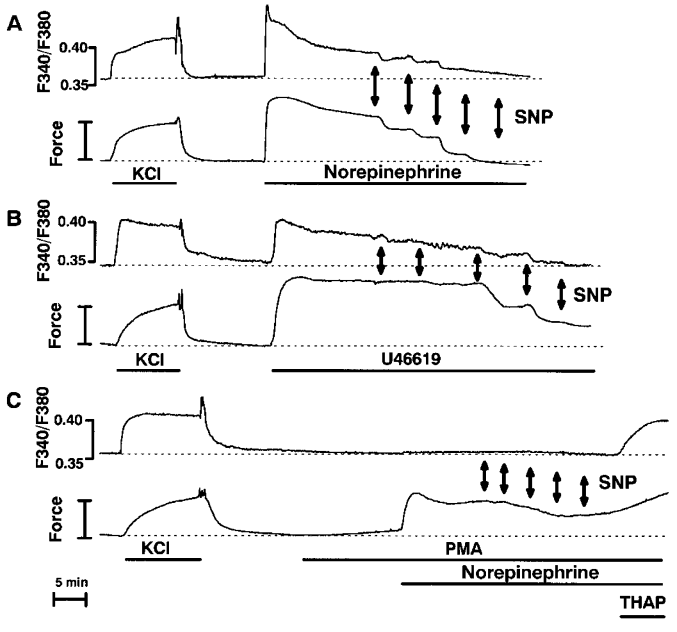

Figure 1. Representative experiments showing the effects of norepinephrine (*A*), U46619 (*B*), and PMA plus norepinephrine (*C*) and the inhibitory effects of nitroprusside on $[Ca^{2+}]_i$ (F340/F380, *upper traces*) and contractile force (*lower traces*) in endothelium-denuded pulmonary arteries. Arteries were initially stimulated by 80 mM KCl, then with norepinephrine (10^{-5} M), U46619 (10^{-7} M), or PMA (10^{-7} M) plus norepinephrine (10^{-5} M), as indicated by the bars, and thereafter a cumulative concentration-response curve to nitroprusside (10^{-8} M, 10^{-7} M, 10^{-6} M, 10^{-5} M, and 3×10^{-5} M) was constructed by cumulative addition of the drug as indicated by the arrows. *C* shows the effects of thapsigargin (*THAP*, 10^{-6} M) when added at the end of the experiment. The vertical calibration bars for force recordings indicate 500 mg. The traces of F340/F380 represent arbitrary units.

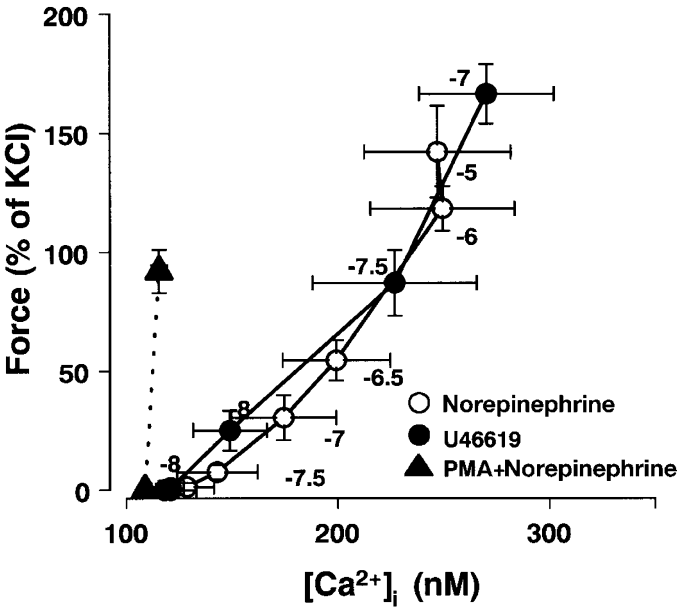

Figure 2. $[Ca^{2+}]_i$–force relationships constructed by increasing concentrations of norepinephrine (10^{-8} M to 10^{-5} M) and U46619 (10^{-9} M to 10^{-7} M) or by a single concentration of PMA (10^{-7} M) plus norepinephrine (10^{-5} M). The logarithm of the concentrations of norepinephrine or U46619 are indicated at each point. The results (mean ± SEM, 4–7 experiments) are expressed as a percentage of the response to 80 mM KCl.

Effects of nitroprusside. Cumulative addition of nitroprusside induced a concentration-dependent reduction in $[Ca^{2+}]_i$ and relaxation (Figs. 1 and 3, Table 1). Nitroprusside reverted the increase in $[Ca^{2+}]_i$ induced by norepinephrine, the TXA_2 mimetic, and PMA plus norepinephrine with a similar potency and efficacy (*i.e.* similar pD_2 and E_{max} values), even when marked differences in the preexisting $[Ca^{2+}]_i$ level were observed in PMA plus norepinephrine-treated arteries (Fig. 3*A*). Nitroprusside relaxed norepinephrine-stimulated arteries in parallel to the reduction in $[Ca^{2+}]_i$ (Fig. 3, *A* and *B*). In contrast, nitroprusside produced only a partial relaxant effect on U46619- and PMA plus norepinephrine-induced contractions ($p < 0.05$ *versus* norepinephrine and $p < 0.05$ *versus* $[Ca^{2+}]_i$). The reduction in E_{max} was accompanied by a significant reduction in the pD_2 value in U46619-treated but not in PMA plus norepinephrine-treated arteries (Table 1). Figure 3*C* shows the plot of the relaxation as a function of $[Ca^{2+}]_i$ reduction induced by nitroprusside, *i.e.* the $[Ca^{2+}]_i$–force relationship. It can be observed that in norepinephrine-stimulated arteries, the $[Ca^{2+}]_i$–force relationship was almost linear. In contrast, in arteries stimulated by U46619, low concentrations of nitroprusside (up to 10^{-7} M) induced smaller relaxant effects for a given reduction in $[Ca^{2+}]_i$ compared with norepinephrine-stimulated arteries, whereas at higher concentrations of nitroprusside the reduction in $[Ca^{2+}]_i$ was accompanied by

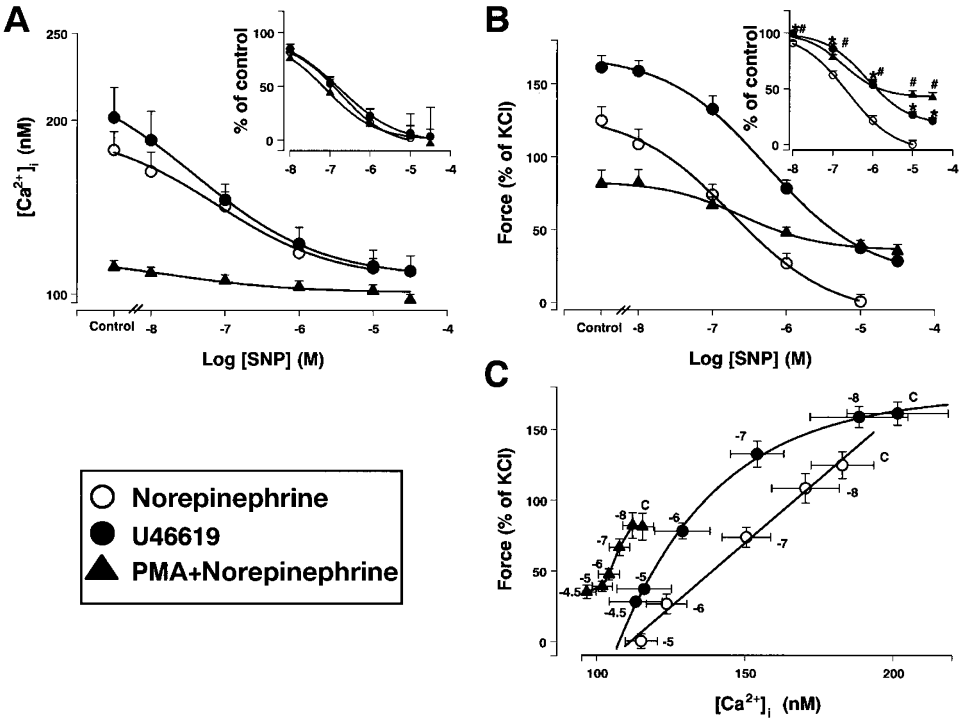

Figure 3. Effects of nitroprusside (10^{-8} M to 3×10^{-5} M) on $[Ca^{2+}]_i$ (*A*) and force (*B*) in endothelium-denuded pulmonary arteries stimulated by norepinephrine (10^{-5} M), U46619 (10^{-7} M), and PMA (10^{-7} M) plus norepinephrine (10^{-5} M). The experiments were performed as shown in Figure 1. The insets in *A* and *B* show the same results expressed as a percentage of control values before the addition of nitroprusside. *C*, $[Ca^{2+}]_i$– force relationship obtained from data in *A* and *B*. Control values before the addition of nitroprusside are indicated by *C*, and the logarithm of the concentration of nitroprusside is at each point. The symbols represent the mean ± SEM of 6–9 experiments. * $p < 0.05$ U46619 *vs* norepinephrine, # $p < 0.05$ PMA plus norepinephrine *vs* norepinephrine.

Table 1. *Variables of the concentration-response curves to nitroprusside (10^{-8} M to 3×10^{-5} M) on $[Ca^{2+}]_i$ and contractile force in pulmonary rings*

		$[Ca^{2+}]_i$		Force	
	n	pD_2	E_{max} (%)	pD_2	E_{max} (%)
Norepinephrine	9	6.88 ± 0.10	103 ± 4	6.61 ± 0.08	105 ± 3
U46619	6	6.96 ± 0.17	97 ± 7	6.19 ± 0.11*	79 ± 3*
PMA + norepinephrine	6	7.02 ± 0.10	105 ± 24	6.66 ± 0.09	60 ± 3*
STAU + U46619	6	6.80 ± 0.16	109 ± 4	6.39 ± 0.10	99 ± 3†
BISI + U46619	5	6.75 ± 0.10	92 ± 3	6.33 ± 0.10	69 ± 4*

Abbreviations: STAU, staurosporine (10^{-7} M); BISI, bisindolylmaleimide (10^{-6} M).

* $p < 0.05$ *vs* norepinephrine.

† $p < 0.05$ *vs* U46619.

E_{max} values represent the reduction in $[Ca^{2+}]_i$ and force expressed as a percentage of the control response. Values are mean ± SEM. The data are calculated from the insets in Figures 3 and 4.

significant relaxation. At 3×10^{-5} M nitroprusside, $[Ca^{2+}]_i$ values were similar to resting levels, but there was still a component (22 ± 3%) of contraction remaining. Therefore, the $[Ca^{2+}]_i$–force relationship showed a nonlinear shape, *i.e.* there was a dissociation between $[Ca^{2+}]_i$ and contractile force. In PMA plus norepinephrine-treated arteries, the $[Ca^{2+}]_i$ values before the addition of nitroprusside were only 9 ± 3% of the response to 80 mM KCl, and after 3×10^{-5} M nitroprusside, they were reduced to −7 ± 3% (not significantly different from baseline), *i.e.* the relaxant effect of nitroprusside was accompanied of absolute changes in $[Ca^{2+}]_i$ much smaller than those observed in norepinephrine- and U46619-stimulated arteries. The $[Ca^{2+}]_i$–force relationship was almost linear, but it was significantly steeper ($p < 0.05$) compared with arteries treated with norepinephrine alone.

Effects of staurosporine and bisindolylmaleimide. The nonselective protein kinase inhibitor staurosporine (10^{-7} M) or the selective protein kinase C inhibitor bisindolylmaleimide (10^{-6} M) had no measurable effect on baseline $[Ca^{2+}]_i$ or force. In the experiments with staurosporine the concentration of U46619 was raised to 2×10^{-7} M to reach an equivalent contractile response to that of 10^{-7} M U46619 in the absence of the drug. The increases in $[Ca^{2+}]_i$ and contractile force induced by U46619 in the presence of the drugs were not significantly different from those induced in their absence, and, therefore, the force relative to $[Ca^{2+}]_i$ was not significantly different (Fig. 4). Cumulative addition of nitroprusside induced a concentration-dependent decrease in $[Ca^{2+}]_i$, which was similar in control and in staurosporine- or bisindolylmaleimide-treated arteries (Fig. 4*B*). However, there was a significant

increase in the maximal relaxant response to nitroprusside in staurosporine- but not in bisindolylmaleimide-treated arteries (Fig. 4*C*, Table 1).

DISCUSSION

In the present study we have analyzed the effects of the NO donor nitroprusside on $[Ca^{2+}]_i$ and contractile force in endothelium-denuded piglet pulmonary arteries activated by the TXA_2-mimetic U46619 or by the protein kinase C activator PMA. Norepinephrine was used as a control vasoconstrictor. The results can be summarized as follows. In arteries stimulated by the TXA_2 mimetic or PMA plus norepinephrine, nitroprusside induced only a partial relaxation despite a similar potency and efficacy in lowering $[Ca^{2+}]_i$ compared with norepinephrine-stimulated arteries. The nonselective protein kinase inhibitor staurosporine but not the selective protein kinase C inhibitor bisindolylmaleimide increased the maximal relaxant response to nitroprusside in the TXA_2 mimetic-stimulated arteries, although neither of them modified the nitroprusside-induced $[Ca^{2+}]_i$-lowering effects.

The regulation of vascular smooth muscle tone depends primarily on changes in $[Ca^{2+}]_i$, which controls myosin light chain kinase activity (25). However, in recent years, Ca^{2+}-independent mechanisms (*i.e.* Ca^{2+} sensitization) involving changes in myosin light chain phosphatase activity or actin-linked regulatory mechanisms have also been reported to play an important role (25, 40). The Ca^{2+} sensitization induced by phorbol esters (which directly stimulate protein kinase C) and receptor agonists such as TXA_2 and norepinephrine have been reported elsewhere (25, 40, 41). Likewise, in the present study, both the TXA_2 mimetic and norepinephrine induced a substantial greater contraction relative to the increase in $[Ca^{2+}]_i$ than 80 mM KCl. The force relative to $[Ca^{2+}]_i$ was similar for the TXA_2 mimetic and norepinephrine (about twofold increase over KCl), *i.e.* they produced similar Ca^{2+} sensitization. Furthermore, the Ca^{2+}–force relationships were similar for the whole concentration-response curves to both agonists. However, in the presence of PMA, norepinephrine induced a weak increase in $[Ca^{2+}]_i$, but the contractile response was not significantly different from that observed in the absence of PMA. Thus, force relative to $[Ca^{2+}]_i$ (Ca^{2+} sensitization) in PMA plus norepinephrine-stimulated arteries was much higher than in the TXA_2 mimetic- or norepinephrine-stimulated arteries.

Nitroprusside causes vascular smooth muscle relaxation by releasing NO, which, in turn, activates guanylate cyclase and increases intracellular cGMP levels (26). cGMP and cGMP-activated protein kinase may control a large number of cellular activities to regulate vascular smooth muscle tone (2, 28). The relative role of these mechanisms of action varies widely depending on the vascular tissue. In piglet pulmonary arteries, nitroprusside-induced relaxation is blunted by inhibition of guanylate cyclase and has been attributed to both $[Ca^{2+}]_i$-dependent and -independent mechanisms (24, 30).

Porcine intrapulmonary arteries stimulated by the TXA_2 mimetic showed smaller relaxant responses to nitroprusside and other agents stimulating the NO/cGMP pathway than arteries stimulated by norepinephrine (24). This effect was specific for piglet pulmonary arteries, inasmuch as it was not seen in piglet mesenteric and coronary arteries and it was specific for the cGMP pathway because similar relaxant re-

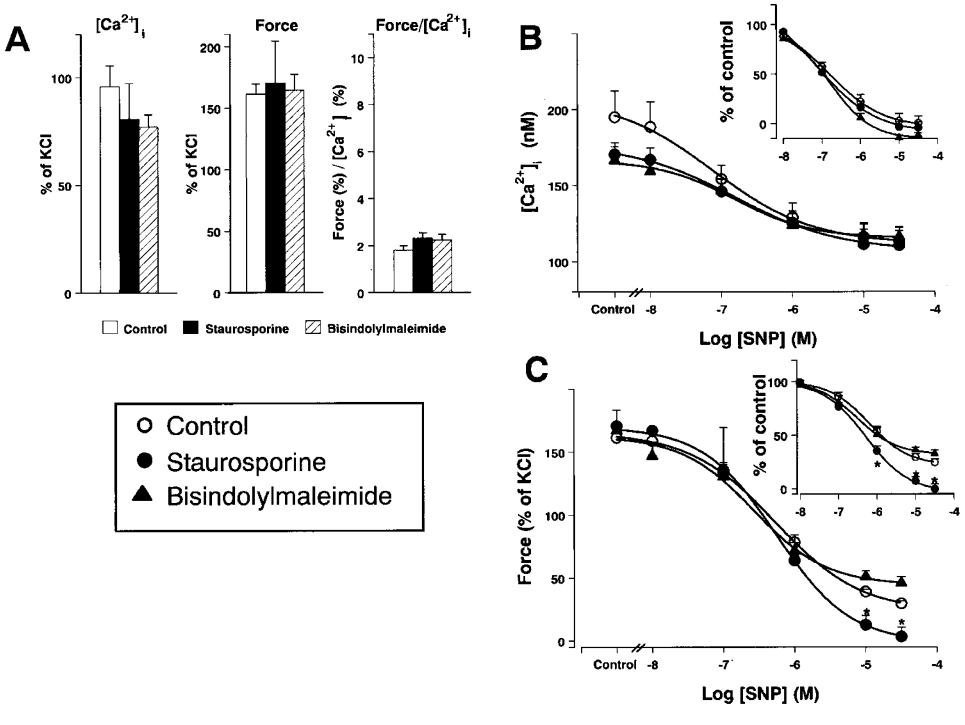

Figure 4. Effects of staurosporine (10^{-7} M) and bisindolylmaleimide (10^{-6} M) on the U46619-induced increase in $[Ca^{2+}]_i$ (*A*; *left*), force (*A*; *middle*), and the ratio of both variables (*A*; *right*) and on nitroprusside-induced $[Ca^{2+}]_i$-lowering (*B*) and relaxation (*C*) in endothelium-denuded pulmonary arteries stimulated by U46619. The insets in *B* and *C* show the same results expressed as a percentage of control values before the addition of nitroprusside. The results (mean ± SEM of 5–6 experiments) are expressed as a percentage of the response to 80 mM KCl. * $p < 0.05$ *vs* control.

sponses were observed for the activator of the cAMP pathway, forskolin, in the TXA_2 mimetic- and norepinephrine-stimulated arteries (24). In the present study, we have demonstrated that the TXA_2 mimetic- and PMA plus norepinephrine-induced vasoconstrictions are relatively resistant to vasodilate in response to nitroprusside, although the ability of this NO donor to lower $[Ca^{2+}]_i$ is similar regardless of the vasoconstrictor agonist. Therefore, the difference in the nature of the stimulation only influences the $[Ca^{2+}]_i$-independent component of NO-induced vasodilation. In the case of PMA plus norepinephrine, this difference in the stimulation process could be attributed to the different degree of Ca^{2+} sensitization produced by PMA plus norepinephrine (*i.e.* the contractile responses occurred with very weak changes in $[Ca^{2+}]_i$) compared with norepinephrine alone. In contrast, norepinephrine and TXA_2 produced similar Ca^{2+} sensitization. Therefore, we attempted to further characterize the signaling pathway of TXA_2 involved in the reduced response to NO by using the protein kinase inhibitors staurosporine and bisindolylmaleimide. The nonselective serine/threonine protein kinase inhibitor staurosporine induced no change in the $[Ca^{2+}]_i$-lowering effect of nitroprusside but increased nitroprusside-induced relaxation, suggesting the involvement of a protein kinase. However, several findings suggest that this serine/threonine protein kinase is not protein kinase C: *1*) the TXA_2 mimetic did not produce as high a sensitization as the protein kinase C stimulator PMA (Fig. 2); *2*) the potency (pD_2) of nitroprusside to relax pulmonary arteries was reduced in the TXA_2 mimetic- but not in PMA plus norepinephrine-induced contractions (Table 1); *3*) a clearly different $[Ca^{2+}]_i$–force relationship for the effects of nitroprusside was observed in arteries stimulated by the TXA_2 mimetic and PMA plus norepinephrine (Fig. 3*C*); and *4*) the selective protein kinase C inhibitor bisindolylmaleimide did not modify the relaxant effects of nitroprusside in the TXA_2 mimetic-stimulated arteries (Fig. 4). Thus, the signaling of TXA_2, involving a staurosporine-sensitive but bisindolylmaleimide-insensitive mechanism, reduces the sensitivity of the $[Ca^{2+}]_i$-independent relaxant effects of NO.

In conclusion, the pulmonary vasoconstriction induced by the TXA_2 mimetic U46619 or by activation of protein kinase C is partially resistant to the relaxant responses induced by the NO donor nitroprusside. However, nitroprusside fully reverted the increase in $[Ca^{2+}]_i$ induced by the vasoconstrictor stimuli. Therefore, the resistance to NO/cGMP-induced vasodilation involves Ca^{2+}-independent mechanisms. The different pattern of the NO/cGMP resistance induced by the TXA_2-mimetic compared with that induced by the protein kinase C activator PMA, together with the lack of effect of the selective protein kinase C inhibitor bisindolylmaleimide, indicates that protein kinase C is not involved and suggests a role for other serine/threonine kinase(s).

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