Inhibitors of myosin light chain kinase and phosphodiesterase reduce ventilator-induced lung injury

JAMES C. PARKER (With the Technical Assistance of Sherri L. Martin)
Department of Physiology, University of South Alabama, Mobile, Alabama 36688
Received 24 February 2000; accepted in final form 1 June 2000

Parker, James C. Inhibitors of myosin light chain kinase and phosphodiesterase reduce ventilator-induced lung injury. J Appl Physiol 89: 2241–2248, 2000.—Alveolar overdistension due to high peak inflation pressures (PIP) is associated with an increased capillary filtration coefficient (Kfc). To determine which signal pathways contribute to this injury, we perfused isolated rat lungs with 5% bovine albumin in Krebs solution and measured Kfc after successive 30-min periods of ventilation with peak inflation pressures (PIP) of 7, 20, 30, and 35 cmH2O. In a high-PIP control group, Kfc increased significantly after ventilation with 30 and 35 cmH2O PIP, but significant increases were prevented by treatment with 100 μM trifluoperazine, an inhibitor of Ca2+/calmodulin, 500 nM ML-7, an inhibitor of myosin light chain kinase (MLCK), a combination of isoproterenol (20 μM) and rolipram (10 μM) to enhance intracellular cAMP levels, and a dose of KT-5720 (2 μM), which inhibits MLCK and protein kinase C. These studies suggest that the Ca2+/calmodulin-MLCK pathway augments capillary fluid leak after a modest high-PIP injury and that this is attenuated by kinase inhibition and increased intracellular cAMP.

Pulmonary barotrauma; mechanical ventilation; capillary permeability; rolipram; isoproterenol; calmodulin

RECENT EXPERIMENTAL STUDIES have confirmed the long-standing suspicion of clinicians that the high airway pressures (Paw) used to maintain adequate oxygenation of patients with acute respiratory distress syndrome may augment leakage of fluid and protein from pulmonary capillaries (4, 27). In fact, a recent large-scale clinical trial demonstrated a 22% reduction in mortality in patients with acute respiratory distress syndrome ventilated with 6 ml/kg vs. the conventional 12 ml/kg (3), but the mechanism of the permeability lesion resulting from mechanical injury remains to be elucidated. Past hypotheses regarding the increased microvascular permeability induced by mechanical stress have attributed capillary leak to a passive failure of vascular structures. Shirley et al. (37) proposed the “stretched-pore phenomenon” due to hydrostatic pressure forcing open endothelial junctions to explain the increased fluid and protein flow from peripheral capillaries subjected to high venous pressures (Ppv). More recently, West et al. (42) proposed that “stress failure” of the alveolar capillary basement membrane accounted for the increased capillary leak and hemorrhage at high pulmonary vascular pressures. In contrast, recent evidence from our laboratory suggests that microvascular permeability increases induced by mechanical stress may have a significant active cellular component involving many of the signal transduction pathways involved in receptor ligand-mediated permeability increases (29).

In previous isolated rat lung studies, Parker and Ivey (28) observed that infusion of isoproterenol attenuated the increase in capillary filtration coefficient (Kfc) induced by high Ppv. The Kfc increase at modest increases in Ppv was attenuated by 64%, whereas Kfc at higher Ppv was attenuated by only 36%. Even though capillary rupture and hemorrhage were apparent in these lungs, extravasation of blood accounted for only ~12% of the 191% total weight gain due to edema accumulation induced by a 31-fold increase in Kfc in the control group. This study indicated a massive fluid flux across capillaries that have an intact basement membrane and an active endothelial cell response. A similar increase in hydraulic conductance in single mesenteric capillaries that occurred at pressures below the threshold for vessel rupture was also reported by Neal and Michel (21). In another isolated rat lung study, we infused gadolinium, an inhibitor of stretch-activated cation channels, and prevented the increase in Kfc after ventilation with the same protocol of high peak inflation pressures (PIP) used in the present study (29). The effectiveness of gadolinium suggests an active, Ca2+-dependent endothelial cell response to mechanical injury similar to receptor ligand- and ischemia-induced increases in permeability observed in endothelial monolayers, single capillaries, and isolated lungs (12, 17).

Mechanical stress-induced permeability responses appear to require Ca2+ entry through mechanogated channels, with formation of transcellular openings rather than intercellular gaps (42). Although the signal transduction pathways for mechanical injury are unknown, a signal cascade has been described by several investigators for the effect of thrombin on cultured endothelial cells that involves an increased intracellular...
lar Ca$^{2+}$, activation of Ca$^{2+}$/calmodulin (CaM), activation of myosin light chain kinase (MLCK), phosphorylation of myosin light chains, and resultant endothelial cell contraction to increased barrier permeability (7, 17, 20, 35, 44). An increased intracellular cAMP has been shown to attenuate these receptor ligand-mediated increases in vascular permeability in endothelial monolayers (28), but the contribution of cAMP and its effector, protein kinase A, to ventilator-induced lung injury has not been characterized (18, 28).

The purpose of the present study was to determine whether pharmacological inhibitors designed to inhibit steps in the Ca$^{2+}$/CaM-MLCK pathway or agents that increase intracellular cAMP would decrease the vascular leak due to ventilator-induced lung injury. Inhibition of Ca$^{2+}$/CaM with trifluoperazine (TFP) or inhibition of MLCK with ML-7 or KT-5720 attenuated the increase produced by high-PIP ventilation. Likewise, the combination of isoproterenol and rolipram to increase cAMP in cells by enhancing production and reducing degradation of cAMP ablated the high-PIP-induced $K_{fc}$ increase.

**METHODS**

**Isolated Rat Lung Preparation**

The isolated rat lung preparation has been previously described (28, 29). Briefly, male Charles River CD rats, weighing 306–476 g, were anesthetized with pentobarbital sodium (65 mg/kg ip), the trachea was cannulated, and the rats were ventilated with 20% O$_2$ and 5% CO$_2$ by use of a rodent ventilator (model 683, Harvard, South Natick, MA) with a tidal volume of 2.5 ml and a positive end-expiratory pressure (PEEP) of 3 cmH$_2$O at 40 breaths/min. This tidal volume resulted in a nominal PIP of 7 cmH$_2$O. The chest was opened, and 300 U of sodium heparin were injected into the right ventricle. The pulmonary artery and left atrium were then cannulated, and the heart and lungs were excised en bloc and suspended from a force transducer. Lungs of four groups were perfused with 5% bovine albumin in Krebs bicarbonate buffer (37°C) at 6 ml/min, and KT-5720-treated lungs were perfused at 6 ml/min-1 g predicted (initial) lung wt$^{-1}$ using a Minipuls 2 roller pump (Gilson, Middleton, WI). Homologous blood (10 ml) was obtained from a donor rat and added to the perfusate to obtain a hematocrit of ~7%, which was measured using a microcentrifuge. Arterial pressure, $P_{pv}$, and $P_{aw}$ were measured using Cobe pressure transducers (Lakewood, CO), and the lung weight was continuously recorded using a polygraph (model 7, Grass, Quincy, MA). Lung weights were measured at the end of each experiment.

$K_{fc}$ and Vascular Resistance

The procedure for measuring $K_{fc}$ in isolated rat lungs has been previously described (28, 29). After an isogravimetric state was obtained, the venous reservoir was raised to obtain the desired $P_{pv}$. For baseline $K_{fc}$ measurements, $P_{pv}$ was increased to 15 cmH$_2$O and maintained for 20 min. Capillary pressure ($P_{pc}$) was measured using the double-occlusion pressure at baseline and at increased $P_{pv}$, and the increase in capillary filtration pressure ($\Delta P_{pc}$) was the change in $P_{pc}$ between vascular pressure states. The rate of weight gain (in g/min) was averaged over the last 2 min of the lung weight-gain curve ($\Delta W_{t-20}$) at increased $P_{pv}$ and used to calculate $K_{fc}$ as follows

$$K_{fc} = \Delta W_{t-20}/\Delta P_{pc}$$  

All $K_{fc}$ values were normalized to 100 g predicted lung weight (PLW), which was based on body weight (BW) as follows

$$PLW = 0.0053 \times BW - 0.48$$

and calculated as ml-min$^{-1}$cmH$_2$O$^{-1}$-100 g PLW$^{-1}$ by assuming a specific gravity of 1.0 for filtered fluid. Perfusion flow ($Q$) was set at 6 ml/min for four groups but was calculated from the PLW and set at 6 ml-min$^{-1}$ g PLW$^{-1}$ for the two KT-5720-treated groups. Pulmonary vascular resistance ($R_z$) was calculated from $P_{pa}$ and $P_{pv}$ as follows

$$R_z = (P_{pa} - P_{pv})/Q$$

**Pharmaceuticals**

The following inhibitor drugs were added to the perfusion system to attain the following concentrations in these studies. TFP (100 μM) was used to inhibit Ca$^{2+}$/CaM. ML-7 (5-iodonaphthalene-1-sulfonyl-homopiperazine, 500 nM) and KT-5720 (67 nM) were used to inhibit MLCK and were obtained from Calbiochem (San Diego, CA) (6). These doses of TFP and ML-7 reversed the increase in $K_{fc}$ induced by ischemia-reperfusion in a similar isolated rat lung preparation (12). Isoproterenol (20 μM) and rolipram (10 μM) were used to induce an increase in intracellular cAMP and were obtained from Sigma Chemical (St. Louis, MO). These doses produce large increases in intracellular cAMP in rat pulmonary artery endothelial cells and reverse the $K_{fc}$ increase induced by ischemia-reperfusion in isolated rat lungs (2, 7, 11).

**Experimental Protocols**

The general protocol consisted of isolation and perfusion of the rat lungs as described above. The general time course of $P_{aw}$ and $P_{pv}$ increases is shown in Fig. 1. After a baseline period of 30 min with ~7 cmH$_2$O PIP, a baseline $K_{fc}$ was determined, then the lungs were ventilated for 30-min periods with 20 cmH$_2$O PIP and 3 cmH$_2$O PEEP, 30 cmH$_2$O PIP and 3 cmH$_2$O PEEP, and 35 cmH$_2$O PIP and 8 cmH$_2$O PEEP, with each ventilation period followed by a $K_{fc}$ measurement for 20 min. Drugs were infused immediately after the baseline $K_{fc}$ measurement. The latter combination of $P_{aw}$ produced a consistent increase in $K_{fc}$ of approximately three- to fourfold at the highest PIP in this preparation. After perfusion was stopped, the lungs were weighed.

Lungs of rats in the high-PIP control group ($n = 7$) were isolated and ventilated with the high-PIP protocol described above. Lungs of rats in the high-PIP group treated with isoproterenol + rolipram ($n = 7$) were prepared as described above. After a baseline $K_{fc}$ measurement, TFP (100 μM) was added to the venous reservoir and the protocol described above was performed.

Lungs of rats in the high-PIP group treated with ML-7 ($n = 7$) were prepared as described above. After a baseline $K_{fc}$ measurement, ML-7 (500 nM) was added to the venous reservoir and the protocol described above was performed.

Lungs of rats in the high-PIP group treated with KT-5720 ($n = 5$) were prepared as described above. After a baseline $K_{fc}$ measurement, KT-5720 (67 nM) was added to the venous reservoir and the protocol described above was performed.
measurement, KT-5720 (6.7 μM) was added to the venous reservoir and the protocol described above was performed. Although low doses of KT-5720 are specific for protein kinase A, this dose of KT-5720 exceeded the inhibition constant (>2 μM) for MLCK and protein kinase C (PKC) (9, 40).

**Statistics**

Values are means ± SE unless otherwise stated. The $K_\text{fc}$ values were compared between groups using an ANOVA with repeated measures and a Newman-Keuls post hoc test with CRUNCH4 statistical software and a Gateway 2000 digital computer. A logarithmic transformation was used to minimize the within-group variance effects, and a significant difference was determined at $P < 0.05$.

**RESULTS**

**Hemodynamics and Lung Weights**

Mean vascular pressure and $P_{aw}$ and $R_t$ for the six groups at each ventilated state are summarized in Table 1. Vascular pressures and $R_t$ are those present at baseline PIP immediately before the $K_\text{fc}$ measurements. There was a small but significant increase in $R_t$ in the high-PIP control, TFP, and ML-7 groups before the last $K_\text{fc}$ measurement. Somewhat higher perfusate flows were used in the KT-5720 group (9.9 ± 0.5 ml/min), as reflected in the lower $R_t$ values in this group.

**Table 1. Vascular and airway pressures at each peak inflation pressure state**

<table>
<thead>
<tr>
<th></th>
<th>PIP, cmH2O</th>
<th>PEEP, cmH2O</th>
<th>$P_{aw}$, cmH2O</th>
<th>$P_{pv}$, cmH2O</th>
<th>$R_t$, ml·min⁻¹·100 g PLW⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>High PIP control</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>6.7 ± 0.3</td>
<td>2.8 ± 0.1</td>
<td>7.4 ± 0.2</td>
<td>3.6 ± 0.2</td>
<td>42.7 ± 3.2</td>
</tr>
<tr>
<td>20 cmH2O PIP</td>
<td>19.9 ± 0.1†</td>
<td>2.3 ± 0.1</td>
<td>7.4 ± 0.2</td>
<td>3.6 ± 0.2</td>
<td>41.9 ± 3.8</td>
</tr>
<tr>
<td>30 cmH2O PIP</td>
<td>29.9 ± 0.4‡</td>
<td>2.8 ± 0.4</td>
<td>7.9 ± 0.4</td>
<td>3.5 ± 0.3</td>
<td>53.3 ± 11.5</td>
</tr>
<tr>
<td>35 cmH2O PIP</td>
<td>34.9 ± 0.3‡</td>
<td>7.1 ± 0.9‡</td>
<td>9.1 ± 0.5‡</td>
<td>3.9 ± 0.1</td>
<td>63.6 ± 12.8‡</td>
</tr>
<tr>
<td><strong>High PIP trifluoperazine</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>7.4 ± 0.2</td>
<td>2.2 ± 0.1</td>
<td>7.2 ± 0.2</td>
<td>3.6 ± 0.1</td>
<td>31.9 ± 0.6</td>
</tr>
<tr>
<td>20 cmH2O PIP</td>
<td>20.0 ± 0.0*</td>
<td>2.1 ± 0.1</td>
<td>7.4 ± 0.3</td>
<td>3.8 ± 0.1</td>
<td>36.6 ± 2.9</td>
</tr>
<tr>
<td>30 cmH2O PIP</td>
<td>30.1 ± 0.1†</td>
<td>2.1 ± 0.1</td>
<td>7.5 ± 0.2</td>
<td>3.9 ± 0.1</td>
<td>37.0 ± 2.4</td>
</tr>
<tr>
<td>35 cmH2O PIP</td>
<td>35.2 ± 0.1‡</td>
<td>6.6 ± 0.2‡</td>
<td>8.6 ± 0.4‡</td>
<td>3.8 ± 0.1</td>
<td>49.1 ± 4.0‡</td>
</tr>
<tr>
<td><strong>High PIP ML-7</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>6.8 ± 0.1</td>
<td>2.0 ± 0.1</td>
<td>7.4 ± 0.3</td>
<td>3.6 ± 0.1</td>
<td>44.3 ± 7.8</td>
</tr>
<tr>
<td>20 cmH2O PIP</td>
<td>19.9 ± 0.1*</td>
<td>2.3 ± 0.1</td>
<td>7.4 ± 0.2</td>
<td>3.6 ± 0.2</td>
<td>47.6 ± 9.1</td>
</tr>
<tr>
<td>30 cmH2O PIP</td>
<td>29.7 ± 0.2†</td>
<td>2.1 ± 0.2</td>
<td>7.4 ± 0.3</td>
<td>3.6 ± 0.2</td>
<td>46.2 ± 7.1</td>
</tr>
<tr>
<td>35 cmH2O PIP</td>
<td>35.0 ± 0.0‡</td>
<td>6.1 ± 0.8‡</td>
<td>8.2 ± 0.3‡</td>
<td>3.5 ± 0.1</td>
<td>57.4 ± 7.9‡</td>
</tr>
<tr>
<td><strong>High PIP KT-5720</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>8.2 ± 0.2</td>
<td>2.7 ± 0.2</td>
<td>8.4 ± 0.7</td>
<td>4.1 ± 0.1</td>
<td>18.6 ± 2.4</td>
</tr>
<tr>
<td>20 cmH2O PIP</td>
<td>19.5 ± 0.3*</td>
<td>2.9 ± 0.3</td>
<td>7.8 ± 0.7</td>
<td>4.0 ± 0.0</td>
<td>16.9 ± 2.9</td>
</tr>
<tr>
<td>30 cmH2O PIP</td>
<td>30.0 ± 0.5†</td>
<td>3.1 ± 0.1</td>
<td>8.0 ± 0.5</td>
<td>4.9 ± 0.1</td>
<td>18.7 ± 2.5</td>
</tr>
<tr>
<td>35 cmH2O PIP</td>
<td>35.0 ± 0.5†</td>
<td>7.6 ± 0.2‡</td>
<td>9.5 ± 0.9</td>
<td>4.1 ± 0.1</td>
<td>25.4 ± 6.5</td>
</tr>
<tr>
<td><strong>High PIP isoproterenol + rolipram</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>7.7 ± 0.2</td>
<td>2.4 ± 0.1</td>
<td>7.7 ± 0.3</td>
<td>3.6 ± 0.2</td>
<td>46.0 ± 5.5</td>
</tr>
<tr>
<td>20 cmH2O PIP</td>
<td>20.3 ± 0.2*</td>
<td>2.2 ± 0.1</td>
<td>7.2 ± 0.3</td>
<td>3.8 ± 0.1</td>
<td>38.4 ± 3.8</td>
</tr>
<tr>
<td>30 cmH2O PIP</td>
<td>30.0 ± 0.0†</td>
<td>2.1 ± 0.1</td>
<td>7.4 ± 0.3</td>
<td>4.1 ± 0.1</td>
<td>38.3 ± 4.1</td>
</tr>
<tr>
<td>35 cmH2O PIP</td>
<td>35.0 ± 0.3‡</td>
<td>6.8 ± 0.2‡</td>
<td>7.6 ± 0.3</td>
<td>3.8 ± 0.3</td>
<td>44.6 ± 5.1</td>
</tr>
</tbody>
</table>

Values are means ± SE. $P_{aw}$, pulmonary arterial pressure; $P_{pv}$, pulmonary venous pressure; PIP, peak inflation pressure; PEEP, positive end-expiratory pressure; $R_t$, pulmonary vascular resistance; PLW, predicted lung weight. *$P < 0.05$ vs. baseline. †$P < 0.05$ vs. preceding 2 pressure states. ‡$P < 0.05$ vs. all preceding pressure states.
Terminal lung weight means for the groups were as follows: 2.92 ± 0.13 g for control, 2.63 ± 0.21 g for TFP, 2.49 ± 0.10 g for ML-7, 2.64 ± 0.15 g for KT-5720, and 1.89 ± 0.06 g for isoproterenol + rolipram. Because there was variation in body weight between groups, the lung weight gains are shown in Fig. 2 as percent weight gain from PLW, which normalizes for body weight differences. Only the weight gain in the isoproterenol + rolipram group was significantly lower than that in the high-PIP control group, although the weight gain of the KT-5720 group approached significance (P < 0.05, t-test).

\( K_{fc} \)

Inhibition of \( Ca^{2+}/CaM \) and MLCK. Figure 3 shows \( K_{fc} \) as a function of PIP in the high-PIP control group and the high-PIP groups treated with ML-7 and TFP. There were no significant differences in \( K_{fc} \) between groups at the baseline PIP of 7 cmH\(_2\)O. \( K_{fc} \) increased significantly from baseline by 2.0-fold at 30 cmH\(_2\)O PIP and 3.4-fold at 35 cmH\(_2\)O PIP in the high-PIP control group but did not increase significantly from baseline in the TFP or ML-7 group after any PIP. Mean \( K_{fc} \) values in the ML-7 and TFP groups were significantly lower than control values after 35 cmH\(_2\)O PIP, but the ML-7 and TFP groups were not significantly different from each other after any PIP ventilation state.

Inhibition of MLCK and PKC. Figure 4 compares the \( K_{fc} \) values of lungs treated with KT-5720 with the high-PIP control group. This dose of KT-5720 has been shown to inhibit MLCK and PKC. \( K_{fc} \) was significantly increased in the high-PIP control group after ventilation with 30 and 35 cmH\(_2\)O PIP. \( K_{fc} \) did not increase significantly from baseline in the KT-5720-treated group and was significantly lower than in the high-PIP control group after ventilation with 30 and 35 cmH\(_2\)O PIP. There were no significant differences in \( K_{fc} \) at any time point between the TFP, ML-7, isoproterenol + rolipram, and KT-5720 groups.

Augmentation of intracellular cAMP. In Fig. 5, the \( K_{fc} \) values of lungs treated with isoproterenol + rolipram are compared with untreated controls at increased PIP. Whereas \( K_{fc} \) in the high-PIP control group increased significantly after ventilation with 30 and 35 cmH\(_2\)O PIP, the \( K_{fc} \) values of lungs treated with KT-5720 did not increase significantly from baseline.

Fig. 2. Percent gain in lung weight from predicted lung weight (PLW) for all groups. TFP, trifluoperazine; Iso, isoproterenol; Roli, rolipram. *P < 0.05 vs. control group. &P < 0.05 vs. control group (by unpaired t-test).

Fig. 3. Capillary filtration coefficients (means ± SE) in the high-PIP control group and high-PIP groups treated with ML-7 and TFP as a function of PIP. *P < 0.05 vs. baseline within the same group. #P < 0.05 vs. all previous states within the same group. §P < 0.05 vs. all other groups at the same PIP.

Fig. 4. Capillary filtration coefficients (means ± SE) in the high-PIP control group and the high-PIP group treated with KT-5720 as a function of PIP. *P < 0.05 vs. baseline within the same group. #P < 0.05 vs. all previous states within the same group. §P < 0.05 vs. all other groups at the same PIP.
cmH₂O PIP, there were no changes in $K_{fc}$ from baseline in the isoproterenol + rolipram group during high-PIP ventilation. $K_{fc}$ was significantly lower than control in the isoproterenol + rolipram group after ventilation with 30 and 35 cmH₂O PIP, and there was a trend for $K_{fc}$ to decrease with ventilation in the isoproterenol + rolipram group.

**DISCUSSION**

The major new findings of this study are that kinase inhibitors, which may prevent Ca²⁺ entry, contraction of the actin-myosin filaments, or release of adhesion proteins, can significantly attenuate a vascular permeability increase after high-PIP ventilation of isolated rat lungs. Inhibition of Ca²⁺/CaM activity with TFP or inhibition of MLCK activity with ML-7 or a high dose of KT-5720 was equally effective in preventing the 3.4-fold increase in $K_{fc}$ induced by ventilation with PIP up to 35 cmH₂O. A treatment regimen known to increase intracellular CAMP by stimulating adenylyl cyclase activity and inhibiting type IV phosphodiesterase activity also prevented any increase in $K_{fc}$ and significantly reduced edema formation during the experiments. The ventilation protocol used here was designed to produce only modest permeability increases with minimal numbers of ruptured capillaries, because large tears that penetrate the basement membrane would be less responsive to active modulation by endothelial cells and other lung cellular elements. Although the PIP values used here are modest, they were sufficient to injure the isolated rat lungs. Rat lungs are more susceptible to ventilator-induced lung injury than dog lungs, and isolated lungs are more easily injured at high PIP than in situ lungs, in which volume increases are limited by the chest wall (8, 15, 29, 32).

Although a major objective of the present study examines the effects of kinase inhibitors that decrease the active contraction of endothelial cells in culture, the possible effects on multiple signal transduction pathways and multiple cell types in intact lungs preclude firm conclusions about the role of specific kinase steps in endothelial contraction or other mechanisms contributing to the increased vascular permeability. As described by Garcia et al. (7), a thrombin-induced retraction of cultured endothelial cells involves inositol triphosphate-induced release of stored Ca²⁺ and an influx of extracellular Ca²⁺ to increase intracellular Ca²⁺. The high cytosolic Ca²⁺ binds to CaM, which activates MLCK to phosphorylate myosin light chain, resulting in contraction of actin-myosin filaments and gap formation. Inhibition of Ca²⁺/CaM using TFP or W-7 or inhibition of MLCK using ML-7 or KT-5926 attenuated thrombin-induced myosin light chain phosphorylation and prevented the increase in albumin transfer across the endothelial monolayers. Wysolmerski and Lagunoff (44, 45) found that Ca²⁺, CaM, ATP, and MLCK were absolutely required for phosphorylation of myosin light chain and retraction of permeabilized endothelial cells.

However, Watanabe et al. (41) showed that ML-9, an inhibitor of MLCK, blocked Ca²⁺ entry through mechanogated (shear stress) and store-operated (bradykinin and thapsigargin) channels. In addition, our group recently reported that inhibition of MLCK with 500 nM ML-7 prevented the mechanogated Ca²⁺ transients in rat pulmonary artery and microvascular endothelial cells and that MLCK inhibition with ML-9 prevented store-operated Ca²⁺ entry in response to thapsigargin (25, 31). Michel and Curry (17) emphasized that an increased intracellular Ca²⁺ is a necessary but not a sufficient requisite for receptor-mediated vascular permeability increases, so inhibition of Ca²⁺ entry has a profound effect on high PIP-induced vascular permeability. An increased intracellular Ca²⁺ has multiple effects on cellular processes that influence permeability, including activation of rho kinases, reorganization of the actin cytoskeleton, remodeling of cadherin junctions and focal adhesions, and increased actin-myosin filament tension (17, 18). Thus the effects of ML-7 and KT-5926 in inhibiting the high-PIP $K_{fc}$ increase may primarily act at the level of Ca²⁺ entry rather than endothelial cell retraction. In support of such a mechanism, Parker et al. (29) infused gadolinium, an inhibitor of stretch-activated cation channels, into isolated rat lungs subjected to the same high-PIP protocol as described in the present study. $K_{fc}$ did not increase above baseline in treated lungs but increased significantly in untreated lungs. Gadolinium also blocked the stretch-activated Ca²⁺ entry in cultured rat pulmonary artery and microvascular endothelial cells induced by fluid jet stimulation (31).

In the present study, infusion of TFP at concentrations inhibitory for Ca²⁺/CaM and infusions of ML-7
and KT-5720 at concentrations inhibitory for MLCK effectively blocked the $K_{fc}$ increase induced by the high-PIP ventilation protocol. Therefore, these studies implicate Ca$^{2+}$/CaM and MLCK in the vascular permeability response to lung overdistension. Although KT-5720 may also inhibit PKC at the dose effective for MLCK (9), the inhibitory dose of ML-7 for MLCK used in the present study is 140 times less than its inhibitory dose for PKC (34). These reductions in the $K_{fc}$ response suggest that the effects of ML-7 and KT-5720 were related to inhibition of MLCK rather than inhibition of other possible kinases. However, as described previously, MLCK inhibition may have multiple effects related to vascular permeability. Likewise, Ca$^{2+}$/CaM may be involved in nitric oxide synthase activation, which could also affect permeability (17). Further confounding the interpretation of these isolated lung experiments in terms of cellular pathways is the presence of other cell types within the lung, which could also influence $K_{fc}$ measurements. These include the alveolar and airway epithelial cells and possibly the contractile cells within pulmonary interstitium and alveolar septa (16).

An additional difficulty in interpreting mechanical permeability increases in terms of cellular signal pathways results from the poor understanding of how the permeability lesion is produced. High vascular pressure and $P_{aw}$ do not form the typical neuropeptide-induced gaps at endothelial intercellular junctions that have been studied by several investigators (17); rather, openings are formed through the cell bodies of endothelial cells with minimal junctional opening (5, 15, 22, 23). Micrographs of these injuries suggest formation of pathways through connected vacuoles or depletion of plasmalemmal membrane in attenuated endothelium to form gaps or breaks (17).

The effect of an increased intracellular cAMP in restoring endothelial barrier function has been well documented in receptor-mediated permeability increases in endothelial monolayers, perfused single capillaries, and lungs injured with a variety of inflammatory mediators including thrombin, histamine, ATP, phorbol 12-myristate 13-acetate, and thapsigargin (2, 7, 17, 18, 20). We previously reported that isoproterenol infusions resulted in 64 and 36% attenuations in the respective $K_{fc}$ increases induced by $P_{aw}$ increases of 31 and 43 cmH$_2$O in isolated rat lungs (28), but the effects of cAMP-increasing agents on the vascular permeability increases caused by high $P_{aw}$ have not previously been demonstrated. In the present study, we used a combination of isoproterenol, which activates adenyl cyclase to increase cAMP production, and rolipram, a type IV phosphodiesterase inhibitor, to prevent cAMP degradation (18). In previous isolated rat lung studies, Barnard et al. (2) found that the combination of isoproterenol and rolipram was more effective than either drug alone in blocking the $K_{fc}$ increase and lung edema induced by ischemia-reperfusion. Radiolabeled cAMP increased in the perfusate in proportion to the dose of isoproterenol or rolipram. Stevens et al. (38) recently reported that adenyl cyclase activation with forskolin, in conjunction with rolipram, increased intracellular cAMP to a greater extent than either drug alone in rat pulmonary artery and microvascular endothelial cells. This increase in intracellular cAMP can reduce MLCK activity through protein kinase A phosphorylation of MLCK near the Ca$^{2+}$/CaM binding site to reduce the affinity of MLCK for Ca$^{2+}$/CaM (24). In cultured endothelial monolayers, an increased intracellular cAMP resulted in significant reductions in myosin light chain phosphorylation and restored the monolayer barrier properties after challenge with thrombin or histamine (7, 19). In the present study, the reduced $K_{fc}$ observed with isoproterenol and rolipram pretreatment may have resulted from inhibition of MLCK activity, preventing Ca$^{2+}$ entry through stretch-activated cation channels (31, 41) or endothelial cell retraction (7, 25, 31). Also, activation of the cyclic nucleotide-gated cation channels, recently described by Wu et al. (43), in rat pulmonary artery endothelial cells would lead to cell depolarization and a decreased electrochemical gradient and would also inhibit Ca$^{2+}$ entry.

Additional antiedemagenic effects of an increased intracellular cAMP that may have contributed to the reduced $K_{fc}$ and lung weight gain include inhibition of Ca$^{2+}$ release from intracellular stores, augmented Ca$^{2+}$ removal from cytoplasm, stabilization of focal adhesions, and prevention of stress fiber formation (7). Upregulation of the epithelial sodium pump in the group treated with isoproterenol + rolipram would be expected to increase alveolar fluid reabsorption throughout the experiment and reduce edema formation (10). Lampugnani et al. (14) reported that increased cAMP levels also increased endothelial cell adhesion, reduced cell mobility, and produced a reversible disruption of actin microfilaments. Recently, Moy et al. (19) proposed that cAMP attenuated thrombin-induced edema formation by accelerating the rate of endothelial cell adhesion rather than by reducing actin-myosin fiber tension. Certainly, endothelial cell adhesion has a prominent role in ventilator-induced lung injury, as evidenced by the prominent endothelial and epithelial blebbing due to separation of cells from their basement membranes in lungs subjected to high-pressure ventilation (4). Weakening of cell tethering due to tyrosine phosphorylation may enhance susceptibility to ventilator-induced lung injury, as observed by Parker et al. (30) in isolated rat lungs treated with the tyrosine phosphatase inhibitor phenylarsine oxide (13), so strengthening of these adhesions may confer protection against mechanical injury.

A direct effect on vascular smooth muscle of Ca$^{2+}$/CaM and MLCK inhibition must also be considered (39). However, vascular relaxation would have a minimal effect on the $K_{fc}$ response observed in the present study, because previous studies in isolated lungs indicate that vasodilators have a minimal effect on the normally low baseline vascular tone or on $K_{fc}$ in the pulmonary circulation (1). $R_{fc}$ was not decreased significantly after drug treatment in any group. The resistances increased significantly in three groups after the
highest PIP ventilation period undoubtedly as a result of cumulative edema formation during the $K_{fc}$ maneuvers. Because infusion of vasoconstrictors did not decrease $K_{fc}$ due to capillary derecruitment (33), only a massive injury much more severe than that observed here could mask a $K_{fc}$ increase by decreasing capillary surface area (36). Therefore, there is little doubt that the $K_{fc}$ increase after high-PIP ventilation represents a vascular permeability increase and that the drug treatments attenuated this increase.

In summary, we have shown that inhibition of Ca$^{2+}$/CaM and MLCK can attenuate the increased transvascular fluid filtration produced by a modest degree of ventilator-induced lung injury. Although the transvascular openings observed after mechanical stress described by West and colleagues (5, 15, 42) do not appear to occur by the release of intercellular junctions, the relationship of these endothelial openings to $K_{fc}$ is unknown. However, the effects of the kinase inhibitors and adenylyl cyclase agonists used here suggest that many of the signaling events affecting vascular permeability following mechanical stress are similar to those involved in receptor-mediated permeability increases (18, 38). Previous work from our laboratory suggests that stretch-activated Ca$^{2+}$ entry may supply the requisite Ca$^{2+}$ transient required for the increased vascular permeability involved in ventilator-induced lung injury (29). Predicting which of the various potential signaling pathways observed in cell studies that can relate to ventilator-induced lung injury is difficult because of the complex micromechanics and difficulty in accurately calculating alveolar and extra-alveolar vesSEL wall stresses (26). In addition, endothelial cells from different vascular segments have markedly different permeability responses to increased intracellular Ca$^{2+}$ (38), and the epithelium can also modulate fluid movements (10). The specific vascular region damaged during ventilator-induced lung injury may also differ depending on whether injury occurs at high or low lung volumes (4). Nevertheless, our studies indicate that capillary leak secondary to lung distension appears to have an active component mediated by the Ca$^{2+}$/CaM-MLCK pathway and that this permeability increase can be modulated by kinase inhibitors and increased intracellular cAMP.

This study was supported by American Heart Association Grant 981018SE.

REFERENCES


