Relaxation of Endothelin-1-Induced Pulmonary Arterial Constriction by Niflumic Acid and NPPB: Mechanism(s) Independent of Chloride Channel Block

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ABSTRACT

We investigated the effects of the Cl\(^{-}\) channel blockers niflumic acid, 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB) and 4,4\(^{-}\)-disothiocyanatostilbene-2,2\(^{-}\}-disulphonic acid (DIDS) on endothelin-1 (ET-1)-induced constriction of rat small pulmonary arteries (diameter 100–400 \(\mu\)m) in vitro, following endothelium removal. ET-1 (30 nM) induced a sustained constriction of rat pulmonary arteries in physiological salt solution. Arteries precontracted with ET-1 were relaxed by niflumic acid (IC\(_{50}\): 35.8 \(\mu\)M) and NPPB (IC\(_{50}\): 21.1 \(\mu\)M) in a reversible and concentration-dependent manner. However, at concentrations known to block Ca\(^{2+}\)-activated Cl\(^{-}\) channels, DIDS (\(\leq\)500 \(\mu\)M) had no effect on the ET-1-induced constriction. Similar results were obtained when pulmonary arteries were preincubated with these Cl\(^{-}\) channel blockers. When L-type Ca\(^{2+}\) channels were blocked by nifedipine (10 \(\mu\)M), the ET-1-induced (30 nM) constriction was inhibited by only 5.8\%. However, niflumic acid (30 \(\mu\)M) and NPPB (30 \(\mu\)M) inhibited the ET-1-induced constriction by \(\sim\)53\% and \(\sim\)60\%, respectively, both in the continued presence of nifedipine and in Ca\(^{2+}\)-free physiological salt solution. The Ca\(^{2+}\) ionophore A23187 (10 \(\mu\)M) also evoked a sustained constriction of pulmonary arteries. Surprisingly, the A23187-induced constriction was also inhibited in a reversible and concentration-dependent manner by niflumic acid (IC\(_{50}\): 18.0 \(\mu\)M) and NPPB (IC\(_{50}\): 8.8 \(\mu\)M), but not by DIDS (\(\leq\)500 \(\mu\)M). Our data suggest that the primary mechanism by which niflumic acid and NPPB inhibit pulmonary artery constriction is independent of Cl\(^{-}\) channel blockade. One possibility is that these compounds may block the Ca\(^{2+}\)-dependent contractile processes.

Although Ca\(^{2+}\)-activated Cl\(^{-}\) channels have been observed in smooth muscle cells in a variety of tissues (see review Large and Wang, 1996), the functional role of Cl\(^{-}\) channels in vascular smooth muscle is still unclear. However, because the equilibrium potential of Cl\(^{-}\) in smooth muscle is regarded to be between −20 and −30 mV (Aickin, 1990), it is generally assumed that activation of Cl\(^{-}\) channels could lead to membrane depolarization and activation of voltage-gated Ca\(^{2+}\) channels (VGCCs), resulting in Ca\(^{2+}\) entry and contraction (Large and Wang, 1996). This is supported by the observation that the Cl\(^{-}\) channel blocker niflumic acid depresses noradrenaline-evoked contraction of rat aorta (Criddle et al., 1996) and by the fact that niflumic acid and DIDS (4,4\(^{-}\)-disothiocyanatostilbene-2,2\(^{-}\)-disulphonic acid) have been shown to inhibit noradrenaline or phenylephrine-induced constriction of rat pulmonary arteries (Wang et al., 1997; Yuan, 1997). In addition, Nelson et al. (1997) showed that the Cl\(^{-}\) channel blockers indanyloxyacetic acid (IAA-94) and DIDS cause hyperpolarization and dilation in pressurized rat cerebral arteries, although they suggested that niflumic acid-sensitive Cl\(^{-}\) channels were unlikely to be involved in this mechanism.

It is well known that endothelin-1 (ET-1) is one of the most potent constrictors of vascular smooth muscle and that it mediates its effects via at least two receptor subtypes, ET\(_{A}\) and ET\(_{B}\) (Yanagisawa et al., 1988; Arai et al., 1990; Leach et al., 1990; Sakurai et al., 1990; Sakamoto et al., 1991; Yoshida et al., 1994; Maguire et al., 1996). In a previous study using the patch-clamp technique, Salter and Kozlowski (1996) showed that ET-1 has three electrophysiological effects on rat pulmonary arterial smooth muscle cells: 1) activation of a Ca\(^{2+}\)-activated Cl\(^{-}\) current (\(I_{\text{Cl(Ca)}}\)); 2) enhancement of a Ca\(^{2+}\)-activated K\(^{+}\) current, both of which are mediated by ET\(_{A}\) receptors; and 3) inhibition of the delayed rectifier K\(^{+}\) current (\(I_{\text{KV}}\)), which is mediated by ET\(_{B}\) receptors. Of these three effects, activation of \(I_{\text{Cl(Ca)}}\) and inhibition of \(I_{\text{KV}}\) have the capacity to induce membrane depolarization in rat small pulmonary arteries. More specifically, ET-1 may induce constriction of pulmonary arterial myocytes, at least in part, by activating \(I_{\text{Cl(Ca)}}\), which may in turn produce membrane depolarization and Ca\(^{2+}\) influx through VGCCs. Several studi

ABBREVIATIONS: ET-1, endothelin-1; STXS6c, sarafotoxin S6c; NPPB, 5-nitro-2-(3-phenylpropylamino)-benzoic acid; DIDS, 4,4\(^{-}\)-disothiocyanatostilbene-2,2\(^{-}\}-disulphonic acid; VGCC, voltage-gated Ca\(^{2+}\); \(I_{\text{Cl(Ca)}}\), Ca\(^{2+}\)-activated Cl\(^{-}\) current; \(I_{\text{KV}}\), delayed rectifier K\(^{+}\) current.
ies have shown that niflumic acid, 5-nitro-2-(3-phenylproplylamino)-benzoic acid (NPPB), and DIDS block activation of $I_{\text{Cl(Ca)}}$ in isolated pulmonary artery smooth muscle cells (e.g., Clapp et al., 1996; Salter and Kozlowski, 1996). However, the relationship between the observed activation of $I_{\text{Cl(Ca)}}$ and the constriction induced by ET-1 has yet to be studied in intact pulmonary arteries.

The purpose of this study was to examine the role of $I_{\text{Cl(Ca)}}$ activation in ET-1-induced constriction of rat small pulmonary arteries. All experiments were conducted using endothelium-denuded arteries to obviate the involvement of the endothelium. Our data suggest that activation of $I_{\text{Cl(Ca)}}$ is unlikely to play an essential role in ET-1-induced constriction of pulmonary arteries. In addition, our data demonstrate that niflumic acid and NPPB may relax these arteries by mechanism(s) independent of Cl channel inhibition.

**Materials and Methods**

**Tissue Isolation and Tension Measurement.** Male Wistar rats (250–350 g) were sacrificed with an overdose of pentobarbitone (40 mg/kg b.wt., i.p.). The heart and lungs were removed and then the small pulmonary arteries (i.d. 100–400 μm; length 1–2 mm) were dissected free of the surrounding connective tissue and adventitia. After the dissection, two arteries were immediately mounted on a Mulvany and Halpern-type Myograph (Mulvany and Halpern, 1977; JP Trading, Aarhus, Denmark). Additional arteries were stored in a refrigerator at 4°C until needed. The arterial endothelium was removed by rubbing the inner surface with thin surgical thread (o.d. −0.1 mm). Removal of the endothelium was assessed by the ability of 10 μM acetylcholine to relax constrictions induced by 1 μM phenylephrine. The experimental bath (volume 10 ml) was maintained at 37 ± 1°C. Physiological salt solution (PSS) contained: NaCl, 145.0 mM; KCl, 5.4 mM; CaCl₂, 1.8 mM; MgCl₂, 1.0 mM; HEPES, 5.0 mM; and glucose, 10.0 mM, pH adjusted to 7.4 with NaOH. For Ca²⁺-free experiments, CaCl₂ was replaced with equimolar MgCl₂ and 1 mM EGTA was added to the solution. Isometric muscle tension was sampled and analyzed by a Macintosh computer (SE 30 and Power Macintosh 7200/90) via a Myo-Interface (model 500A; JP Trading, Aarhus, Denmark) and a MacLab interface (AD Instruments Pty Ltd, Castle Hill, Australia). Pulmonary artery rings were subjected to an initial tension of 25 mm Hg (3.3 kPa). Before experiments were carried out, arteries were constricted by high K (50 mM) several times to verify that the tissue was viable and to allow for tissue equilibration. All drugs were applied directly to the bath solution, of niflumic acid (100 mM) and NPPB (10 mM) were made up in pure water with approximately equivalent amounts of NaOH. Solutions of niflumic acid (100 mM) and NPPB (10 mM) were made up in pure water with approximately equivalent amounts of NaOH. Stock solutions of A23187 (100 mM), nifedipine (100 mM), and DIDS (100 mM) were dissolved in DMSO. DIDS was prepared before every experiment. The concentration of DMSO was 0.5% throughout in this study. Vehicle controls showed that at the highest concentrations used, DMSO had no effect on resting tone or on the ET-1-induced constriction.

**Results**

**Effect of Niflumic Acid, NPPB, and DIDS on Arteries Preconstricted with ET-1.** Initial experiments were carried out to obtain a concentration-response curve for ET-1. This revealed that a submaximal response would be elicited by 30 nM ET-1, the tension developed being 10.9 ± 2.1 mN/mm² ($n = 6$). This concentration of ET-1 was routinely used in the experiments described below, unless stated otherwise.

ET-1 (30 nM) produced two types of constriction in rat small pulmonary arteries. In 40 out of 53 preparations a slowly developing constriction was induced, which reached a maximum that was sustained for the duration of exposure to ET-1 (up to 2 h; Fig. 1a). In the remaining preparations ($n = 13$), the ET-1-induced constriction reached a maximum and then declined by 14.2 ± 3.1% to a plateau (Fig. 4b). The ET-1-induced constriction reversed on washing, but the rate of reversal was slow and incomplete even after 5 h.

Figure 1, b and c show the effects of cumulative application of nitric acid (1–300 μM; $n = 7$) and NPPB (0.3–100 μM; $n = 8$) on arteries preconstricted with 30 nM ET-1. Clearly, niflumic acid and NPPB induced a concentration-dependent relaxation. The inhibitory effect of both niflumic acid and NPPB was reversed on washing, although it took over 1 h to restore the tension to control levels (Fig. 1, b and c, inset). The effects of a third and structurally distinct Cl⁻ channel blocker were, however, quite different. Figure 1d shows that application of DIDS (10–500 μM) had little or no effect on the ET-1-induced constriction ($n = 5$). Figure 2 shows a plot describing the concentration-relaxation relationship for nitric acid, NPPB, and DIDS ($n = 5–8$). The data for niflumic acid and NPPB, but not DIDS, were well fitted by the Hill equation (see Materials and Methods), which gave an $R_{\text{max}}$, IC₅₀, and Hill coefficient of 68.4 ± 2.1%, 35.8 ± 5.5 μM, and 2.2, respectively, for niflumic acid, and 79.8 ± 5.7%, 21.1 ± 1.1 μM, and 3.5, respectively, for NPPB.

**Effect of Pretreatment with Niflumic Acid, NPPB, and DIDS on ET-1-Induced Constriction.** Application of Cl⁻ channel blockers to ET-1 preconstricted arteries gives (see above) an indication of their effects on the steady-state...
constriction, at which point the Ca
$^{2+}$-activated Cl
$^{-}$ current ($I_{\text{Cl(Ca)}}$) may have less influence than at the point of initiation of constriction, especially when one considers that ET-1 rapidly activates $I_{\text{Cl(Ca)}}$ in isolated pulmonary artery smooth muscle (~30 s; Salter and Kozlowski, 1996). We therefore studied the effects of preincubating the arteries with the Cl
$^{-}$ channel blockers on the initial rising phase of a subsequent constriction to ET-1. Arteries were pretreated with niflumic acid, NPPB, or DIDS, respectively, for 10 min before ET-1 (30 nM) application. In general, the Cl
$^{-}$ channel blockers had little effect on the resting tone, although two out of six arteries showed a small increase in resting tone following the application of 100 μM NPPB (Fig. 3b). Pretreatment with niflumic acid (10, 30, and 100 μM) and NPPB (3, 10, 30, and 100 μM) attenuated ET-1-induced muscle constriction in a concentration-dependent and reversible manner (Fig. 3, a and b). These results are summarized in Table 1. Neither niflumic acid nor NPPB had any effect on the time to half-maximum ($T_{1/2}$) for the ET-1-induced constriction when compared with control. Furthermore, the degree of inhibition of the maximum constriction observed was similar to that observed when these blockers were tested against arteries preconstricted with ET-1 (Fig. 3d). In contrast, pretreatment with 500 μM DIDS had no effect on the maximum response to ET-1 but the $T_{1/2}$ was reduced from 192.5 ± 55.8 s to 68.2 ± 16.6 s (n=6). This is, however, likely due to a nonspecific effect, because block of $I_{\text{Cl(Ca)}}$ would be expected to slow the rising phase of constriction.

**Effect of Ibtx and Glibenclamide on Niflumic Acid and NPPB-Mediated Relaxation.** Previous reports have suggested that niflumic acid may activate large conductance Ca
$^{2+}$-activated K
$^{+}$ (BKCa) channels (Ottolia and Toro, 1994; Greenwood and Large, 1995) and that NPPB may also activate ATP-sensitive K
$^{+}$ (KATP) channels (Kirkup et al., 1996). We therefore investigated whether the inhibitory effects of niflumic acid and NPPB were due to BKCa or KATP channel activation by selectively blocking these channels with Ibtx and glibenclamide, respectively. Figure 4a shows that in ET-1 (30 nM) preconstricted arteries 100 nM Ibtx failed to reverse the relaxation induced by 100 μM niflumic acid (n=8). Ibtx itself had no effect on resting muscle tone (Fig. 4a, inset), whereas in two out of four muscles preconstricted with ET-1, Ibtx produced further constriction (Fig. 4b). Preapplication of Ibtx (Fig. 4b; n=4) or glibenclamide 30 μM (Fig. 4c; n=4) also failed to prevent the relaxation observed with 100 μM NPPB. It should be noted that glibenclamide itself (30 μM) and the combined application of glibenclamide and Ibtx (100 nM) had no effect on resting muscle tone (Fig. 4c, inset). These data suggest that the relaxations induced by niflumic acid and NPPB occur independently of either BKCa or KATP channel activation.
Niflumic acid

(i) Niflumic acid 10 μM

(ii) 100 μM

ET-1 30 nM

Wash

5 mN/mm²

30 min

NPPB

(i) NPPB 3 μM

(ii) 100 μM

ET-1 30 nM

Wash

5 mN/mm²

30 min

d

DIDS 500 μM

ET-1 30 nM

Wash

% of control

Concentration (μM)

Effect of Niflumic Acid, NPPB, and DIDS on A23187-Induced Constriction. To establish whether the relaxation induced by niflumic acid and NPPB was mediated via inhibition of signal transduction before or subsequent to any elevation of the intracellular free Ca²⁺ concentration, the effects of these agents on constriction induced by the Ca²⁺ ionophore, A23187, were examined. For the purposes of these studies, arteries were first pretreated with A23187 (10 μM) in Ca²⁺-free PSS for 5 min followed by application of Ca²⁺ to yield a final Ca²⁺ concentration of 2 mM (see Materials and Methods). A23187 itself had virtually no effect on the resting tone in Ca²⁺-free PSS. However, Fig. 6a shows that when Ca²⁺ was readmitted in the continued presence of A23187 (10 μM), pulmonary arteries constricted, generating a maximum tension of 9.4 ± 2.0 mN/mm² (n = 4), relaxed pulmonary arteries preconstricted with 30 nM ET-1 (Fig. 6, b-d). Figure 7 shows the concentration-relaxation relationship for niflumic acid, NPPB, and DIDS. The data for niflumic acid and NPPB, but not DIDS, fit well to the Hill equation (see Materials and Methods), which gave values for $R_{max}$, IC₅₀, and the Hill coefficient of 75.1 ± 4.3%, 18.0 ± 2.3 μM, and 1.3, respectively, for niflumic acid and 72.1 ± 8.2%, 8.8 ± 2.0 μM, and 1.8, respectively, for NPPB.

Nonselective Relaxation Effect of Niflumic Acid on Sarafotoxin S6c (STXs6c), UTP-, and Angiotensin II-Induced Constriction. Figure 8a shows that 30 pM STXs6c, a selective ET₁ receptor agonist (Williams et al., 1991), induced a constriction of 12.2 ± 1.1 mN/mm² (n = 6), equivalent in magnitude to that produced by 30 nM ET-1. In contrast to the constriction induced by ET-1, the STXs6c-induced constriction was not sustained but gradually decayed with a $T_{1/2}$ of 58.4 ± 7.0 min. To avoid overestimating the magnitude of any relaxation effects, we therefore used only a single concentration of the chloride channel blockers. Figure 8, b-d show that nifedipine (10 μM) partially relaxed arteries preconstricted with 30 pM STXs6c to 75.0 ± 2.8% of

6.2 and 23.0 ± 1.5% of the control, respectively (Fig. 5, ai, aii, and c). These results suggest that relaxation induced by niflumic acid and NPPB are unlikely to be due to the inhibition of Ca²⁺ influx via VGCCs. We also investigated the effect of nifedipine (10 μM) pretreatment on the ET-1-induced constriction (Fig. 5aiii). Surprisingly, nifedipine (10 mM) did not alter the maximum tension or the $T_{1/2}$ of the ET-1-induced constriction. The values for maximum tension were 11.0 ± 0.9 and 10.9 ± 2.1 mN/mm² and for $T_{1/2}$ were 236.7 ± 36.5 and 192.5 ± 55.8 in the presence and absence of nifedipine (10 μM), respectively. This suggests that the contribution of voltage-dependent Ca²⁺ influx is small even during the rising phase of the response.

Support for the above conclusion was provided by experiments carried out in Ca²⁺-free PSS (containing 1 mM EGTA), i.e., in the absence of any Ca²⁺ influx. In these experiments, 100 nM ET-1 was used to preconstrict the pulmonary arteries, because insufficient tension could be generated by 30 nM ET-1. The maximum constriction evoked by 100 nM ET-1 was 3.7 ± 0.6 mN/mm². Figure 5, b and c show that even in the absence of induced Ca²⁺ influx, both niflumic acid (100 μM, n = 5) and NPPB (30 μM, n = 5) relaxed the constriction induced by ET-1 (100 nM) to 41.5 ± 9.6 and 40.1 ± 1.3% of control, respectively.

**Effect of Niflumic Acid and Ca²⁺-Free PSS on Niflumic Acid and NPPB-Mediated Relaxation.** One of the most common mechanisms by which an agonist causes constriction of vascular smooth muscle is by inducing membrane depolarization and Ca²⁺ influx via VGCCs. If ET-1-mediated activation of Cl⁻ channels produces sufficient membrane depolarization to open VGCCs, and niflumic acid and NPPB induce relaxation by blocking Cl⁻ channels, then the relaxation induced by Cl⁻ channel blockers should be similar to that produced by Ca²⁺ channel antagonists such as nifedipine. This assumption would also apply if niflumic acid and NPPB had the capacity to block VGCCs directly. Figure 5, a and c show that application of nifedipine (10 μM) reduced constriction induced by 30 nM ET-1 to only 94.2 ± 2.3% of the control (n = 9). Moreover, application of either niflumic acid (30 μM, n = 5) or NPPB (30 μM, n = 4) in the continued presence of nifedipine caused a further relaxation to 46.9 ±...
control (n = 8). Even in the continued presence of nifedipine (10 μM), however, both niflumic acid (30 μM; Fig. 8b) and NPPB (30 μM; Fig. 8d) relaxed pulmonary arteries preconstricted with STXS6c to 16.0 ± 5.1% (n = 4) and 5.6 ± 2.3% (n = 4) of control, respectively (Fig. 8d). In contrast, DIDS (500 μM) had no effect on the STXS6c-induced constriction (n = 4; data not shown).

To verify whether the effects of niflumic acid and NPPB used in this study were agonist specific or not, we examined their effects on UTP- and angiotensin II-induced constriction. UTP (1 mM) induced a sustained constriction of rat small pulmonary arteries. Figure 9, a and c show that niflumic acid (30 μM) and NPPB (30 μM) reduced the UTP-induced constriction to 66.1 ± 5.5% of control (n = 6). Their potency was higher than that of nifedipine, which reduced UTP-induced constriction to 66.1 ± 13.8% of control (n = 5, data not shown). Niflumic acid and NPPB also relaxed constriction evoked by 30 nM angiotensin II (Fig. 9, b and d). Nicardipine (1 μM) reduced the angiotensin II-induced constriction to 50.8 ± 6.3% of control (n = 10). The nicardipine-insensitive component of the angiotensin II-induced constriction was significantly inhibited by niflumic acid (n = 6) and NPPB (n = 4) to 3.2 ± 1.3 and 2.7 ± 1.6% of control in rat small pulmonary arteries.

**Discussion**

The purpose of the present study was to determine whether Cl− channel blockers play a significant role in ET-1- and STXS6c-induced constriction of rat small pulmonary arteries. Our findings suggest that Cl− channel activation does not play a significant role in mediating the constriction evoked by either ET-1 or STXS6c in this tissue. Furthermore, we conclude that the Cl− channel blockers niflumic acid and NPPB relax the ET-1- and STXS6c-induced constriction of pulmonary arteries by a mechanism independent of Cl− channel inhibition.

Cumulative application of niflumic acid and NPPB relaxed rat pulmonary arteries preconstricted with ET-1 in a concentration-dependent and reversible manner. The threshold concentration of the induced relaxation was approximately 10 μM for both niflumic acid and NPPB. This is within the concentration range of niflumic acid used to confirm the involvement of I_{Ca,Cl} in agonist-induced constriction of pul-

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**Table 1**

Effect of pretreatment with Cl− channel blockers on 30 nM ET-1-induced constriction

<table>
<thead>
<tr>
<th></th>
<th>Maximum tension in</th>
<th>Maximum tension after wash</th>
<th>% of control</th>
<th>T_{1/2} Max (A)</th>
<th>No.</th>
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<tr>
<td></td>
<td>mN/mm²</td>
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<tr>
<td>Control</td>
<td>10.9 ± 2.1</td>
<td>12.3 ± 3.2</td>
<td>91.3 ± 6.3</td>
<td>192.5 ± 55.8</td>
<td>6</td>
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<td>Niflumic acid (μM)</td>
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<tr>
<td>10</td>
<td>10.5 ± 1.9</td>
<td>10.8 ± 1.3</td>
<td>65.9 ± 12.2</td>
<td>130.9 ± 31.4</td>
<td>6</td>
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<tr>
<td>30</td>
<td>5.6 ± 1.1^{a,c}</td>
<td>10.1 ± 1.6</td>
<td>41.4 ± 6.5</td>
<td>135.0 ± 14.8</td>
<td>6</td>
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<td>NPPB (μM)</td>
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<td>3</td>
<td>9.2 ± 2.1</td>
<td>9.8 ± 2.4</td>
<td>95.0 ± 2.9</td>
<td>138.0 ± 19.9</td>
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<tr>
<td>10</td>
<td>8.4 ± 1.4</td>
<td>10.7 ± 1.0</td>
<td>77.3 ± 8.8</td>
<td>206.0 ± 41.7</td>
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<tr>
<td>30</td>
<td>4.3 ± 0.6^{b,d}</td>
<td>9.4 ± 1.2</td>
<td>45.3 ± 3.6</td>
<td>118.2 ± 16.0</td>
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</tr>
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<td>100</td>
<td>2.6 ± 1.0^{b,d}</td>
<td>8.1 ± 1.1</td>
<td>27.5 ± 9.0</td>
<td>249.1 ± 17.5</td>
<td>6</td>
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<tr>
<td>DIDS 500 μM</td>
<td>12.7 ± 1.8</td>
<td>11.7 ± 1.7</td>
<td>109.7 ± 4.4</td>
<td>68.2 ± 16.8</td>
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* P < .05 and ** P < .01 compared with control (ET-1, 30 nM).

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**Fig. 4.** The K+ channel blockers Ibtx and glibenclamide do not block the niflumic acid- and NPPB-induced relaxation. a, application of Ibtx (100 nM) failed to reverse the relaxation induced by niflumic acid (100 μM). b, preapplication of Ibtx (100 nM) failed to prevent the NPPB-induced relaxation. c, preapplication of glibenclamide (30 μM) failed to prevent NPPB-induced relaxation. Insets show that 100 nM Ibtx (b) and 30 μM glibenclamide (c) had no effect on resting tension. In a, b, and c the arteries were preconstricted with ET-1 (30 nM). The vertical arrows and horizontal bars indicate the point and duration of drug application, respectively. The horizontal and vertical bars in all scales show 20 min and 5 mN/mm², respectively. All of the traces in this figure were obtained from different preparations. Note: as shown in b, application of Ibtx to the muscle preconstricted with ET-1 produced a further constriction in two out of four preparations.
monary arteries in previous studies (Wang et al. 1997; Yuan, 1997). Another Cl\textsuperscript{-} channel blocker, DIDS, was ineffective in spite of the fact that it has previously been shown to block \(I_{\text{Cl(Ca)}}\) in smooth muscle cells from a variety of tissues including rat pulmonary arteries (Amédée et al., 1990; Clapp et al., 1996; Large and Wang, 1996; Yuan, 1997). In contrast, however, DIDS has been shown to relax the phenylephrine-induced constriction of rat small pulmonary arteries (Yuan, 1997).

**Fig. 5.** Effects of niflumic acid and NPPB on ET-1-induced constriction in the presence of nifedipine and in Ca\textsuperscript{2+}-free PSS. a, Nifedipine (10 \(\mu M\)) partially relaxed the ET-1-induced (30 nM) constriction. In the continued presence of nifedipine (10 \(\mu M\)), application of 30 \(\mu M\) niflumic acid (ai) and 30 \(\mu M\) NPPB (aii) induced further relaxation. aiii, pretreatment with nifedipine (10 \(\mu M\)) had no effect on the ET-1-induced constriction. bi, niflumic acid (100 \(\mu M\)) and bii, NPPB (30 \(\mu M\)) relaxed arteries preconstricted with ET-1 (100 nM) in Ca\textsuperscript{2+}-free PSS. The vertical arrows and horizontal bars indicate the point and duration of drug application, respectively. The horizontal and vertical bars in all scales show 20 min and 5 mN/mm\textsuperscript{2}, respectively. All of the traces in this figure were obtained from different preparations. c, bar graph showing mean ± S.E.M. (\(n = 4–5\)) for the relaxation of arteries preconstricted with 30 nM ET-1 by 10 \(\mu M\) nifedipine alone (open columns), by 30 \(\mu M\) niflumic acid (solid column), and by 30 \(\mu M\) NPPB (solid column) in the continued presence of 10 \(\mu M\) nifedipine in normal PSS. And the relaxation of arteries preconstricted 100 nM ET-1 by 100 \(\mu M\) niflumic acid and 30 \(\mu M\) NPPB in Ca\textsuperscript{2+}-free PSS (hatched columns). **\(P < .01\) statistical difference with nifedipine-treated tension. #\(P < .05\) statistical difference with control.

**Fig. 6.** Effects of niflumic acid, NPPB, and DIDS on the A23187-induced constriction. a, typical constriction produced by A23187 (10 \(\mu M\)) in the presence of 2 mM extracellular Ca\textsuperscript{2+} (see Materials and Methods for detail). Niflumic acid (b) and NPPB (c) relaxed arteries preconstricted with 10 \(\mu M\) A23187 in a concentration-dependent manner but DIDS (d) did not. Insets in b and c show reversal of both niflumic acid- and NPPB-induced relaxation on washing. The vertical arrows and horizontal bars indicate the point and duration of Ca\textsuperscript{2+} and drug application, respectively. The horizontal and vertical bars in all scales show 30 min and 5 mN/mm\textsuperscript{2}, respectively. All of the traces in this figure were obtained from different preparations.
arteries were preincubated with the Cl\(^{-}\) channel blockers before ET-1 application. In this series of experiments, niflumic acid and NPPB failed to alter the \(T_{1/2}\) for ET-1-induced constriction. However, the ET-1-induced constriction was inhibited to a similar degree that observed in arteries preconstricted with ET-1. Again, in marked contrast, preincubation with DIDS did not inhibit the ET-1-induced constriction of rat pulmonary arteries: observations that suggest that \(I_{\text{Cl(Ca)}}\) activation is not involved in ET-1-induced constriction and that NPPB and niflumic acid may inhibit constriction through some other means. In support of this notion, the effects of niflumic acid and NPPB seem to be unrelated to the nature of the agonist used and, therefore, the membrane receptor activated since niflumic acid and NPPB relax STX6c-\(-\), UTP-, and angiotensin II-induced constriction in rat small pulmonary arteries.

Additional support for a mechanism distinct from the block of \(I_{\text{Cl(Ca)}}\) comes from the markedly different recovery times for the effects of niflumic acid and NPPB. Generally, it takes approximately 5 min for complete recovery of \(I_{\text{Cl(Ca)}}\) after washing off niflumic acid and NPPB (Kirkup et al., 1996; Salter and Kozlowski, 1997; Wang et al., 1997), whereas in the present investigation it took around 1 h to recover the ET-1-induced constriction after washing off these Cl\(^{-}\) channel blockers. This slow time course of recovery from inhibition by niflumic acid has also been observed in studies of the role of \(I_{\text{Cl(Ca)}}\) in constriction evoked by other agonists (Criddle et al., 1996; Yuan, 1997), where complete recovery was not observed following 25 min of washing. Of further interest in this respect are the findings of Wang et al. (1997), who showed that niflumic acid not only inhibits agonist-induced pulmonary artery constriction, but also inhibits constriction induced by elevating extracellular K\(^{+}\) (Wang et al., 1977). These authors conclude that inhibition of the K\(^{+}\)-induced constriction was due either to the block of some contribution of \(I_{\text{Cl(Ca)}}\) to depolarization evoked contraction or to nonspecific effects of niflumic acid. In contrast, Criddle et al. (1996) observed no inhibition of K\(^{+}\)-induced constriction of rat aorta with 10 \(\mu\)M niflumic acid, and Yuan (1997) showed that niflumic acid (50 \(\mu\)M) had no effect on high K\(^{+}\)-induced contraction of rat pulmonary arteries. These discrepancies may be due to the incubation time required (~5 min) before the onset of niflumic acid-induced inhibition.

It is arguable that the niflumic acid- and NPPB-induced relaxation of preconstricted pulmonary arteries could be mediated via the activation of K\(^{+}\) channels. Previous electrophysiological studies (Ottolia and Toro, 1994; Greenwood and Large, 1995) have demonstrated that the fenamate family, which includes niflumic acid, can activate BK\(_{\text{Ca}}\) channels. This effect is also exhibited by NPPB, which has been shown to activate K\(_{\text{ATP}}\) channels in rat portal vein (Kirkup et al., 1996). Such effects could produce membrane hyperpolarization, resulting in closure of VGCCs and ultimately relaxation of smooth muscle. However, in the present investigation, Ibtx, one of the most potent and specific BK\(_{\text{Ca}}\) channel block-

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**Fig. 7.** Concentration-relaxation relationship for niflumic acid, NPPB, and DIDS in arteries preconstricted with A23187. Each point indicates the averaged data obtained from Fig. 6 for niflumic acid (C), NPPB (D), and DIDS (L), whereas the vertical bars show the S.E.M. (n = 4–6). The horizontal and vertical axes show drug concentration and percentage of control (see Fig. 2), respectively. The solid lines for niflumic acid and NPPB show the best fit to the Hill equation (see Materials and Methods), which gave values for \(R_{\text{max}}\), \(I_{\text{C(50)}}\), and Hill coefficient of 75.1 ± 4.3\%, 18.0 ± 2.3 \(\mu\)M, and 1.3, respectively, for niflumic acid and 72.1 ± 8.2\%, 8.8 ± 2.0 \(\mu\)M, and 1.5, respectively, for NPPB.

**Fig. 8.** Effect of niflumic acid, NPPB, and nifedipine on STX6c-induced constriction. a, a typical constriction to STX6c (30 \(\mu\)M). Nifedipine (10 \(\mu\)M) partially relaxed arteries preconstricted with 30 \(\mu\)M STX6c (b and c). b, niflumic acid (30 \(\mu\)M) and c, NPPB (30 \(\mu\)M) caused a further relaxation in the continued presence of nifedipine (10 \(\mu\)M). The vertical arrows and horizontal bars indicate the point and duration of drug application, respectively. d, bar graph showing mean ± S.E.M. (n = 4–6) for the percentage of control of 10 \(\mu\)M nifedipine alone (open columns), 30 \(\mu\)M niflumic acid (solid column), and 30 \(\mu\)M NPPB (solid column) in the continued presence of nifedipine (10 \(\mu\)M). The horizontal and vertical bars in all scales show 30 min and 5 mN/mm\(^2\), respectively. All of the traces in this figure were obtained from different preparations. **\(P < .01\) statistical difference with nifedipine.
ers, had no effect on the relaxation induced by niflumic acid or NPPB. Moreover, the NPPB-induced relaxation was unaffected by glibenclamide. Thus, it is unlikely that the niflumic acid and NPPB-induced relaxation of pulmonary arterial smooth muscle involves activation of either BK<sub>Ca</sub> or K<sub>ATP</sub> channels.

It is possible that the niflumic acid- and NPPB-induced relaxation of preconstricted rat pulmonary arteries involves either direct or indirect inhibition of VGCCs. To test this possibility, we studied the effects of nifedipine on ET-1-induced constriction and on the inhibitory action of niflumic acid and NPPB. However, we discovered that nifedipine had only a marginal effect on the ET-1-induced constriction, and that application of niflumic acid and NPPB in the continued presence of nifedipine produced a much greater degree of relaxation. Although it is clear from our previous studies (Salter and Kozlowski, 1996) and those of others (Miyoshi et al., 1983; Leach et al., 1992; Evans et al., 1996) that ET-1-induced membrane depolarization in smooth muscle cells may activate L-type Ca<sup>2+</sup> channels, it is unlikely that this mechanism plays a primary role in mediating ET-1-induced constriction of rat pulmonary arteries. Furthermore, when pulmonary arteries were bathed in Ca<sup>2+</sup>-free PSS to eliminate the possibility that ET-1-stimulated Ca<sup>2+</sup> influx contributed to the constriction, niflumic acid and NPPB were still able to produce a substantial relaxation of ET-1-preconstricted arteries. These data, however, do not rule out the possibility that niflumic acid and NPPB may act as antagonists of ET receptors, or inhibitors of ET-receptor mediated activation of second messenger pathways. We therefore used the Ca<sup>2+</sup> ionophore, A23187, to induce constriction in a manner that would bypass receptor activation and any receptor-dependent signal transduction pathways. Surprisingly, niflumic acid and NPPB, but not DIDS, relaxed pulmonary arteries preconstricted with A23187 to a similar degree to that following ET-1-induced constriction. Comparison of the Hill coefficients for niflumic acid (~1) and NPPB (~2) under these conditions, suggests that a less-complex mechanism than that observed in ET-1-preconstricted arteries may be involved. In fact, niflumic acid may affect only one Ca<sup>2+</sup>-dependent contractile process, given that the fitted Hill coefficient is close to unity. Taken together, these observations suggest that niflumic acid and NPPB inhibit contraction downstream of Ca<sup>2+</sup> influx and intracellular Ca<sup>2+</sup> release pathways.

Our observations clearly differ from a recent study in rat cerebral arteries, which showed that IAA-94 and DIDS, but not niflumic acid, cause hyperpolarization and dilatation of pressurized (80 mm Hg) cerebral arteries (Nelson et al., 1997). Nelson et al. (1997) concluded that the observed vasodilation is due to Cl<sup>-</sup> channel block, and that Cl<sup>-</sup> channels are active and contribute to the membrane potential in cerebral artery smooth muscle under quasi-physiological transmural pressure (around 80 mm Hg). Furthermore, they conclude that the inhibition of Cl<sup>-</sup> channels by IAA-94 or DIDS promotes the observed membrane hyperpolarization, which would lead to the closure of VGCCs, reduced Ca<sup>2+</sup> influx, and dilation. Our findings suggest that neither I<sub>Cl(Ca)</sub> nor any other DIDS-sensitive Cl<sup>-</sup> current is active in pulmonary artery smooth muscle under the quasi-physiological transmural pressures (25 mm Hg) used in the present study (because DIDS had no effect on resting or ET-1-induced tone). It is possible that these discrepancies may be explained by differences in the basic physiology of pulmonary and cerebral blood vessels. Indeed, under physiological conditions, the intravascular pressure of pulmonary arteries is markedly lower (15–30 mm Hg) than that of cerebral arteries (Grover et al., 1983; Leach et al., 1992; Evans et al., 1996). Furthermore, although previous electrophysiological studies have demonstrated that ET-1 may activate I<sub>Cl(Ca)</sub> and thereby promote membrane depolarization in isolated rat pulmonary artery smooth muscle cells, it is unlikely that this depolarization plays an important role in the ET-1-induced constriction. However, it is clear that Cl<sup>-</sup> channels may play some role in mediating pulmonary artery constriction in response to other agonists (Wang et al. 1997; Yuan, 1997).
In conclusion, we propose that Cl\textsuperscript{−} channel activation does not play an essential role in either ET-1- or STXS6c-induced constriction of rat small pulmonary arteries. Our findings also suggest that, at least in rat pulmonary arteries, the Cl\textsuperscript{−} channel blockers niflumic acid and NPPB, but not DIDS may induce a vasorelaxation by inhibiting Ca\textsuperscript{2+}-dependent activation of the contractile process. This result raises serious doubts over the suitability of agents like niflumic acid and NPPB as pharmacological tools for examining Cl\textsuperscript{−} channel involvement in physiological processes in the absence of rigorous control experiments.

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