Effect of dehydroepiandrosterone on hypoxic pulmonary vasoconstriction: a Ca\(^{2+}\)-activated K\(^+\)-channel opener

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Farrukh, Imad S., Wei Peng, Urszula Orlinska, and John R. Hoidal. Effect of dehydroepiandrosterone on hypoxic pulmonary vasoconstriction: a Ca\(^{2+}\)-activated K\(^+\)-channel opener. Am. J. Physiol. 274 (Lung Cell. Mol. Physiol. 18): L186–L195, 1998.—In the present study, we investigated the effects of the naturally occurring hormone dehydroepiandrosterone (DHEA) on hypoxic pulmonary vasoconstriction (HPVC) in isolated ferret lungs and on K\(^+\) currents in isolated and cultured ferret pulmonary arterial smooth muscle cells (FPSMCs). Severe alveolar hypoxia (3% \(\mathrm{O}_2\)-5% \(\mathrm{CO}_2\)-92% \(\mathrm{N}_2\)) caused an initial increase in pulmonary arterial pressure \(P_{\text{pa}}\) that was followed by a reversal in pulmonary hypertension. Maintaining alveolar hypoxia caused a sustained secondary increase in \(P_{\text{pa}}\). Pretreating the lungs with the \(K^+\)-channel inhibitor tetraethylammonium (TEA) caused a small increase in baseline \(P_{\text{pa}}\), potentiated HPVC, and prevented the reversal of HPVC during the sustained alveolar hypoxia. Treating the lungs with DHEA caused a near-complete reversal of HPVC in control lungs and in lungs that were pretreated with TEA. DHEA also reversed the KC1-induced increase in \(P_{\text{pa}}\). In FPSMCs, DHEA caused an adenosine 3',5'-cyclic monophosphate- and guanosine 3',5'-cyclic monophosphate-independent increase in activity of the Ca\(^{2+}\)-activated K\(^+\) (K\(_{\text{Ca}}\)) current. In a cell-attached configuration, DHEA caused a mean shift of −22 mV in the voltage-dependent activation of the K\(_{\text{Ca}}\) channel. We conclude that DHEA is a novel K\(_{\text{Ca}}\)-channel opener of the pulmonary vasculature.

pulmonary circulation; pulmonary hypertension; calcium-activated potassium-channel agonist; potassium-channel opener; potassium currents; tetraethylammonium

HYPOXIC AND PRIMARY pulmonary hypertension cause substantial morbidity and mortality. Current therapy for pulmonary hypertension is ineffective, in part, because of inadequate understanding of basic mechanisms that regulate pulmonary vascular tone. Alveolar hypoxia was reported to cause membrane depolarization of pulmonary arterial smooth muscle cells (SMCs) (14, 27, 29, 35). Several types of membrane ion channels were reported to be involved in mediating SMC depolarization and hypoxic pulmonary vasoconstriction (15). Post et al. (29) demonstrated in canine pulmonary arterial SMCs that lowering \(\mathrm{PO}_2\) caused membrane depolarization by decreasing Ca\(^{2+}\)-dependent whole cell outward currents. Yuan et al. (35) reported in rat pulmonary arterial SMCs that sodium dithionite-induced hypoxia inhibited a voltage-gated K\(^+\) channel. More recently, it was reported that alveolar hypoxia inhibited a delayed rectifier K\(^+\) (\(K_{\text{DR}}\)) channel (2) or ATP-dependent K\(^+\) (K\(_{\text{ATP}}\)) channel (31) of rat small pulmonary arterial SMCs. Previous observations by Peng and colleagues (26, 27) in human pulmonary SMCs and previous reports (6, 30, 35) on animal pulmonary vasculature suggested that Ca\(^{2+}\)-activated K\(^+\) (K\(_{\text{Ca}}\)) channels can act as a negative feedback mechanism to counteract the acute effect of hypoxia and other agonist-induced membrane depolarizations. In vascular SMCs, the membrane potential \(E_m\) is a major determinant of vascular tone. The \(E_m\) of vascular SMCs is controlled to a large degree by K\(_{\text{Ca}}\), K\(_{\text{dr}}\), and K\(_{\text{ATP}}\) channels (6, 8, 28).

K\(_{\text{Ca}}\) channels are large-conductance channels and carry ionic currents that mediate membrane hyperpolarization and thus regulate important cellular functions including vascular relaxation. K\(_{\text{Ca}}\)-channel activity is regulated by several physiological parameters including \(E_m\), cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)), and channel phosphorylation (6). The probability of the K\(_{\text{Ca}}\) channel being open is relatively low in resting vascular SMCs. However, the opening state of the channels is increased by membrane depolarization. Thus it is believed that the K\(_{\text{Ca}}\) channel acts as a relaxing negative feedback mechanism after agonist-induced membrane depolarization (6, 27). The purified toxin peptides such as charybdotoxin (CTX), iberiotoxin, and external tetraethylammonium (TEA) are known inhibitors of the K\(_{\text{Ca}}\) channel (7). However, little is known about K\(_{\text{Ca}}\)-channel agonists.

Dehydroepiandrosterone (3β-hydroxy-5-androsten-17-one; DHEA) and its sulfate ester are major secretory products of the adrenal cortex. The exact physiological role of DHEA is unknown. However, epidemiologic studies demonstrated that in humans a high level of DHEA is an independent predictor of a significantly reduced risk for fatal coronary vascular disease (3) and is inversely associated with the development of accelerated coronary allograft vasculopathy (16). In addition, DHEA was reported to cause a concentration-dependent in vivo and in vitro inhibition of platelet aggregation (17). The observed platelet-aggregation inhibition was associated with a reduction in thromboxane B\(_2\) production (17). Farrukh et al. (10) previously reported that administration of corticosteroid caused a marked inhibition of lipid peroxide- or thromboxane A\(_2\)-mediated pulmonary vasoconstriction. Progesterone, a derivative of the parent molecule pregnenolone and the precursor of DHEA, was reported to cause smooth muscle relaxation (5). In addition, deoxycorticosterone, another derivative of the parent molecule pregnenolone, was reported to cause both an acute and a chronic alteration in membrane ion transport of aortic SMCs (19). These observations suggest that the steroid-
induced pulmonary vasorelaxation is mediated via membrane hyperpolarization.

In this study, we investigated the effect of DHEA on hypoxic pulmonary vasoconstriction in an isolated, ventilated, and perfused ferret lung preparation. We also determined in cultured ferret pulmonary arterial SMCs (FPSMCs) whether the effects of DHEA on pulmonary vascular tone were induced via modulating K⁺ channels.

METHODS AND MATERIALS

Isolated Lung Preparation

The isolated lung preparations were performed as previously described (12) and as approved by the Institutional Animal Care and Use Committee at the University of Utah. Briefly, male ferrets (Marshall Farm, North Rose, NY) weighing 3.5–4 kg were maintained on formula and water ad libitum. On the day of the experiment, the ferrets were anesthetized with an intraperitoneal injection of pentobarbital. On the day of the experiment, the ferrets were given 3,000 units of heparin by intracardiac injection and killed by rapid exsanguination from the left ventricle. Right heart pressures were monitored with Gould Statham P23ID radiometer MK2 blood gas analyzer at 37°C.

The lungs were ventilated via a tracheotomy with a Harvard animal respirator (Harvard Apparatus, Millis, MA) maintained at 7.35–7.40 by adding sodium bicarbonate to the perfusate. The lungs were perfused with a mixture of 70 ± 5 ml of autologous blood and 70 ± 5 ml of Krebs-Henseleit buffer in a recirculating manner at a constant rate of 85 ml/min at 37°C. The chemical composition of the Krebs-Henseleit buffer was: (in mM) 137 NaCl, 4.7 KCl, 2.5 CaCl₂, 2H₂O, 1.3 KH₂PO₄, 1.2 MgSO₄, 11 dextrose, and 18 NaHCO₃. The temperature of the perfusate was kept between 37 and 38°C. The pH was maintained at 7.35–7.40 by adding sodium bicarbonate to the reservoir as needed. The venous reservoir was placed below the lowermost portion of the lung.

The lungs were ventilated via a tracheotomy with a Harvard animal respirator (Harvard Apparatus, Millis, MA) with either a normoxic gas mixture of 5% CO₂, 21% O₂, and 74% N₂ or a hypoxic gas mixture of 5% CO₂, 3% O₂, and 92% N₂. The animals were ventilated with a tidal volume of 30 ml at a rate of 12 breaths/min and with 2 cmH₂O positive end-expiratory pressure.

Measurements. Pulmonary arterial, left atrial, and tracheal pressures were monitored with Gould Statham P23ID pressure transducers connected to the inflow circulation and recorded on a Gould polygraph (model RS 3400, Gould, Valley View, OH). Arterial blood gases and pH were measured on a radiometer MK2 blood gas analyzer at 37°C.

Interventions. TEA (Sigma Chemical, St. Louis, MO) was dissolved in modified Krebs-Henseleit buffer and added directly to the perfusion reservoir. DHEA (Pharmadigm, Salt View, OH) was dissolved in modified Krebs-Henseleit buffer and added directly to the perfusion reservoir to achieve the desired final concentration. In pilot experiments, this amount of DMSO had no effect on hypoxic pulmonary vasoconstriction.

FPSMC Culture

FPSMCs were cultured from explants of ferret pulmonary arteries as previously described (11). Briefly, the arteries were separated from their adventitia and endothelium and then minced into 1- to 3-mm² pieces with sterile scalpels. The tissues were mounted in tissue culture wells with 0.05 ml of chicken plasma (Sigma Chemical) plus 0.05 ml of chick embryo extract (Sigma Chemical). The tissue, and membrane of the FPSMCs, was then plated with the same SMC culture media, SmoG (Clonetics, San Diego, CA) that was supplemented with 5% fetal bovine serum (HyClone, Logan, UT) and 10% bovine calf serum (HyClone) plus dexamethasone (0.39 mg; Sigma Chemical) and antimicrobial agents (50 µg/ml of gentamicin, 25 µg/ml of amphotericin B, 120 U/ml of penicillin, and 0.12 mg/ml of streptomycin; Sigma Chemical). Experiments were performed in primary FPSMCs. FPSMCs were assessed by light microscopy, electron microscopy, and indirect immunofluorescent staining of α-actin. Transmission electron micrographs of the FPSMCs revealed the characteristics of vascular SMCs, including the contractile filaments and the elongated or cigar-shaped mitochondria. The indirect immunofluorescent staining of α-actin [the primary antibody for the immunofluorescent staining was mouse monoclonal anti-α-smooth muscle actin (Sigma Chemical), and the secondary antibody was rhodamine-conjugated anti-mouse F(ab')₂ fragment] demonstrated the lacy and longitudinal pattern of fluorescence of actin that is characteristic of SMCs and distinct from fibroblasts (11).

Electrophysiological Measurements

Single cells were voltage clamped, and membrane currents were recorded with cell-attached inside-out and outside-out configurations of the patch-clamp technique (13, 26). The patch-clamp micropipettes were made of borosilicate glass capillaries and had a resistance of 5–10 MΩ. Voltage-clamp potentials were applied to membrane patches, and membrane currents were recorded with an Axopatch amplifier (Axopatch 200A, Axon, Foster City, CA). The current data were filtered at 1 kHz and digitized at 5 kHz with a Digidata 1200 interface (Axon). Data were acquired and analyzed with the pClamp software (Axon). Single-channel currents were calculated by Gaussian distribution fitted to a current-amplitude histogram. Mean values for the state of channel open probability and mean open times were obtained from a 5- to 15-min steady-state recording time, and the channel open probability is expressed as NPₒ where N is the number of functional channels in the membrane patch and Pₒ is the state of open probability. Average channel activity (NPₒ) in membrane patches was determined as follows: NPₒ = (Σ[Np]T)/T, where T is the duration of recording and tₒ is the time spent with j = 1, 2, 3, . . . N channels open.

Solutions and Chemicals

The pipette and bath solutions were composed of (in mM) 140 KCl, 2 MgCl₂, 3 ethylene glycol-bis(-aminoethyl ether)-N,N,N',N’-tetraacetic acid, 10 N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid, and 2.29 CaCl₂; pH was adjusted to 7.4. The estimated free Ca²⁺ concentration was 300 nM as computed by Fabiato’s (9) computer program. The FPSMCs and membrane patches were continuously perfused with this solution. CTX was obtained from Alomone Laboratories. The cyclic nucleotide-dependent protein kinase inhibitor N-[2-
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Statistical Analysis
Values are means ± SE. The data were analyzed with analysis of variance for repeated measures (Scheffé’s F-test significance at 95%) and Student’s t-test. Results were considered statistically significant when P < 0.05.

RESULTS

Effect of Severe Alveolar Hypoxia on Pulmonary Arterial Pressure
In a group of animals (n = 4), we tested the effect of severe hypoxia (3% O2) on pulmonary arterial pressure (Ppa). During an initial stabilizing period, the lungs were ventilated with a normoxic gas mixture (5% CO2-21% O2-74% N2) for 20 min. Switching the ventilation to a hypoxic gas mixture (5% CO2-3% O2-92% N2) caused a sharp increase in Ppa [change in (Δ)Ppa = 11 ± 2 mmHg; P < 0.05]. Because the perfusion flow rate and left atrial pressure were held constant and there was no significant change in airway pressures, the increase in Ppa represents an active increase in pulmonary vascular resistance in response to alveolar hypoxia. The increase in Ppa was followed by a near-complete reversal of hypoxia-induced pulmonary vasoconstriction (Fig. 1). Maintaining alveolar hypoxia caused another slow and sustained increase in Ppa (ΔPpa = 8 ± 2 mmHg; P < 0.05; Fig. 1).

Effect of K+-Channel Blocker (TEA) on Ppa During Severe Alveolar Hypoxia
It was suggested in isolated ferret lungs that the secondary pulmonary vasodilatation during severe hypoxia was most likely mediated by opening K+ channels (34). Therefore, we tested the effect of pretreating the lungs (n = 3) with TEA (10 mM) on the vascular response to severe alveolar hypoxia. TEA, when applied externally, has a relatively high-affinity blockade of the KCa channel [dissociation constant (Kd) = 150-300 µM] (6, 28). In vascular smooth muscle, high concentrations of TEA (5–20 mM) can also inhibit the Kdr channel (6, 28). Pretreating the lungs with TEA (10 mM) caused a small baseline increase in Ppa (ΔPpa = 1.0 ± 0.2 mmHg; P > 0.05), potentiated the initial pulmonary vascular response to hypoxia (ΔPpa = 14 ± 1.5 vs. 11 ± 2 mmHg for control lungs; P < 0.05), and prevented the reversal of the pulmonary vasocostriction during the sustained severe alveolar hypoxia (Fig. 2). This suggested that the secondary hypoxia-induced pulmonary vasodilatation is mediated via activating TEA-sensitive K+ channels.

Effect of DHEA on Ppa Responses During Severe Alveolar Hypoxia
Administration of DHEA to human volunteers to achieve serum concentrations of 0.1, 0.2, and 0.3 mM had no reported side effects (17). In two groups of experiments, we tested the effect of DHEA on the acute and the delayed severe hypoxia-induced pulmonary vasoconstriction. In the first group of ferrets, DHEA (0.14 or 0.2 mM; n = 3 each) was administered at about the peak of the acute hypoxic pulmonary vasoconstriction. Treating the lung with DHEA caused an acute and complete reversal of the hypoxic pulmonary vasoconstriction and prevented the secondary hypoxic vasoconstriction (Fig. 3). Treating the hypoxic lungs with vehicle (DMSO) alone had no effect on the hypoxic pulmonary vasoconstriction. We also tested the effect of DHEA on lungs that were pretreated with 10 mM TEA before exposure to hypoxia. As illustrated earlier (Fig. 2), pretreating the lungs with TEA potentiated the hypoxic pulmonary vasoconstriction and blocked the secondary pulmonary vasodilatation during the sustained severe hypoxia. After the sustained hypoxic pulmonary vasoconstriction was established, the addition of DHEA (0.14 or 0.2 mM; n = 3 ferrets each) dramatically reversed the effects of TEA treatment plus alveolar hypoxia (Fig. 2). The vasodilating effects of 0.14 and 2.0 mM DHEA were maintained throughout the 75 and 150 min, respectively, of sustained hypoxia. Because the effects of both TEA and hypoxia are believed to be mediated via SMC membrane depolarization, the observations in this group of experiments suggested that the DHEA-induced pulmonary vasodilatation was mediated via membrane hyperpolarization.

Effect of DHEA on KCl-Induced Pulmonary Vasocostriction
To investigate further the mechanism of DHEA-induced pulmonary vasodilatation, we tested the effect...
of DHEA on KCl-induced pulmonary vasoconstriction. Infusion of 30 mM KCl into the pulmonary arteries of ferret lungs (n = 3) caused an initial transient hyperacute increase in Ppa that was followed by a sustained increase in Ppa (Fig. 4). Posttreating the lungs with DHEA (0.14 mM) completely reversed the effect of KCl on Ppa and reduced the Ppa below baseline (Fig. 4). This observation further supports the hypothesis that DHEA-induced pulmonary vasodilatation is mediated via SMC membrane hyperpolarization.

Effect of DHEA on E_m in Intact FPSMCs

The effect of DHEA on the resting E_m was examined in primary FPSMCs by using whole cell current-clamp recordings. Treating the cells with DHEA (50 µM) caused significant baseline membrane hyperpolarization (E_m = -69.0 ± 1.23 mV for DHEA-treated cells vs. -53.6 ± 1.38 mV for baseline control cells; P < 0.0001; n = 6 cells). The effect of DHEA was reversible after 12–15 min of washing (Fig. 5).

Effect of DHEA on K^+ Channels in Intact FPSMCs

To determine whether the DHEA-induced reversal of hypoxic pulmonary vasoconstriction was mediated by membrane hyperpolarization, we investigated the effect of DHEA on the major K^+ channels in intact cells. The two major components of the K^+ current (I_K) of pulmonary arterial SMCs have previously been characterized (28). The first component or K_dr current [I_{K(dr)}] is a low-amplitude and low-noise current that is voltage and time dependent and insensitive to Ca^{2+} or low concentrations of TEA but can be inhibited with high-dose TEA (2–2 mM) and 4-aminopyridine (4-AP). The second component or Ca^{2+}-activated K^+ current [I_{K(Ca)}] is a high-amplitude and large-noise current that is voltage dependent. I_{K(Ca)} is sensitive to Ca^{2+}, CTX, and low concentrations of TEA (26–28). Single channels were recorded in a cell-attached configuration before and after FPSMCs were exposed to DHEA. DHEA (50 µM) exposure caused a significant increase in KCa-channel activity (NP_o = 0.147 ± 0.007 vs. 0.029 ± 0.001 for control cells; P < 0.0001; n = 10 cells; Fig. 6). DHEA had no significant effect on the K_dr channel (NP_o = 0.026 ± 0.002 vs. 0.022 ± 0.001 for control cells; P > 0.05; n = 10 cells). The effect of DHEA on K_ca-channel activity was reversible after washout (data not shown). The DHEA-induced increase in channel activity was not associated with a change in unitary current (mean single unitary current after DHEA was 9.6 ± 0.16 vs. 9.4 ± 0.1 pA for control cells; P > 0.05; n = 10 cells).
To investigate further whether the action of DHEA was selective for the KCa channel, we tested its effect on outward K+ currents (I\textsubscript{Ko}) from FPSMCs that were pretreated with a selective KCa-channel inhibitor, CTX (2, 26, 35). In a cell-attached configuration, CTX (100 nM in the pipette solution) blocked the KCa channel and prevented the DHEA-induced activation of the KCa channel (Fig. 6A). In the presence of CTX, DHEA had no effect on the low-conductance K+ currents (Fig. 6A). This indicates that DHEA selectively activates the KCa channel.

In another group of experiments, we tested the effect of 4-AP and glibenclamide on the DHEA-induced increase in KCa-channel activity. 4-AP (2 mM) blocked the low-amplitude or KC\textsubscript{dr} channel but had no effect on the DHEA-induced increase in KCa-channel activity (NP\textsubscript{0} = 0.037 ± 0.001 for control baseline activity vs. 0.19 ± 0.007 for DHEA + 4-AP-induced activity; P < 0.0001; n = 6 cells each; Fig. 6B). To determine whether the KC\textsubscript{ATP} channel is involved in the DHEA-induced increase in IKo, we tested the effect of the KC\textsubscript{ATP}-channel inhibitor glibenclamide (6, 8) on the DHEA-induced activation of the KCa channel. Glibenclamide (50 µM) had no effect on the DHEA-mediated increase in KCa-channel activity (NP\textsubscript{0} = 0.037 ± 0.001 for control baseline activity vs. 0.177 ± 0.006 for DHEA + glibenclamide-induced activity; P < 0.05; n = 10 cells; Fig. 6B).

Role of Guanosine 3',5'-Cyclic Monophosphate and Adenosine 3',5'-Cyclic Monophosphate in DHEA-Mediated KCa-Channel Activation in Intact FPSMCs

Because the mechanism of vascular agonist-induced increase in KCa-channel activity is reported to be mediated via guanosine 3',5'-cyclic monophosphate (cGMP) or adenosine 3',5'-cyclic monophosphate (cAMP) (26, 30), we investigated the possible role of cGMP and/or cAMP in DHEA-mediated KCa-channel activation. We tested the effect of antagonizing the cyclic nucleotides with Rp-cGMPS, Rp-8-pCPT-cGMPS, and Rp-cAMPS on the DHEA-induced increase in KCa-channel activity. FPSMCs were incubated with one of the Rp isomers (1 × 10\textsuperscript{-4} M) for 30 min and were then perfused with bath solution containing the same concentration of the isomer.

Rp-cGMPS, Rp-8-CPT-cGMPS, and Rp-cAMPS reduced baseline activity of the KCa channel (NP\textsubscript{0} = 0.029 ± 0.001 for mean control activity vs. 0.007 ± 0.0016, 0.008 ± 0.0003, and 0.011 ± 0.001 for Rp-cGMPS-, Rp-cAMPS-, and Rp-8-pCPT-cGMPS-induced activity, respectively; P < 0.0001; n = 6 cells each; Fig. 7). The addition of DHEA (50 µM) to cells pretreated with one of the Rp isomers caused a significant increase in KCa-channel activity (NP\textsubscript{0} = 0.029 ± 0.001 for mean control activity vs. 0.15 ± 0.001, 0.16 ± 0.008, and 0.171 ± 0.009 for Rp-cGMPS + DHEA-, Rp-cAMPS + DHEA-, and Rp-8-pCPT-cGMPS + DHEA-induced activity, respectively; P < 0.0001; n = 6 cells each; Fig. 7). This suggests that the DHEA-induced activation of the KCa channel is not mediated via the generation of cGMP or cAMP.

To test further whether the DHEA-induced K+ activation was mediated via cyclic nucleotide phosphorylation, we studied the effect of the cyclic nucleotide-dependent protein kinase inhibitor H-8 on the DHEA-induced increase in KCa-channel activity. In inside-out excised patches, applying H-8 (0.45 µM) to the inner surface of the membrane caused a significant decrease in baseline KCa-channel activity (NP\textsubscript{0} = 0.034 ± 0.003 vs. 0.078 ± 0.006 for control baseline activity; P < 0.0001).
0.001; n = 6 patches; Fig. 8). In the presence of H-8, DHEA caused a significant increase in Ca\(^{2+}\)-activated K\(^+\) (K\(_{Ca}\)) channel activity (P < 0.0001). Effect of DHEA on K\(_{Ca}\)-channel activity was blocked by charybtoxin (CTX; 100 nM). DHEA had no effect on low-amplitude or delayed rectifier K\(^+\) (K\(_{dr}\)) channel. The effects of H-8 and DHEA were reversible after the patches were washed with the bath solution (Fig. 8).

### Effect of DHEA on K\(^+\)-Channel Sensitivity to E\(_m\)

To investigate further the mechanism by which DHEA activates the K\(_{Ca}\) channel, we determined its effect on the sensitivity of K\(_{Ca}\) channels to E\(_m\). In a cell-attached configuration with a perfusate Ca\(^{2+}\) concentration of 300 nM and at an E\(_m\) range of -20 to +60 mV, DHEA caused a significant increase in K\(_{Ca}\)-channel activity at all E\(_m\) values (P < 0.0001; n = 6 cells; Fig. 9). The linear fit of ln \([N_P_o/(1 - N_P_o)]\) vs. E\(_m\) demonstrated a -21- to -24-mV (or leftward) shift in the voltage-dependent activation of the K\(_{Ca}\) channel (Fig. 9). This was equivalent to an ~1.4-fold increase in K\(_{Ca}\)-channel activity at any E\(_m\). This suggests that in FPSMCs DHEA increases K\(_{Ca}\)-channel activity by increasing the sensitivity of the channel to E\(_m\).

### DISCUSSION

The principal observation in this study is that severe alveolar hypoxia in ferret lungs causes a triphasic vascular response: an initial acute pulmonary vasoconstriction that is followed by a transient vasorelaxation and then a progressive and sustained vasoconstriction. The transient vasorelaxation is mediated via activating TEA-sensitive K\(^+\) channels. DHEA, a naturally produced hormone, reverses the acute hypoxic pulmonary vasoconstriction and prevents the development of the
secondary increase in $P_{pa}$ during sustained alveolar hypoxia. The mechanism of DHEA-induced pulmonary vasorelaxation is mediated, in part, via an increase in $K_{Ca}$-channel activity.

Effect of Severe Alveolar Hypoxia

The response of the pulmonary vasculature to alveolar hypoxia is dependent on the degree of hypoxia. Previous work from our laboratory (12) and by other investigators (32) demonstrated that lowering alveolar oxygen to 5–7% caused a sustained increase in $P_{pa}$. In this investigation, a further decrease in alveolar $O_2$ to 3% caused the observed triphasic changes in pulmonary vasculature (Fig. 1). It was also reported that ventilating the pulmonary vasculature or isolated lungs with 5% $CO_2$-95% $N_2$ further decreased arterial $P_{O_2}$ and caused a biphasic response of a brief initial vasoconstriction followed by a predominant vasodilatation (32, 34).

The multiphasic response to acute and severe hypoxia

Fig. 7. Channel recordings obtained from FPSMCs in a cell-attached configuration and at $E_{m} = +50$ mV. Rp isomers of guanosine 3',5'-cyclic monophosphothioate (Rp-cGMPS; A) and adenosine 3',5'-cyclic monophosphothioate (Rp-cAMPS; B) reduced baseline $K_{Ca}$-channel activity. Neither of the cyclic nucleotide antagonists affected DHEA-induced increase in $K_{Ca}$-channel activity.

Fig. 8. Change with time of $K_{Ca}$-channel activity in inside-out patches at $E_{m}$ of +50 mV (measured as channel open probability ($NP_{o}$)). Dotted line, period during which inner surface of membrane was exposed to protein kinase C antagonist (0.45 µM H-8); solid line, period during which DHEA (50 µM) was added to inner surface of membrane patch. H-8 had no significant effect on DHEA-induced increase in $K_{Ca}$-channel activity.

Fig. 9. Effect of DHEA on pulmonary vascular reactivity.

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was also observed in isolated pig lungs (31) and isolated proximal large and small rat pulmonary arteries that were precontracted with vascular agonists (4, 18, 23). Intrapulmonary ferret and porcine arterial rings were reported to have predominant biphasic responses to acute and severe hypoxia, characterized by an initial vasoconstriction followed by a predominant vasodilatation (20, 33). The discrepancies in the reported observations indicate that hypoxic pulmonary vasoconstriction is species and arterial size specific.

The mechanism of hypoxia-induced secondary pulmonary vasodilatation is not fully understood. Recent observations suggested that the secondary relaxation is endothelium independent and is unaffected by inhibitors of endothelium-derived relaxing factors, by inhibitors of the cyclooxygenase pathway, or by blockers of adrenergic receptors (18, 23). In the present study, we observed that pretreating ferret lungs with TEA potentiated the hypoxic pulmonary vasoconstriction and blocked the secondary vasodilatation during severe alveolar hypoxia. Previous reports from our laboratory concluded that the vasodilatation during severe hypoxia was mediated by activation of both KATP- and adrenergic receptors (18, 23). In the present study, we report that the hypoxic vasodilatation observed in isolated rat proximal pulmonary arterial rings is species and arterial size specific.

Role of K_{Ca}-Channel Agonist

The second important observation of the present investigation is that the naturally occurring steroid DHEA reversed acute hypoxic pulmonary vasoconstriction, prevented the development of secondary hypoxic vasoconstriction, and reversed the effect of TEA-induced potentiation of hypoxic vasoconstriction. The mechanism of DHEA-induced pulmonary vasodilatation is most likely mediated via activation of the K_{Ca} channel. This conclusion is supported by our physiological and electrophysiological observations. In the isolated ferret lungs, DHEA reversed the KCl-induced increase in P_{aO2} and reversed the effect of TEA on hypoxic pulmonary vasculature. It is not fully clear to us why, in the isolated ferret lung experiments, TEA did not affect the DHEA-induced vasodilatation. One explanation is that external TEA causes a flickery block, and the rate constants for blocking and unblocking the K_{Ca} channel were reported to be 380 and 73 mM/ms, respectively (21). Thus it is possible that applying a potent activator (like DHEA), which has the ability to activate K_{Ca} channels from inside and outside the membrane (Figs. 6–8), can reverse the effect of TEA, probably by affecting its K_{d} to the K_{Ca} channel or by modulating the interaction between the α- and β-subunits of the channels. The fact that DHEA activated the channel from inside the membrane suggests that the DHEA-induced activation of the channel was mediated, in part, via the β-subunit or the regulatory subunit of the channel.

It is unlikely that the DHEA-mediated pulmonary vasorelaxation is mediated via inhibition of L-type Ca^{2+} channels. L-type Ca^{2+}-channel blockers are known to inhibit the K_{Ca} channel (6, 28). Contrary to this, DHEA caused FPSMC membrane hyperpolarization and an increase in K_{Ca}-channel activity. Accordingly, we do not believe that the DHEA-induced vasorelaxation could be mediated via inhibiting L-type Ca^{2+} channels. With the use of the patch-clamp technique in a cell-attached configuration, DHEA increased the K_{Ca}-channel activity of FPSMCs. The DHEA-induced increase in K_{Ca}-channel activity was blocked by a specific K_{Ca}-channel inhibitor, CTX, and was not affected by 4-AP or glibenclamide, inhibitors of the K_{ATP} and K_{Ca} channels, respectively. It is not clear how DHEA increases K_{Ca} activity. In an intact FPSMC, cell-attached configuration, cGMP and cAMP did not block the effect of DHEA on the K_{Ca} channel. In addition, in excised
increase in the KCa channel is mediated by increasing activity at any E_m, equivalent to an 1.4-fold increase in K Ca-channel activity at any E_m value in the range of −20 to +60 mV. These observations suggest that the DHEA-mediated increase in the K Ca channel is mediated by increasing the sensitivity of the channels to E_m. This represents a pathway for K Ca-channel activation that has not been previously described and suggests that DHEA is a novel K Ca-channel opener. Although we have strong evidence to indicate that DHEA acts as a K Ca-channel agonist in FSPMCs, the mechanism of DHEA-mediated pulmonary vasodilatation in hypoxic ferret lungs needs further investigation. Additional experiments are needed to explore the effect(s) of DHEA on isolated microvascular endothelial cells and SMCs.

The previously described K+ channel openers cromakalim and pinacidil are believed to mediate their vasorelaxing effect via activating K ATP channels (8). Limited information is available about K Ca-channel openers. Soyasaponins, which were isolated from a medicinal herb, activate K Ca channels from inside the cell (24). However, their poor membrane permeation limits their therapeutic use (24). Other agents like the synthesized imidazopyrazine derivatives were reported to cause aortic and tracheal smooth muscle relaxation, in part, via activating the K Ca channel (22, 25). These conclusions were reached because SCA40, the most potent imidazopyrazine derivative, inhibited the effect of an intermediate KCl concentration (20 mM) on rat isolated aortic vessels (22). The vasorelaxing effect of SCA40 was not inhibited by the K ATP-channel inhibitor glibenclamide but was antagonized by CTX (25). Although these studies suggested that SCA40-induced vasorelaxation was mediated via activation of the K Ca channel, the investigators provided no electrophysiological evidence to demonstrate that this agent actually increases the activity of the K Ca channel.

Openers of K Ca channels have great therapeutic potential and serve as important investigational tools. K Ca channels have large unitary conductances, thus the opening of only a few K Ca channels has a significant impact on E_m (6, 26, 28). The ability of DHEA to open the K Ca channel of pulmonary SMCs and relax constricted pulmonary vasculature may provide a novel therapeutic agent for pathological conditions such as chronic hypoxia in which the pulmonary vascular E_m is depolarized. The pulmonary vasorelaxing effects of DHEA were observed at concentrations (0.1–0.2 mM) that were previously shown to have no systemic side effects in healthy human volunteers (17). If this holds true in future clinical trials, unlike the other available vasodilators, DHEA will have the advantage of being a potent pulmonary vasodilator without significant systemic side effects. Nonetheless, because K Ca channels are ubiquitous to pulmonary and systemic circulations, further studies are needed to characterize the effect(s) of DHEA on the systemic circulation.

In summary, severe alveolar hypoxia caused an initial acute pulmonary vasoconstriction that was followed by a transient vasorelaxation and then a progressive and sustained vasoconstriction. The transient vasorelaxation was mediated via activating a TEA-sensitive K+ channel. DHEA, a naturally produced hormone, reversed the acute hypoxic pulmonary vasoconstriction and prevented the development of the secondary increase in Ppa during sustained hypoxia. DHEA also reversed the effects of KCl and TEA on the pulmonary vasculature. In FSPMCs, DHEA increased the activity of the K Ca channel. The DHEA-induced increase in K Ca-channel activity was not mediated via cAMP or cGMP and was not blocked by the protein kinase inhibitor H-8. The mechanism of DHEA-mediated increase in K Ca-channel activity was probably mediated via increasing channel sensitivity to E_m.

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