Delayed rectifier K⁺ current of dog bronchial myocytes: effect of pollen sensitization and PKC activation

Gareth J. Waldron, Stefan B. Sigurdsson, Ernesto A. Aiello, Andrew J. Halayko, Newman L. Stephens, and William C. Cole. Delayed rectifier K⁺ current of dog bronchial myocytes: effect of pollen sensitization and PKC activation. Am. J. Physiol. 275 (Lung Cell. Mol. Physiol. 19): L336–L347, 1998.—The properties of delayed rectifier K⁺ current [I_{K(dr)}] of canine airway smooth muscle cells isolated from small bronchi and its modulation by protein kinase C (PKC) were studied by whole cell patch clamp. I_{K(dr)} activated positive to −40 mV, with half-maximal activation at −16 ± 1.2 mV (n = 15) and average current density of 31 ± 2.6 pA/pF (n = 15) at +30 mV. The capacitive surface area, current density, and voltage dependence of activation of I_{K(dr)} of myocytes of ragweed pollen-sensitized dogs were not different from age-matched control dogs. However, the sensitization reduced the availability of I_{K(dr)} between −40 and −20 mV due to a hyperpolarizing shift in the voltage dependence of steady-state inactivation (−29.9 ± 1.2 in sensitized versus −26.0 ± 0.7 mV in control dogs, n = 9 and 11, respectively; P < 0.05). PKC activation with diacylglycerol analog or phorbol ester depressed I_{K(dr)} amplitude, whereas an inactive diacylglycerol analog had no effect. The hyperpolarizing shift in voltage dependence of inactivation and/or modulation of I_{K(dr)} by PKC may be two mechanisms that contribute to the enhanced reactivity of bronchial tissues from ragweed pollen-sensitized dogs.

THE LEVEL OF CONTRACTILE tone exhibited by smooth muscle cells is dependent on intrinsic (myogenic) and extrinsic (neuronal, epithelial, and hormonal) factors that regulate plasmalemmal ion channel activities, Ca²⁺ release from the endoplasmic reticulum, intracellular Ca²⁺ concentration, and the interaction of actin and myosin filaments, i.e., cross-bridge cycling (17). Dysfunctional control of smooth muscle tone contributes to a variety of pathological conditions. For example, asthma has been attributed to alterations in control of airway smooth muscle contractility; anaphylactic bronchospasm is associated with a marked hyper-reactivity of airway smooth muscle to a variety of pharmacological and physical stimuli, including histamine (8, 33).

The level of resting membrane potential is important for the control of airway smooth muscle tone (13, 33). Action potentials are not observed in bronchial muscle. Rather, contractile tone is postulated to result from maintained, nonregenerative depolarizations that increase the open probability of voltage-dependent L-type Ca²⁺ channels (13, 14, 33). Sustained Ca²⁺ influx due to steady-state activation of L-type Ca²⁺ channels occurs over a narrow range of membrane potentials between −50 and −20 mV and is known to cause significant elevations in intracellular Ca²⁺ levels in isolated airway myocytes (13, 14). A role for 4-aminopyridine (4-AP)-sensitive delayed rectifier K⁺ channels (K_{dr}) in contributing to control of membrane potential, Ca²⁺ channel activity, and tone is indicated for the airways; exposure of intact tissues and/or isolated tracheal/bronchial myocytes of dogs, ferrets, and humans to 4-AP leads to depolarization and contraction (1, 15, 23).

The biophysical properties of delayed rectifier K⁺ current [I_{K(dr)}] are well described for tracheal myocytes; however, little is known concerning this conductance in smaller-caliber airways. This is potentially significant since the site of airway dysfunction in pathological conditions, such as asthma, is either the central (2nd to 6th order) bronchi (immediate asthmatic response) or the peripheral bronchi (delayed asthmatic response; see Ref. 36).

Previous studies using a canine model of bronchial hyperreactivity demonstrated that tracheal smooth muscle of dogs sensitized with ovalbumin possessed an enhanced contractile response to histamine mediated by H₁ receptors, as well as spontaneous phasic contractile activity and an increased myogenic response (4, 5). Similar findings were subsequently obtained for airway smooth muscle from second- to sixth-order bronchi of ragweed-sensitized dogs, including an increased capacity and velocity of shortening of the smooth muscle and increased release of histamine to ragweed pollen antigen (8, 22). The second- to sixth-order bronchi were also shown to be more sensitive to ragweed pollen compared with tracheal smooth muscle by several orders of magnitude (39). The characteristics of bronchial smooth muscle of ragweed-sensitized dogs have been reviewed (43).

Protein kinase C (PKC) of airway and nonairway smooth muscles is activated by a variety of contractile agonists, including acetylcholine and histamine, and enhanced PKC activity has been implicated to contribute to the pathogenesis of asthma (17, 34, 35). We recently obtained evidence that I_{K(dr)} of vascular smooth muscle cells is suppressed by PKC (2, 10, 11). Whether I_{K(dr)} of bronchial myocytes is similarly regulated by PKC is unknown.
Accordingly, in this study, we employed freshly isolated myocytes from bronchi of age-matched, control, and ragweed pollen-sensitized dogs and the standard whole cell patch-clamp technique to 1) characterize the properties of \( I_{K\text{dr}} \) in cells isolated from a small-airway preparation, 2) determine whether the magnitude or properties of 4-AP-sensitive \( I_{K\text{dr}} \) were altered in myocytes from bronchial tissues from ragweed pollen-sensitized dogs, and 3) assess the effect on the magnitude and properties of \( I_{K\text{dr}} \) of activation of PKC.

**METHODS**

Animal sensitization. Dogs were housed, sensitized, and then killed with a lethal injection of pentobarbital sodium (30 mg/kg body wt iv) according to a research protocol consistent with the Canadian Council on Animal Care and approved by local animal care committees of the Medical Research Council of Canada. Newborn mongrel dogs were immunized within 24 h of birth by intraperitoneal injection of 500 µg of ragweed (Ambrosia elatior) pollen extract as previously described (24). Booster injections were given on a weekly basis for 8 wk and then at a biweekly interval for an additional 8 wk. Age-matched, littermate control dogs received a similar schedule of intraperitoneal injections with adjuvant alone. Sensitization to ragweed pollen extract was confirmed by homologous ragweed antibody titer of equal to or greater than 256 dilutions. We employed tissues from 9 sensitized and 10 control littermate dogs from five different litters. Tissues required (within 5 h). Myocytes were liberated from the digested tissue pieces by gentle trituration. Viable myocytes used in the patch-clamp experiments were spindle shaped, optically refractive, and relaxed, as is apparent for the representative myocyte shown in Fig. 1.

Solutions. The Krebs-Henseleit solution for dissection and overnight storage of intact bronchial tissues contained (in mM) 115 NaCl, 25 NaHCO3, 1.38 KH2PO4, 2.51 KCl, 2.46 MgCl2, 1.91 CaCl2, and 5.56 dextrose (pH 7.4 when gassed with 95% O2-5% CO2 at room temperature, 20–22°C), supplemented with collagenase (564 U/ml Sigma type 1A; Sigma Chemical), protease (10 U/ml Sigma type XXVII), and elastase (81 U/ml Sigma type IV).

The standard bath solution employed in the whole cell voltage-clamp experiments contained the following (in mM): 120 NaCl, 25 NaHCO3, 4.2 KCl, 0.6 KH2PO4, 1.2 MgCl2, and 0.01 CaCl2 (pH 7.4 when bubbled with 95% O2-5% CO2 at room temperature, 20–22°C), supplemented with collagenase (564 U/ml Sigma type 1A; Sigma Chemical), protease (10 U/ml Sigma type XXVII), and elastase (81 U/ml Sigma type IV).

**Fig. 1.** Light micrograph of a single bronchial myocyte isolated from a control dog. Scale bar indicates 20 µm.
1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA), as stated in RESULTS, to produce varied levels of internal Ca\(^{2+}\) buffering (pH adjusted to 7.2 with KOH).

Electrophysiological recordings. Isolated myocytes were placed in a 300-µl bath and allowed to settle to the bottom of the chamber before a constant flow of bath solution (~0.5 ml/min) at room temperature (20–22°C) was initiated. A standard whole cell patch-clamp recording technique was employed in the experiments (16). Pipettes were prepared from capillary glass (7052 glass; Richland Glass) using a Sutter P-87 puller (Sutter Instruments) and MF-03 microforge (Narashige Scientific Instrument Laboratory). Tip resistances of 1–3 MΩ were obtained when filled with pipette solution. Recordings were performed using either an Axopatch 1-D or 200A amplifier (Axon Instruments). Pipette potential and capacitance were nulled and a 5- to 15-GΩ seal formed with the membrane. To correct for junction potential, 20 pipettes were nulled in pipette solution and then transferred to bath solution. A consistent value of 10 mV for the junction potential was obtained and employed to correct all voltage-clamp protocols and current recordings. Whole cell voltage-clamp protocols were applied using pClamp software (Axon Instruments). Data were filtered at 2 kHz by an on-board 8-pole Bessel filter before digitization with a Labmaster TL-1–125 or Digidata 1200 analog-to-digital converter (Axon Instruments) and stored on hard disk in a 486 PC clone. Data were displayed and analyzed off-line using pClamp (Axon Instruments) and Origin software (MicroCal Software).

Values for net and tail currents due to \(I_{K(dr)}\) were normalized for cell capacitance and expressed as picoamperes per picofarad. Cell capacitance was determined by integration of the capacity transient evoked by 20-mV command steps. In this manner, net current due to \(I_{K(dr)}\), with minimal contamination by other conductances, was measured at the end of 250-ms pulses and plotted as a function of command step voltage between ~80 and +30 mV. Tail currents evoked by repolarization to ~40 mV following steps to between ~80 and ~30 mV and due to deactivating \(K_{dr}\) exclusively were calculated as the difference between the peak amplitude of the tail and sustained current levels at 200 ms. Drug-sensitive difference currents were determined by digital subtraction of current traces obtained in the presence of drug from those recorded in its absence.

Dyes - 48-Fluorescein 12,13-dibutyrate (PDBu), Na\(_2\)ATP, EGTA, BAPTA, tetraethylammonium chloride (TEA\(^{-}\)), and 4-AP were obtained from Sigma Chemical; 1,2- and 1,3-isomers of dioctanoyl-sn-glycerol (diC\(_2\)) were obtained from Serdary Research Laboratories. A stock solution of PDBu (10 mM) was prepared in dimethyl sulfoxide. Diacylglycerol (DAG) analogs were dissolved in hexane. All were added directly to the bath solution at the desired concentration on the day of use. Vehicle controls had no effect on currents recorded. 4-AP was dissolved in bath solution, and the pH was readjusted to 7.4.

Statistics. All values in the text and Figs. 1–9 are presented as means ± SE. Data were compared by paired or unpaired Student’s t-test for single comparisons. Individual data points in the I-V relations for myocytes exposed to single treatments were compared with a paired Student’s t-test, and in experiments where multiple treatments were applied to single cells, the data points were compared by repeated-measures ANOVA followed by Bonferroni’s test for multiple comparisons. A level of \(P < 0.05\) was considered to be statistically significant.

RESULTS

Morphological studies. Bronchial myocytes isolated from control and sensitized dogs and employed in the whole cell patch-clamp studies were identical to the representative cell shown in Fig. 1. Myocytes from control dogs had an average length of 228 ± 36 µm (range 155–307 µm, \(n = 59\) from three separate cell isolations). There was no difference in the capacitive cell surface area of myocytes from control and sensitized dogs: values of 4,210 ± 290 (\(n = 16\)) and 3,710 ± 336 (\(n = 8\)) µm\(^2\) (\(P > 0.05\)) were obtained based on a specific membrane capacitance of 1 µF/cm\(^2\) and average cell capacitances of 42.1 ± 2.9 and 37.1 ± 3.4 pF (\(P > 0.05\)), respectively. The cells were relaxed on isolation and were capable of repeated contractions to elevated external K\(^{+}\) or histamine (data not shown).

Identification of macroscopic \(K^{+}\) currents. The identity and properties of macroscopic \(K^{+}\) currents of canine bronchial myocytes have not been characterized in detail. For this reason, we conducted a series of preliminary experiments to identify conditions that would permit selective recordings of \(I_{K(dr)}\). These initial experiments demonstrated the presence of 1) inward L-type \(Ca^{2+}\) currents that showed rundown within the first 2–3 min of recording and 2) two dominant components of macroscopic outward \(K^{+}\) current in bronchial myocytes of adult and young control or ragweed pollen-sensitized dogs. In the presence of minimal intracellular \(Ca^{2+}\) chelation with 0.05 mM EGTA in the pipette solution, command steps from a holding potential of –60 mV to between –80 and +30 mV every 10 s evoked net outward \(K^{+}\) current positive to ~40 mV, which displayed voltage- and time-dependent activation and was “noisy” positive to 0 mV (Fig. 2A). Outward current evoked during voltage steps increased exponentially with applied voltage and on average reached a density of 58 ± 4 pA/pF at +30 mV (Fig. 2B).

Voltage ramps of 16 s in duration were used to assess the quasi-steady-state current of bronchial myocytes at membrane potentials between –90 and +30 mV. A prominent “hump” and noisy appearance were noted for net outward current between ~40 and 0 mV and positive to approximately ~10 mV with minimal intracellular \(Ca^{2+}\) chelation, respectively (Figs. 2C and 3A). \(TEA^{-}\) was employed at a concentration of 0.1 mM to selectively inhibit large-conductance \(Ca^{2+}\)-activated \(K^{+}\) channels (\(BK_{Ca}\); see Ref. 31). This agent caused a marked inhibition of net outward current positive to ~20 mV during 8-s voltage ramps (shorter duration ramp was employed in these experiments to increase the amplitude of the noisy component), but this blocker had little effect on quasi-steady-state current negative to ~20 mV (Fig. 3A). The selective block of current at positive potentials was apparent in the \(TEA^{-}\)-sensitive difference currents determined by digital subtraction of traces recorded in the presence of \(TEA^{-}\) from those obtained in control conditions (Fig. 3B). These data are consistent with previous studies on macroscopic \(K^{+}\) currents of smooth muscle cells, which attributed the noisy component to \(BK_{Ca}\) and the hump to 4-AP-sensitive \(K_{dr}\) activity (28, 30).
To minimize the contribution of BKCa to macroscopic K\textsuperscript{+} current and permit selective recordings of I_{K_{dr}} of bronchial myocytes, we employed
1) a pipette solution containing 10 mM BAPTA to provide a high level of internal Ca\textsuperscript{2+} chelation,
2) depolarizing voltage steps to potentials equal or negative to +30 mV to avoid voltage-dependent activation of BKCa, and
3) recordings of slowly deactivating tail currents at −40 mV. Figure 4 shows representative families of currents and average I-V relations for end-pulse and tail current amplitudes from three myocytes in the absence and presence of 10 mM 4-AP. Under control conditions, a quiet, slowly activating outward current was observed at all potentials positive to −40 mV. Upon repolarization to −40 mV, slowly deactivating tail currents were recorded.

End-pulse current amplitude increased in a linear fashion, and tail currents had a uniform amplitude positive to 0 mV, suggesting maximal channel activation (Fig. 4B). Application of 4-AP caused a complete inhibition of the tail currents over the entire voltage range tested. End-pulse current was completely suppressed negative to 0 mV, and there was minimal 4-AP-resistant outward current positive to this potential (Fig. 4B). The 4-AP-sensitive difference current shown in Fig. 4A is typical for I_{K_{dr}}, with slow inactivation during the command step. These data suggest that the tail currents were exclusively due to deactivating K_{dr} and that net current at the end of the command pulses was almost completely due to I_{K_{dr}} with little contamination from 4-AP-resistant components.

No evidence was obtained for inward rectification at negative potentials (data not shown) or for any fast-inactivating, transient outward current (see, for example, Figs. 2, 4, 6, 8, and 9). These data, therefore, describe the major outward currents of canine bronchial smooth muscle cells as I_{K_{dr}} and large-conductance Ca\textsuperscript{2+}-activated K\textsuperscript{+} current (I_{BKCa}).

Characteristics of I_{K_{dr}} in bronchial myocytes from control dogs. The kinetics and voltage dependence of activation, deactivation, and inactivation of bronchial smooth muscle I_{K_{dr}} were determined. Activation during a 250-ms test pulse to +20 mV and subsequent deactivation at −40 mV were both best fit with two exponentials (Table 1). Slow inactivation of I_{K_{dr}} was apparent during command pulses to potentials positive to 0 mV. Longer-duration, 4- to 10-s steps to +20 mV
were applied to bronchial myocytes to examine the time dependence of inactivation. \( I_{K_{\text{dr}}} \) decayed relatively rapidly during the first 4 s of the command pulses and thereafter exhibited only a very slow decline in amplitude. A biexponential function was found to best de-

Table 1. Properties of \( I_{K_{\text{dr}}} \) of bronchial myocytes from age-matched control and ragweed pollen-sensitized dogs

<table>
<thead>
<tr>
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<th>Control</th>
<th>Sensitized</th>
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<tr>
<td>End-pulse current density</td>
<td></td>
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<tr>
<td>at +30 mV, pA/pF</td>
<td>31 ± 2.6</td>
<td>34.5 ± 4.0</td>
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<td>at −40 mV after steps to</td>
<td>6.0 ± 0.7</td>
<td>7.5 ± 1.1</td>
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<tr>
<td>activation time constants</td>
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<td>at +20 mV, ms</td>
<td>3.9 ± 0.3</td>
<td>4.1 ± 0.3</td>
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<tr>
<td>Deactivation time constants at −40 mV after steps to +20 mV, ms</td>
<td>16.9 ± 0.9</td>
<td>18.4 ± 1.3</td>
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<tr>
<td>Inactivation time constants at +20 mV, s</td>
<td>13.7 ± 1.5</td>
<td>13.2 ± 1.6</td>
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<tr>
<td>Voltage dependence of activation</td>
<td>3.69 ± 0.66</td>
<td>3.36 ± 0.88</td>
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<tr>
<td>Voltage dependence of inactivation</td>
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<tr>
<td>( V_{0.5} )</td>
<td>−15.7 ± 1.2</td>
<td>−17.7 ± 1.3</td>
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<tr>
<td>( k )</td>
<td>7.8 ± 0.4</td>
<td>7.6 ± 0.4</td>
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Data are means ± SE; \( n \), no. of cells. \( I_{K_{\text{dr}}} \), delayed rectifier K\(^{+}\) current; \( \tau_{1} \) and \( \tau_{2} \), fast and slow time constants, respectively; \( V_{0.5} \), voltage at half-maximal activation or inactivation; \( k \), slope. *Significantly different from corresponding value in myocytes from control dogs by unpaired Student’s t-test (\( P < 0.05 \)).
conditions to those described for cells from control dogs. Representative families of $I_{K(\text{dr})}$ of bronchial myocytes of control and ragweed pollen-sensitized dogs and average I-V relations for end-pulse and tail current amplitude of 15 and 13 control and sensitized myocytes, respectively, are shown in Fig. 6. Plots of average normalized current versus voltage for activation and inactivation of $I_{K(\text{dr})}$ in myocytes from sensitized dogs are shown in Fig. 5. Average values for time constants of activation, deactivation, and inactivation, as well as the voltages for half-maximal activation and inactivation, and values for slope factors are given in Table 1. No statistically significant difference was noted for the voltages for half-maximal activation and inactivation, or kinetics of activation, deactivation, and inactivation, as well as the voltage dependence of availability of $I_{K(\text{dr})}$ in a bronchial myocyte from a control dog. Prepulse steps of 4-s duration to between -110 and +20 mV in 10-mV intervals were applied from holding potential of -60 mV every 15 s and followed by a brief 5-ms step to -130 mV and a constant 200-ms depolarizing test pulse to +20 mV. C: activation (circles) and inactivation (squares) for myocytes from control (open symbols) and ragweed pollen-sensitized (closed symbols) dogs. Each point is the mean ± SE of $n = 9$–15 myocytes. Activation data were obtained from protocols as in A; peak tail amplitudes were normalized to maximal amplitude and plotted against the voltage of the test pulse. Inactivation data were obtained in protocols as in B; the fraction of inactivating current was plotted against the voltage of the 4-s conditioning step. Solid lines passing through the activation and inactivation data points are the best fits of Boltzmann distribution functions obtained by a Leven-Marquardt nonlinear least squares fitting algorithm: activation $Y = \frac{1 + \exp(V - V_{0.5})}{k}$, where the voltage at half-maximal activation ($V_{0.5}$) and slope ($k$) were -15.7 and 7.8 mV versus -17.7 and 7.6 mV in myocytes from control and sensitized dogs, respectively, and $V$ is the voltage of the prepulse step. Inactivation curves $Y = \frac{1 + \exp(V - V_{0.5})}{k}$, where $V_{0.5}$ and $k$ were 26.0 and 6.3 mV versus -29.9 and 5.9 mV in myocytes from control and sensitized dogs, respectively. *Value in myocytes from sensitized animals was significantly different ($P < 0.05$) from that in control dogs by unpaired Student's t-test.

Inhibition of $I_{K(\text{dr})}$ by PKC activation. Activation of PKC is thought to play an important role in the signal transduction pathway of histamine, which contracts airway smooth muscle, and alterations in the activity of PKC isoenzymes have been implicated in the pathogenesis of asthma and bronchospasm of hyperresponsive airways of sensitized animals (4, 17, 35). Accordingly, we determined the effects on $I_{K(\text{dr})}$ of direct PKC activation with an analog of DAG, 1,2-diC$_8$, and the phorbol ester PDBu. Eleven myocytes from control and sensitized dogs were used (6 and 5 myocytes, respectively), and a similar change in $I_{K(\text{dr})}$ during PKC activation was observed in all cells tested. Figure 7 shows the effects of the inactive DAG analog 1,3-diC$_8$ followed by 1,2-diC$_8$ on $I_{K(\text{dr})}$ amplitude. To monitor the time course of effects of the DAG analogs on $I_{K(\text{dr})}$ amplitude, depolarizing steps to +10 mV followed by a brief step to
-40 mV were applied every 15 s. Figure 7A shows a representative example of the change in normalized end-pulse current amplitude under control conditions and during sequential exposure to the inactive DAG analog 1,3-diC8 (10 µM) followed by active DAG analog 1,2-diC8 (1 and 10 µM). The representative current recordings shown in Fig. 7, insets, were obtained at the four time points indicated by the arrows. No effect on \( I_{K_{\text{dr}}} \) amplitude was evident with 1,3-diC8, but end-pulse amplitude slowly declined in a concentration-dependent fashion upon exposure to 1,2-diC8. Figure 7B shows superimposed, representative difference current traces obtained by digital subtraction of traces recorded at 30-s intervals during exposure to 1,2-diC8. The onset of inhibition of \( I_{K_{\text{dr}}} \) required ~60 s, so the initial difference currents showed no change. However, the amplitude of the outward 1,2-diC8-sensitive difference current increased with time, reaching a peak sustained level within 3–5 min. The difference currents at peak inhibition by 1,2-diC8 were very similar to those determined for 4-AP inhibition (Fig. 4).

Figure 8 shows representative families of whole cell currents (Fig. 8A) and average I-V relations for three to seven cells (Fig. 8B) obtained in the absence (control) and presence of either 10 µM 1,3-diC8, 10 µM 1,2-diC8, or after washout of active analog. End-pulse and tail current amplitudes for \( I_{K_{\text{dr}}} \) were unaffected by 1,3-diC8 after 8–10 min of exposure, and, on average, end-pulse and tail current amplitudes were not significantly different from those recorded under control conditions. However, treatment with the active analog produced an average decline in end-pulse and tail current amplitudes of ~60 and 75%, respectively, which was fully reversed if the myocyte was held under whole cell voltage-clamp conditions for longer than 15 min washout. Note also the enhanced rate of inactivation of \( I_{K_{\text{dr}}} \) during activation of PKC in Fig. 8, giving the current a transient appearance similar to that observed in vascular smooth muscle cells during treatment with the DAG analog, PDBu or angiotensin II (2, 10).

A similar inhibition of \( I_{K_{\text{dr}}} \) was observed after treatment of four myocytes with 100 nM of the phorbol ester PDBu compared with 1,2-diC8. Figure 9 illustrates representative changes in a family of whole cell \( K_{\text{dr}} \) and average changes in \( I_{K_{\text{dr}}} \) end-pulse and tail current amplitudes. Average end-pulse current at +30 mV amplitude was reduced by 30.3 ± 1.9% (P < 0.05), and the decline was fully reversible upon washout of phorbol ester in two myocytes, but this required some 20–30 min (data not shown).

**DISCUSSION**

In this study, the whole cell voltage-clamp technique was used to characterize the properties of \( I_{K_{\text{dr}}} \), as well as to determine the effect of ragweed pollen sensitization and direct activation of PKC with a DAG analog or phorbol ester on this conductance of isolated canine bronchial myocytes. We show for the first time that 1) the voltage dependence of inactivation for \( I_{K_{\text{dr}}} \) in bronchial myocytes is different from that in the trachea, 2) inactivation is shifted to more negative poten-
I$_K$($dr$) in canine bronchial smooth muscle was studied using 10 mM BAPTA to chelate intracellular Ca$^{2+}$ and was selectively inhibited with 0.1 mM TEA$^+$. In contrast, macroscopic current due to K$_{dr}$ was insensitive to low TEA$^+$ and high levels of intracellular Ca$^{2+}$ chelation, but it was readily blocked by 4-AP and displayed slow, time-dependent inactivation. The presence of these two channel types in bronchial myocytes is consistent with previous studies of airway myocytes of dog, ferret, pig (7, 26, 29), and humans (1, 18, 41). I$_K$($dr$) in canine bronchial myocytes was studied using 10 mM BAPTA in the pipette to provide for substantial chelation of intracellular Ca$^{2+}$ and minimal contamination of whole cell currents by Ca$^{2+}$-activated conductances, such as BK$_{Ca}$, Ca$^{2+}$-activated Cl$^-$, and Ca$^{2+}$-dependent nonselective cation currents. Additionally, depolarizing voltage-clamp steps were limited to potentials of $+30$ mV or less to avoid voltage-dependent activation of BK$_{Ca}$. The ability of 4-AP to substantially inhibit tail currents produced during deactivation of K$_{dr}$ at $-40$ mV as well as end-pulse current amplitude is consistent with minimal contamination by non-K$_{dr}$ channel activity. The presence of I$_K$($dr$) in all canine bronchial myocytes studied here differs from that reported for human myocytes in which the current was only present in 17% of cells employed (41). However, Janssen (18) described I$_K$($dr$) in human bronchial smooth muscle that was sensitive to 4-AP, and Adda et al. (1) described the presence of mRNA encoding the voltage-gated K$^+$ channels Kv1.1, Kv1.2, and Kv1.5 in human airway muscle.

The fittings of the kinetics of inactivation and deactivation are consistent with other smooth muscle preparations, both being best described by biexponential functions with two time constants on the order of 0.2–0.5 and 2–5 s (inactivation) and 10 and 60 ms (deactivation; see Refs. 2, 3, 7, 26, 28, 40, 46). However, few studies have quoted values for activation time constants due to the overlap with activation of transient outward currents. We did not observe evidence in any cell for the presence of “A”-type outward current (note the transient current in the presence of 1,2-diC$_8$ resulted from the change in I$_K$($dr$); see Refs. 2 and 10) indicating its absence or complete inactivation at the holding potential ($-60$ mV) employed. A dual-exponential fit of I$_K$($dr$) activation in bronchial myocytes is consistent with that of cloned rabbit vascular Kv1.5 (O. Clément-Chomienne, K. Ishii, M. Walsh, and W. Cole, unpublished observation). This study shows that I$_K$($dr$) of canine bronchial myocytes is activated at voltages to approximately $-45$ mV and that it displays time- and voltage-dependent inactivation. The overlap of voltage ranges for activation and inactivation suggests that steady-state K$_{dr}$ window current may be present between approximately $-45$ and $0$ mV, with peak outward I$_K$($dr$) at approximately $-25$ mV. These properties are similar to those previously reported for I$_K$($dr$) of ferret tracheal myocytes; K$_{dr}$ window current was observed between approximately $-50$ and $-10$ mV at $37°C$ (15). This more negative activation voltage of $-50$ mV compared with the present study is likely a function of the bath temperature used to record the ferret tracheal I$_K$($dr$) activation, but not inactivation, of I$_K$($dr$) of smooth muscle cells is known to occur over a more negative range at higher temperatures (e.g., see Ref. 10). It is significant that the voltage for half-maximal availability of I$_K$($dr$) of bronchial myocytes was less negative compared with the value reported for canine and porcine tracheal cells: $-26$ mV versus $-46$ and $-53$ mV for whole cell and for single K$_{dr}$ channel
currents in the trachea (7). It would seem unlikely that differences in recording conditions in the two studies can explain the divergent values for $V_{0.5}$ of inactivation (only slightly different pipette solutions were employed, and both were done at room temperature). Moreover, under identical recording conditions (temperature, bath and pipette solutions, protocol) to those employed in this study, $V_{0.5}$ for inactivation of $I_{\text{K(dr)}}$ of

![Diagram](https://via.placeholder.com/150)

Fig. 8. Protein kinase C (PKC) activation with diacylglycerol analog reversibly inhibits bronchial smooth muscle $I_{\text{K(dr)}}$. A: representative families of whole cell currents evoked by 250-ms pulses to between $-80$ and $+30$ mV in 10-mV steps followed by repolarization to $-40$ mV during sequential exposure of a single bronchial myocyte to control bath solution (Control), 10 µM 1,3-diC$_8$, 10 µM 1,2-diC$_8$, and wash with control bath solution (Wash). B: average I-V relations for end-pulse ($I_{\text{net}}$) and tail ($I_{\text{tail}}$) current amplitude normalized to cell capacitance in the absence of diacylglycerol analog (Con; open square; $n = 7$), presence of 1,3-diC$_8$ (open circle; $n = 5$) or 1,2-diC$_8$ (closed circle; $n = 7$), and after 10–15 min wash in control solution (open diamond; $n = 3$). Note: 7 myocytes were exposed to active analog, but only 5 of these had prior exposure to inactive analog, and only 3 myocytes were held for a sufficient period to exhibit washout. *Significantly different from the value determined for control condition ($P < 0.05$) by repeated measures ANOVA followed by Bonferroni's test for comparisons of individual groups.

![Diagram](https://via.placeholder.com/150)

Fig. 9. PKC activation with the phorbol ester 4β-phorbol 12,13-dibutyrate (PDBu; 100 nM) inhibits bronchial smooth muscle $I_{\text{K(dr)}}$. A: representative families of whole cell currents evoked by 250-ms pulses to between $-80$ and $+30$ mV in 10-mV steps followed by repolarization to $-40$ mV during sequential exposure of a single bronchial myocyte to control bath solution (Control) and 100 nM PDBu. B: average I-V relations for end-pulse ($I_{\text{net}}$; circles) and tail ($I_{\text{tail}}$; squares) current amplitude normalized to cell capacitance in the absence of PDBu (Con; open symbols; $n = 4$) or presence of PDBu (closed symbols; $n = 4$). *Significantly different from the value determined for control condition ($P < 0.05$) by paired Student's t-test.
rabbit portal vein myocytes was also more negative (approximately −40 mV) than that observed in the present study, even though the $V_{0.5}$ for activation was similar: approximately −16 and −11 mV in bronchial and portal vein myocytes, respectively (2, 10). The physiological implication of the less negative voltage dependence of inactivation is that $I_{K(d)}$ of bronchial myocytes will contribute steady-state current over a wider range of membrane potentials and, therefore, its importance to control of membrane potential and contractile tone may be greater than in the trachea. The role of $I_{K(d)}$ was similarly concluded to be enhanced in small versus large pulmonary arterial vessels (6). In this case, however, smaller vessels were found to possess more cells with $I_{K(d)}$ (6).

We also found that the availability of $I_{K(d)}$ between −50 and −20 mV was decreased in tissues from sensitized compared with control dogs because of a negative shift in voltage dependence of inactivation. Although small in magnitude, this hyperpolarizing shift in availability of $I_{K(d)}$ is of potential significance to the electrical behavior of the hyperresponsive airways of ragweed pollen-sensitized dogs. The shift in the inactivation curve predicts that the amplitude of steady-state $I_{K(d)}$ will be reduced over the critical range of membrane potentials between −50 and −20 mV over which the L-type Ca$^{2+}$-window current also occurs (13, 14). Such small changes in current amplitude will have large effects on membrane potential in cells with a large input resistance, as is the case for smooth muscle cells (1−15 GΩ; see Ref. 31).

It is possible that the difference in $V_{0.5}$ for inactivation between tracheal and bronchial myocytes, as well as the negative shift in availability of $K_{dr}$ channels in the myocytes of sensitized dogs, may arise from the expression of different $K_{dr}$ channels in the two tissues. Voltage-gated K$^+$ channels consist of pore-forming α- and modulatory β-subunits. The voltage dependence of inactivation of voltage-gated K$^+$ channels is known to be affected by association with some β-subunits. For example, Kv1.5, which is the dominant Kv present in human bronchi (1), displays a more negative voltage dependence of inactivation but no change in kinetics when expressed in the presence of Kvβ2.1 (45). Alternatively, the formation of heterotetramers between different α-subunits of the Kv1 family, which possess distinct inactivation properties, can give rise to channels with novel intermediate properties, including differences in voltage dependence of inactivation (32). Therefore, differences in the expression of a β-subunit and/or increased formation of heteromultimers of Kv1.5 and Kv1.1, which are expressed in human airways (1), could be the cellular basis for the difference in inactivation of $I_{K(d)}$ in myocytes from trachea and bronchi, as well as the negative shift in availability of $I_{K(d)}$ in myocytes of sensitized compared with control dogs. Further studies will be required to address these issues.

In this study, we employed two structurally different analogs of DAG and a phorbol ester to determine the effect of activation of PKC on $I_{K(d)}$ of bronchial smooth muscle. The active DAG analog 1,2-diC8 and the phorbol ester PDBu were found to cause a decrease in the amplitude of $I_{K(d)}$, but the inactive analog 1,3-diC8 was without effect. This indicates that nonspecific effects of 1,2-diC8, for example, changes in membrane fluidity or direct channel block, were not involved. The activity of BK$_{Ca}$, L-type Ca$^{2+}$-, ATP-sensitive K$^+$, and nonselective cation channels in smooth muscle were previously reported to be affected by PKC activation (see Ref. 27 for a review). A change in these conductances also cannot account for the present data for the following reasons: 1) the effects of PKC activation on $K_{dr}$ were determined under conditions that minimized the contribution of BK$_{Ca}$ and Ca$^{2+}$-activated Cl$^-$ current to macroscopic current (high intracellular Ca$^{2+}$ chelation with 10 mM BAPTA and command potentials negative to +30 mV); 2) 1,2-diC8 had no effect on time-independent current under the recording conditions employed, suggesting that changes in ATP-sensitive K$^+$ and nonselective cation currents were not involved; and 3) activation of PKC was associated with a decline in $K_{dr}$ tail current amplitude at −40 mV where little contamination of macroscopic current by Ca$^{2+}$-current would be expected.

The ability of PKC activation to suppress $I_{K(d)}$ of bronchial myocytes is consistent with our previous observations on rabbit portal vein (2, 10). A family of at least 11 related PKC isoforms possessing different properties has been characterized, and at least five isoforms are known to be expressed in smooth muscle tissues (9, 17). The effects of 1,2-diC8 and PDBu on $I_{K(d)}$ of bronchial myocytes can be attributed to PKCδ, ε, or θ for the following reasons. PKCδ, β1, δ, ε, ζ, and θ were identified in immunoblots using isoform-specific antibodies from canine bronchial preparations (12). The low intracellular Ca$^{2+}$ level under our recording conditions (due to dialysis with pipette solution containing 10 mM BAPTA) likely precludes a role for the Ca$^{2+}$-dependent isoforms in the suppression of $I_{K(d)}$ in our experiments, and the Ca$^{2+}$-independent isoform PKCε is insensitive to DAG and phorbol esters (9). In rabbit portal vein, the effect of PKC on $I_{K(d)}$ was attributed to PKCc (10). Inhibition of $I_{K(d)}$ due to activation of PKC may contribute to dysfunctional control of membrane potential in asthma. This view is based on the following: 1) Histamine release is elevated in bronchi of sensitized animals (8). 2) Histamine and other contractile agonists are known to stimulate DAG production, leading to PKC activation in airway smooth muscle (35). 3) Activation of PKC causes a tonic increase in tone in human airway tissue; 100 nM PDBu produces a sustained contraction that is −40% of the maximal response to acetylcholine (34). A role for changes in membrane potential and activation of voltage-gated Ca$^{2+}$-channels is suggested by the fact that phorbol ester-induced contractions are dependent on extracellular Ca$^{2+}$ influx and inhibited by treatment with dihydropyridines (34). 4) PKC activation has been implicated in the pathogenesis of airway smooth muscle hyperreactivity (33, 35). In light of the ability of DAG analog and phorbol ester to depress $I_{K(d)}$, the lack of a difference in current density in bronchial myocytes of sensitized versus control dogs argues against an intrinsic difference in basal PKC activity in the two groups.
However, as noted above, the bronchi of the sensitized dog are known to release more histamine in response to antigen challenge compared with control animals (8). This difference in histamine release may be expected to produce 1) an extrinsic change in PKC activity and 2) an enhanced depression of $I_{Kdr}$ in situ that would not be observed in the isolated myocytes in the absence of antigen, mast cells, and/or histamine.

The changes in $I_{Kdr}$ of bronchial myocytes associated with ragweed pollen sensitization or PKC activation identified in this study may be two contributing factors to the mechanism(s) responsible for dysfunctional airway smooth muscle contractility in asthma. This view is based on compelling evidence that $K_{dr}$ contributes to the control of membrane potential and the level of depolarization of smooth muscle during exposure to contractile or relaxant stimuli (1, 10, 13, 25, 28, 31, 38). Previous studies on intact airways from ferrets and humans employing 4-AP and charybdotoxin to inhibit $K_{dr}$ and $BK_{Ca}$, respectively, indicated that $I_{Kdr}$ contributes to resting $K^+$ conductance but that $BK_{Ca}$ is not as important under basal conditions (1, 15). Treatment with 4-AP (in the presence of tetrodotoxin and atropine) caused depolarization of tracheal smooth muscle membrane potential by at least 5 mV and produced tonic contraction, the latter being sensitive to inhibition of L-type $Ca^{2+}$ channels with dihydropyridines (1, 15, 23). Tonic contractions due to steady-state $Ca^{2+}$ influx in airway smooth muscle are possible because of the presence of a small L-type $Ca^{2+}$ window current, over the same voltage range as that of $K_{dr}$ window current, which is capable of producing marked increases in intracellular $Ca^{2+}$ concentration (13). $I_{Kdr}$ activation has been postulated to function as a voltage-dependent, feedback mechanism for control of $Ca^{2+}$ influx in smooth muscle (11, 31); depolarization due to activation of inward currents in bronchial myocytes carried by T- or L-type $Ca^{2+}$, $Cl^-$, and/or nonselective cation channels (13, 19, 20, 21) is offset by the activation of outward $K^+$ current. In cerebral resistance arteries, inhibition of $I_{Kdr}$ with 4-AP causes enhanced myogenic reactivity to increased intraluminal pressure (25). $K_{dr}$ may function in parallel with a $Ca^{2+}$-dependent feedback mechanism involving $BK_{Ca}$ activation in response to changes in intracellular $Ca^{2+}$ concentration (31). Precise changes in the amplitude of outward current via these voltage- and $Ca^{2+}$-dependent feedback mechanisms would prevent regenerative $Ca^{2+}$ influx and permit the graded depolarizations and tone development observed in bronchi. Further studies are required to determine whether alterations in $BK_{Ca}$ channels also contribute to airway hyperresponsiveness.

This study identifies two different mechanisms by which the contribution of $K_{dr}$ to control of membrane potential may be reduced in airway smooth muscle in asthma: 1) a negative shift in voltage dependence of inactivation resulting in reduced steady-state $I_{Kdr}$ between $-40$ and $-20$ mV and 2) inhibition by PKC activation. Because the level of membrane potential is determined by a balance between inward and outward currents in smooth muscle (31), a decline in steady-state $I_{Kdr}$ due to a change in availability or suppression by PKC activation would result in depolarization and may contribute to the reported shift in membrane potential to more positive potentials in sensitized canine airway tissues (42). A reduction in steady-state $I_{Kdr}$ may also contribute to the development of airway hyperreactivity that is observed in asthma; contractile agonists are known to activate inward conductances, for example, L-type $Ca^{2+}$ (44), $Cl^-$, and nonselective cation currents (20, 21), and depolarize tracheal myocytes. A decline in the voltage-dependent feedback control of membrane potential may therefore result in enhanced depolarization, greater L-type $Ca^{2+}$ channel activation, and an increased rise in intracellular $Ca^{2+}$ concentration during exposure to contractile agonists. The net result would be greater development of tone and increased peak force, which are typical of hyperreactive airway tissues (4, 8, 33, 35). Further experiments on bronchial smooth muscle are required to assess 1) the role of $I_{Kdr}$ in depolarization of intact bronchial tissues to specific contractile agonists and 2) whether activation of phospholipase C-coupled receptors can produce a PKC-dependent decline in $K_{dr}$ channel activity in bronchial myocytes.

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