Inhibition of Hypoxic Pulmonary Vasoconstriction by Calcium Antagonists in Isolated Rat Lungs

IVAN F. MCMURTRY, PH.D., ALLAN B. DAVIDSON, B.S., JOHN T. REEVES, M.D., AND ROBERT F. GROVER, M.D., PH.D.

SUMMARY The role of a transmembrane calcium influx in hypoxic pulmonary vasoconstriction was studied in isolated, blood-perfused, rat lungs. We reasoned that, if the influx of extracellular calcium mediated the hypoxic mechanism, pressor responses to alveolar hypoxia (2.5% O₂) would be susceptible to inhibition by the calcium antagonists verapamil (2 × 10⁻⁵ to 2 × 10⁻⁴ M) and SKF 525 A (2.6 to 260 nM). Susceptibility of hypoxic pressor responses to inhibition by these calcium antagonists was contrasted to that of pressor responses elicited by the humoral vasoconstrictors angiotensin II (1 or 0.5 μg) and prostaglandin F₂α (10 μg). Since neither saralasin (0.5 μM), a competitive antagonist of angiotensin II, nor meclofenamate (6.8 μM), an inhibitor of prostaglandin synthesis, depressed hypoxic pressor responses, it was concluded that these humoral transmitters were not directly involved in the hypoxic mechanism, and therefore served as independent reference agonists. The order of susceptibility of pulmonary pressor responses to inhibition by verapamil was hypoxia > angiotensin II > prostaglandin F₂α. SKF 525 A also reduced pressor responses to hypoxia more readily than those to angiotensin II. The greater inhibition of hypoxic pulmonary vasoconstriction by both calcium antagonists suggested that the hypoxic mechanism was critically dependent on the transmembrane influx of extracellular calcium. Mediation of the hypoxic response by this type of excitation-contraction coupling is consistent with the idea that hypoxia has a direct depolarizing effect on the vascular smooth muscle. It also provides a unifying explanation for inhibition of the hypoxic mechanism by various agents that have depressant or stabilizing actions on membranes in addition to other pharmacological effects.

THE MECHANISM by which alveolar hypoxia acts within the lung to elicit pulmonary arterial vasoconstriction is unknown. Based on current understanding of the roles of extra- and intracellular calcium ions in excitation-contraction coupling of vascular smooth muscle, hypoxic vasoconstriction might involve membrane depolarization and transmembrane influx of extracellular calcium, or transmitter-induced release of calcium from an intracellular pool, or both. If the mechanism depends on the influx of extracellular calcium, then the pressor response to hypoxia should be readily inhibited by agents that reduce calcium entry across the cell membrane.

Verapamil, 2-6 α-isopropyl-α-(N-methyl-N-homoveratryl)-γ-aminopropyl]-3,4-dimethoxphenylacetonyl nitrite HCl, and SKF 525 A, 6, 9, 10 2-diethylaminoethyl-2,2-diphenylvalerate HCl, are described as calcium antagonists and neither agent is known to have α- or β-adrenergic activity or to act as a competitive inhibitor of any humoral transmitter. Studies with isolated vascular strips have shown that these calcium antagonists block contractile responses to K⁺-induced membrane depolarization and spontaneous spike electrogensis more readily than they block responses to humoral transmitters such as norepinephrine, histamine, or angiotensin II. It is believed that, by reducing membrane permeability to calcium, verapamil and SKF 525 A can directly block contractile responses mediated by membrane depolarization and transmembrane calcium influx. These agents probably also depress the rate at which intracellular stores of calcium are replenished from extracellular sources, and thereby interfere indirectly with responses elicited by transmitter-induced release of intracellular calcium. 11, 12 Thus, it is apparent that the susceptibility of a given vasoconstrictive response to inhibition by verapamil and SKF 525 A is determined by the extent to which that response is mediated by membrane depolarization and transmembrane calcium influx.

In this study we used verapamil and SKF 525 A to test the possibility that pulmonary vasoconstriction induced by hypoxia in isolated, blood-perfused, rat lungs is mediated by the transmembrane influx of extracellular calcium. The susceptibility of the hypoxic vasoconstriction to inhibition by these calcium antagonists was contrasted to that of responses elicited by the humoral vasoconstrictors angiotensin II and prostaglandin F₂α. To determine whether either of these humoral vasoconstrictors played an integral role in the hypoxic pressor response 13, 14 and therefore was not suitable as an independent reference agonist, we also performed experiments with saralasin acetate, l-Sar-8-Ala-angiotensin II acetate, a competitive inhibitor of angiotensin II, 15 and sodium meclofenamate, N-(2,6-dichloro-m-tolyl)anthranilate sodium, an inhibitor of prostaglandin synthesis. 16

Methods

ISOLATED LUNG PREPARATION

Lungs removed from male Sprague-Dawley rats weighing 300-400 g were perfused with homologous blood and ventilated by methods adapted from those described by Hauge. 15 Approximately 30 minutes before isolation of lungs, 25-40 ml of blood were collected in heparinized syringes by cardiac puncture of three male rats weighing 400-600 g and anesthetized with ether. The blood was placed in a water-jacketed reservoir at 39°C and pumped...
through the perfusion circuit with a Holter model RL175 peristaltic pump. Lung-donor rats were anesthetized with pentobarbital (10 mg/100 g, ip). The trachea was cannulated and the chest opened during positive-pressure ventilation with a Harvard model 666 respirator at 65 breaths/min. Peak inspiratory pressure was maintained at 9 cm H₂O and expiratory pressure at 2.5 cm H₂O. Ventilatory gas was warmed and humidified by passage through water at 39°C. After the rat had been heparinized (100 IU in 0.5 ml of saline, iv), the main pulmonary artery was cannulated through an incision in the right ventricle, a cannula was tied into the left ventricle, the aorta was ligated, and the lungs and heart were removed from the thoracic cavity. After interruption of the rat’s circulation, ventilatory gas was changed from room air to a mixture of 21% O₂, 5% CO₂, and 74% N₂ (normoxia). Lungs were suspended in a humidified, water-jacketed chamber at 39°C by the tracheal, arterial, and ventricular cannulae. Mean pulmonary arterial inflow pressure was measured with a Statham model P23AA transducer on a Sanborn model 151 recorder. Perfusion was begun and the cannulas were manipulated until resistance to flow was less than 1 mm Hg/ml per min and the left atrium was mildly distended. The approximate time from interruption of the rat’s circulation to initiation of perfusion was 10 minutes. Blood flows ranged from 12 to 15 ml/min at perfusion pressures of 10 to 15 mm Hg. Once the flow for a given lung was established, it was held constant throughout the experiment. Baseline perfusion pressure increased at a rate of approximately 1 mm Hg/hr over 4-5 hours of perfusion. During the experiment, samples of effluent blood were collected at the left ventricular cannula for determination of Po₂, PCO₂, and pH at 39°C with a Radiometer type E5021a microelectrode system. During normoxic ventilation effluent blood gas values were approximately 110 mm Hg for Po₂, and 30 mm Hg for PCO₂. These values did not change with duration of perfusion. The pH was maintained between 7.3 and 7.4 by periodic addition of sodium bicarbonate.

TESTING OF PRESSOR RESPONSES

The isolated, artificially perfused and ventilated lungs were equilibrated for 30 minutes before initiation of pressor responses with alveolar hypoxia, angiotensin II, or prostaglandin F₂α. Because perfusion rate, resistance to effluent flow, and ventilatory rate and pressures were held constant, an increase in perfusion pressure represented an increase in pulmonary vascular resistance. We tested pulmonary pressor responses to alveolar hypoxia by switching from the normoxic gas mixture to one of 2.5% O₂, 5% CO₂, and 92.5% N₂ (hypoxia). The magnitude of the response was measured after 4 minutes of hypoxic ventilation. Effluent blood gas values during the 3rd minute of hypoxia were approximately 22 mm Hg for Po₂, 30 mm Hg for PCO₂, and 7.41 for pH. These values were consistent within and among experiments. Pressor responses to synthetic angiotensin II amide (Hypertensin, Ciba) were elicited during normoxic ventilation by a close arterial injection of 0.1 ml of saline containing 0.5 or 1 µg of angiotensin II. The magnitude of the response was measured at its peak which occurred within a few seconds after injection. These doses of angiotensin II elicited pressor responses that were similar in magnitude to those induced by hypoxia. Pressor responses to 10 µg of prostaglandin F₂α (Upjohn) in 0.1 ml of saline were also elicited by close arterial injections during normoxia. The response to this agonist was more prolonged than that to angiotensin II, and its magnitude was measured at the highest point which occurred within 2-3 minutes after the injection. Although the responses to 10 µg of prostaglandin F₂α were smaller than those elicited by hypoxia and angiotensin II, larger doses were not used because of the prolonged duration of the response and a tendency for baseline perfusion resistance to increase more rapidly than usual after repeated injections.

The vascular responsiveness of the isolated rat lung varies with duration of perfusion, and the temporal pattern of pressor responses must be established before the effects of pharmacological modifiers are examined. In our study, preliminary experiments with lungs stimulated by hypoxia at 20-minute intervals indicated that the magnitude of the pressor responses usually increased progressively until the fourth challenge and then slowly declined to about 80% of maximum by the 10th test. Alternate stimulation with angiotensin II did not alter the pattern of responsiveness to hypoxia. Pressor responses to angiotensin II increased progressively until a relative plateau was reached at about the sixth challenge. Experiments with alternate stimulation by hypoxia and prostaglandin F₂α indicated that the pattern of responsiveness to hypoxia was normal and that pressor responses to the prostaglandin usually declined gradually from a peak at the first challenge. On the basis of these preliminary experiments, we used procedures of alternate stimulation by hypoxia and angiotensin II and by hypoxia and prostaglandin F₂α to differentiate the effects of various inhibitory agents on the pressor responses to hypoxia and the two humoral agonists. The inhibitors were added to the perfusate after the pressor responses had reached relative plateaus. The effects of the various inhibitory agents were measured by expressing the pressor responses after administration of the inhibitor as a percentage of the respective response before administration of the inhibitor.

ADMINISTRATION OF INHIBITORY AGENTS

A variety of inhibitory agents was used to compare or contrast pulmonary pressor responses induced by alveolar hypoxia, angiotensin II, and prostaglandin F₂α. After control pressor responses had been established, saralasin acetate, sodium meclofenamate, verapamil, or SKF 525 A were dissolved in saline and added in volumes of 0.2-1 ml to the perfusate reservoir to achieve the desired concentrations. A tendency for these agents to lower the pH of the perfusate was counteracted by the addition of sodium bicarbonate to the reservoir.

STATISTICS

Data are expressed as mean ± SEM. The significance of differences in mean values before and after administration of inhibitory agents was determined by a paired t-test. Differences were considered significant if P < 0.05.
Results

INHIBITORY EFFECT OF SARALASIN

To test the possibility that interaction of angiotensin II with its membrane receptor plays an essential role in mediation of the hypoxic pressor response, we determined the effects of saralasin acetate, a specific, long-lasting antagonist of angiotensin II, on alternate pressor responses to hypoxia and the humoral agonist. In four experiments, 0.5 μM saralasin acetate reduced the next two responses to 1 μg of angiotensin II to 4 ± 4% of control (P < 0.05), but had no effect on the hypoxic responses. A gradual recovery of the response to angiotensin II was selectively counteracted by periodic addition of saralasin to the perfusate. In one lung stimulated only with hypoxia at 20-minute intervals, four doses of 1 μM saralasin, one after every second pressor response, did not inhibit the hypoxic mechanism.

POTENTIATING EFFECT OF MECLOFENAMATE

The possibility that an intrapulmonary synthesis and release of prostaglandin mediated the pulmonary pressor response to alveolar hypoxia was examined by determining the effect of sodium meclofenamate, a potent inhibitor of prostaglandin synthesis, on pressor responses to hypoxia in nine isolated lungs. Meclofenamate (6.8 μM) was added to the perfusate after responses had reached a plateau or had started to decline spontaneously. The first, second, and third hypoxic responses at 10, 20-30, and 50 minutes after meclofenamate were increased to 115 ± 7%, 135 ± 13%, and 134 ± 13% of control (P < 0.05). There was a positive correlation between degree of potentiation of the second post-meclofenamate response and magnitude of the pre-meclofenamate response (r = 0.90, n = 9, P < 0.001). Meclofenamate also enhanced the rate at which baseline perfusion pressure increased with time, from 1.3 ± 0.3 to 4.6 ± 0.9 mm Hg/hr (P < 0.001).

INHIBITORY EFFECTS OF VERAPAMIL

To determine the relative susceptibility of pressor responses induced by hypoxia and 1 μg of angiotensin II to inhibition by verapamil, the calcium antagonist was added to the perfusate reservoir after control pressor responses had been established (Fig. 1). The inhibitory effects of verapamil in concentrations ranging from 2 x 10^-5 to 2 x 10^-3 M are shown in Figure 2. Threshold levels of inhibition by verapamil appeared to be about 10^-5 and 10^-4 M for hypoxia- and angiotensin II-induced responses, respectively. Pressor responses to hypoxia were inhibited to a greater extent than those to angiotensin II. In addition, inhibition of hypoxic responses was sustained, whereas inhibition of responses to angiotensin II became less with time.

A similar procedure was used to compare the inhibitory effects of verapamil on alternate pulmonary pressor responses to hypoxia and 10 μg of prostaglandin F_2α (Fig. 3). The differential inhibitory effects of verapamil in 0.02
and 0.2 mM concentrations are shown in Figure 4. Whereas responses to hypoxia were reduced to about 10% of control, those to prostaglandin \( F_{2\alpha} \) were not significantly affected.

**INHIBITORY EFFECTS OF SKF 525A**

The procedure used to determine the relative inhibitory effects of SKF 525A on pressor responses to hypoxia and angiotensin II was similar to that used in the case of verapamil. One difference was that the magnitudes of the hypoxia- and angiotensin II-induced responses were matched more closely by injecting 0.5 \( \mu \)g of angiotensin II instead of 1 \( \mu \)g. Differential inhibitory effects of SKF 525A in concentrations ranging from 2.6 to 280 mM are shown in Figure 5. At a given effective level of SKF 525A, responses to hypoxia were reduced by a greater degree than were the responses to angiotensin II. The inhibitory effects of SKF 525A on pressor responses to both hypoxia and angiotensin II declined more rapidly with time than did those of verapamil.

**Discussion**

We reasoned that if a transmembrane calcium influx was critically involved in the mechanism of hypoxia-induced pulmonary vasoconstriction, then pressor responses to alveolar hypoxia would be more susceptible to blockade by verapamil and SKF 525A than were those to the humoral pulmonary vasoconstrictors angiotensin II\(^{11,14}\) and prostaglandin \( F_{2\alpha} \).\(^{12,26}\) This premise required that neither reference agonist was involved in the hypoxic pressor response. However, it has recently been suggested that these vasoconstrictors do play an important role in the mediating mechanism.\(^{12,14}\) It was therefore necessary to examine this possibility in our study.

Inhibition of pulmonary pressor responses to exogenous angiotensin II (but not of responses to hypoxia) by saralasin acetate, a potent, competitive antagonist of angiotensin II,\(^{16}\) suggested that interaction of this vasoconstrictor with its membrane receptor was not involved in the hypoxic mechanism. Although this result implies that angiotensin II is not a specific vasoconstrictive mediator of the hypoxic response, it does not eliminate the possibility that angiotensin II has some nonpressor activity\(^{16}\) that potentiates the response in lungs perfused with a physiological salt solution.\(^{19}\) Prostaglandins are probably synthesized de novo rather than stored and then released,\(^{21}\) therefore mediation of hypoxic pressor responses by prostaglandin \( F_{2\alpha} \) would likely require its synthesis during the hypoxic challenge. Thus, potentiation of the pressor response by meclofenamate, a potent inhibitor of prostaglandin synthesis,\(^{18}\) indicated that during hypoxia there was synthesis of a prostaglandin that modulated rather than mediated the ongoing vasoconstriction.\(^{22,23}\) The positive correlation between magnitude of pre-meclofenamate response and degree of potentiation by meclofenamate suggested that the amount of vasodilator prostaglandin synthesized was related to magnitude of vasoconstriction. Since baseline perfusion pressure increased more rapidly than usual following meclofenamate, it was possible that synthesis of vasodilatory prostaglandin also was involved in maintaining normoxic pulmonary vascular tone. These results suggested that neither angiotensin II nor prostaglandin \( F_{2\alpha} \) was directly involved in mediating the pulmonary vasoconstrictive response to alveolar hypoxia. In addition, studies with isolated smooth muscle have shown that contractile responses to both angiotensin II\(^{18,12,14}\) and prostaglandin \( F_{2\alpha} \)\(^{24}\) are relatively resistant to blockade by calcium antagonists. We therefore concluded that these agents could be used as reference agonists with which to determine the relative susceptibility of hypoxic pulmonary vasoconstriction to inhibition by verapamil and SKF 525A.

Pulmonary vasoconstrictive responses to alveolar hypoxia were more susceptible to inhibition by verapamil than were those responses to either angiotensin II or prostaglandin \( F_{2\alpha} \). The order of susceptibility to inhibition by verapamil was hypoxia > angiotensin II > prostaglandin \( F_{2\alpha} \). It was observed further that SKF 525A also reduced hypoxic pressor responses more readily than it reduced the responses to angiotensin II. Verapamil was a more potent inhibitor than SKF 525A. This finding was similar to that for

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**Figure 3** Relative susceptibilities of hypoxia- and prostaglandin \( F_{2\alpha} \)-induced pressor responses to inhibition by verapamil, in isolated rat lung.

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**Figure 4** Differential inhibition by verapamil of pressor responses to alternate stimulation by hypoxia and prostaglandin \( F_{2\alpha} \) (PGF\(_{2\alpha}\)) in isolated rat lung. Three responses to each stimulus are means ± SEM of five experiments at 0 mm verapamil, four experiments at 0.02 mm, and five experiments at 0.2 mm.
isolated vascular strips reported by Massingham, who found that verapamil was about 100 times more effective than SKF 525A in inhibiting contractile responses to K+-induced membrane depolarization. Because inhibition by verapamil and SKF 525A became greater with continued incubation of isolated vascular strips, perhaps the temporal diminution in the isolated, blood-perfused lung was due to uptake or metabolism by nonvascular tissues. Whatever the explanation, the lesser recovery of the hypoxic pressor response and the more stable inhibition by verapamil emphasized, respectively, the greater susceptibility of hypoxic vasoconstriction to blockade and the more potent inhibitory effects of verapamil.

The susceptibility of the hypoxic pressor response to inhibition by verapamil and SKF 525A is similar to that of vascular contractions that are associated with membrane depolarization. For example, in the isolated rat aorta exposure for 20 minutes to 0.02 mM verapamil reduces contractile responses to K+-induced membrane depolarization to 15% of control without depressing responses to norepinephrine. Verapamil reduces calcium-induced contractions of K+-depolarized strips of rabbit pulmonary artery more readily than it reduces norepinephrine-induced contractions of polarized strips. In guinea pig portal vein, verapamil blocks spontaneous mechanical activity, abolishes contractile responses to K+-induced membrane depolarization, and reduces responses to norepinephrine to 40% of control. SKF 525A blocks K+-induced contractions of rabbit aortic strips without inhibiting contractile responses to norepinephrine or angiotensin II. Contractions elicited by calcium in K+-depolarized strips are almost completely abolished by SKF 525A. These differential inhibitory effects of verapamil and SKF 525A are explained by their ability to reduce membrane permeability to calcium, the critical dependence of electrical excitation on the transmembrane influx of extracellular calcium for coupling to smooth muscle contraction, and the fact that some humoral agonists can elicit contraction, at least in part, by nonelectrical means. Thus, the susceptibility of hypoxia-induced pulmonary vasoconstriction to inhibition by verapamil and SKF 525A suggests that the mediating mechanism is critically dependent on membrane depolarization and transmembrane calcium influx.

If it is assumed that inhibition of the hypoxic pressor response by verapamil and SKF 525A is due to a direct effect on pulmonary vascular smooth muscle, then it can be argued that the results support the idea that hypoxia acts directly to depolarize the smooth muscle cell and initiate transmembrane calcium influx. However, other interpretations are possible because, unlike the case of vascular strips, this assumption is not necessarily true for isolated lungs, in which tissues other than vascular smooth muscle could be involved. Verapamil and SKF 525A might have reduced the hypoxic responses by inhibiting the release of a humoral transmitter from some extravascular tissue. This possibility is based on arguments that there are intrapulmonary, secretory-type cells that are stimulated by alveolar hypoxia to release pulmonary vasoconstrictors, and on the theory that extracellular calcium plays a role in excitation-transmitter release. However, it should be noted that excitation-contraction coupling in vascular smooth muscle is more susceptible to inhibition by verapamil than excitation-secretion coupling in adrenergic nerve terminals, and a suitable humoral mediator of the hypoxic pressor response has not yet been found. If some, as yet unidentified, transmitter is involved in the hypoxic pressor response, it is also possible that its contractile effect is mediated largely by the influx of extracellular calcium and is therefore inhibited by verapamil and SKF 525A.

Regardless of the exact mechanism, it is clear that the pulmonary vasoconstrictive response to alveolar hypoxia is susceptible to inhibition by the calcium antagonists verapamil and SKF 525A. This susceptibility suggests a unifying explanation for inhibition of hypoxic pulmonary vasocon-
striction by numerous substances having nonspecific, depressant or stabilizing effects on cell membranes in addition to the pharmacological actions for which they are best known. For example, nonspecific effects suggestive of membrane stabilization are demonstrated by \( \alpha \)-adrenergic blockers, \( \delta \)-adrenergic antihistamines, and compound 48/80. Inhibition of hypoxic presor responses by relatively high levels of these agents might be related to calcium antagonism or hyperpolarization rather than to \( \alpha \)-adrenergic blockade, \( \gamma \)-histamine antagonism, and histamine depletion. Similarly, inhibition of hypoxic vasoconstriction in intact dogs by increased plasma levels of \( MgCl_2 \) and in isolated lungs by decreased perfusate temperature is possibly related, in the first case, to calcium antagonism by magnesium and, in the latter, to a decrease in membrane permeability to calcium induced by cooling. In summary, a critical dependence of the hypoxic mechanism for pulmonary vasoconstriction on membrane depolarization and transmembrane calcium influx would explain inhibition of this response by agents that hyperpolarize the plasma membrane or reduce membrane permeability to calcium.

Acknowledgments

We thank Mrs. Eva Toyos and Miss Mary Munroe for performing the blood gas analyses and preparing the figures, and Mrs. Dianna Smith for typing the manuscript. Verapamil was supplied by Dr. M.L. Grozier of Knoll Pharmaceutical Co., SKF 525A by Mr. A.C. Cotter of Smith Kline and French Laboratories, saralasin by Dr. A.W. Castellon of Norwich Pharmaceutical Co., and meclofenamate by Dr. A.W. Kinkel of Parke, Davis and Co.

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Inhibition of hypoxic pulmonary vasoconstriction by calcium antagonists in isolated rat lungs.

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doi: 10.1161/01.RES.38.2.99

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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