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

GARETH J. WALDRON,1 STEFAN B. SIGURDSSON,2 ERNESTO A. AIELLO,3 ANDREW J. HALAYKO,4 NEWMAN L. STEPHENS,4 AND WILLIAM C. COLE1

1Smooth Muscle Research Group, Faculty of Medicine, University of Calgary, Calgary, Alberta T2N 4N1; 2Department of Physiology, Faculty of Medicine, University of Manitoba, Winnipeg, Manitoba, Canada R3T 2N2; 3Department of Physiology, Faculty of Medicine, University of Iceland, Reykjavik, Iceland IS-101; and 4Centro de Investigaciones Cardiovasculares, Facultad de Medicina, Universidad Nacional de La Plata, 1900 La Plata, Argentina

Waldron, Gareth J., 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.

Airway smooth muscle; asthma; ragweed pollen sensitization; voltage-gated potassium channel; protein kinase C

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 hyperreactivity 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_d) 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 \( I_{K_{dr}} \) in cells isolated from a small-airway preparation, 2) determine whether the magnitude or properties of 4-AP-sensitive \( I_{K_{dr}} \) were altered in myocytes from bronchial tissues from ragweed pollen-sensitized dogs, and 3) assess the effect on the magnitude and properties of \( I_{K_{dr}} \) 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 passive cutaneous anaphylaxis in normal adult dogs. For the preliminary experiments to characterize the outward \( K^{+} \) conductance \( I_{K_{dr}} \) of bronchial myocytes, tissues were isolated from three adult mongrel dogs with a serum immunoglobulin E 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 from sensitized dogs were selected according to the method of Shioya et al. (37). The smooth muscle layer was dissected free of adherent epithelial cells and adventitia under a dissection microscope using fine iris scissors, placed in fresh ice-cold nominally \( Ca^{2+} \)-free Krebs-Henseleit solution, and stored overnight until tissue digestion the next day. Results of preliminary experiments, performed using myocytes dispersed from tissue on the same day as they were obtained, were not different from those in which the tissues were stored overnight. Freshly dispersed, relaxed single myocytes were prepared from tissue pieces of \( \sim 3 \) mm\(^2\) based on a method previously described (30). Briefly, bronchial smooth muscle tissues were placed in a low-\( Ca^{2+} \) physiological salt solution containing collagenase, protease, and elastase (for composition see below) and gently bubbled with 95\% \( O_2 \)-5\% \( CO_2 \) at 33–35°C for 20–35 min. After removal from the digestion solution, the pieces were washed several times in low-\( Ca^{2+} \) solution and stored at 4°C until 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.

**Fig. 1.** Light micrograph of a single bronchial myocyte isolated from a control dog. Scale bar indicates 20 µm.
K_{dr} CURRENT IN CANINE BRONCHIAL SMOOTH MUSCLE

1,2-bis(2-aminophenoxy)ethane-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. Current-voltage (I-V) relations for net and tail currents were determined in the following manner: net current due to I_{K(dr)} 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.

Drugs: 1,2-bis(2-aminophenoxy)ethane-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. Current-voltage (I-V) relations for net and tail currents were determined in the following manner: net current due to I_{K(dr)} 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.

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 ± 338 (n = 13) µm² (P > 0.05) were obtained based on a specific membrane capacitance of 1 µF/cm² 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 (a 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+ current and permit selective recordings of \( I_{\text{K}}(\text{dr}) \) of bronchial myocytes, we employed 1) a pipette solution containing 10 mM BAPTA to provide a high level of internal Ca\(^{2+}\) 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. 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 \( I_{\text{K}}(\text{dr}) \), with slow inactivation during the command step. These data suggest that the tail currents were exclusively due to deactivating \( K_{\text{dr}} \) and that net current at the end of the command pulses was almost completely due to \( I_{\text{K}}(\text{dr}) \) 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 \( I_{\text{K}}(\text{dr}) \) and large-conductance Ca\(^{2+}\)-activated K+ current (\( I_{\text{BKCa}} \)).

Characteristics of \( I_{\text{K}}(\text{dr}) \) in bronchial myocytes from control dogs. The kinetics and voltage dependence of activation, deactivation, and inactivation of bronchial smooth muscle \( I_{\text{K}}(\text{dr}) \) 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 \( I_{\text{K}}(\text{dr}) \) was apparent during command pulses to potentials positive to \(0\) mV. Longer-duration, 4- to 10-s steps to \(+20\) mV...

Fig. 2. Macroscopic currents of canine bronchial myocytes recorded with minimal internal Ca\(^{2+}\) chelation (0.05 mM EGTA in pipette solution). A: representative family of whole cell currents recorded from a bronchial myocyte of a control dog during 250-ms pulses to between \(-80\) and \(+30\) mV in 10-mV intervals followed by repolarizing steps to \(-40\) mV applied every 10 s. Arrow indicates end-pulse current amplitude used in current-voltage (I-V) plot of B. Dashed line in this and other figures represents zero current. B: average I-V relation for macroscopic current in four bronchial myocytes from control dogs recorded in the presence of minimal internal Ca\(^{2+}\) chelation. Data points are mean end-pulse current amplitudes normalized to cell capacitance \(\pm\) SE. C: representative quasi-steady-state macroscopic current recorded during a 16-s voltage-ramp protocol applied between \(-90\) and \(+30\) mV.

Fig. 3. Inhibition of noisy macroscopic current due to large-conductance Ca\(^{2+}\)-activated K+ channels with tetraethylammonium chloride (TEA\(^{+}\)). A: representative quasi-steady-state macroscopic currents evoked by an 8-s voltage-ramp protocol (to increase amplitude of noisy current) between \(-90\) and \(+30\) mV in the absence [control (Con)] and presence of 0.1 mM TEA\(^{+}\). B: TEA\(^{+}\)-sensitive difference current obtained by digital subtraction of current trace recorded in the presence of TEA\(^{+}\) from control trace.
were applied to bronchial myocytes to examine the time dependence of inactivation. \( I_{K(dr)} \) 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 describe the inactivation kinetics, and, at +20 mV, the average values for the fast and slow time constants (Table 1) were similar to those previously reported for other smooth muscle cells (e.g., 0.14 and 1.1 s at +5 mV for canine tracheal myocytes; see Ref. 7). The voltage dependence of \( I_{K(dr)} \) activation and inactivation was studied. Figure 5 illustrates representative examples of 1) tail currents recorded at −40 mV after steps to between −80 and +30 mV (Fig. 5A and 2) whole cell currents evoked by a double-pulse protocol that employed 4-s conditioning pulses to between −110 and +20 mV followed by command steps of 200-ms duration to +20 mV (Fig. 5B). Figure 5C shows the average values for normalized peak tail current versus voltage and normalized \( I_{K(dr)} \) availability as a function of command and conditioning step voltages, respectively. Peak tail current amplitude at −40 mV after command steps to between −80 and +30 mV was measured (i.e., peak minus sustained amplitude after 200 ms), normalized to the maximal amplitude, and plotted as a function of command step voltage. Availability after a 4-s conditioning pulse was determined from the amplitude of current present at the end of 200-ms pulses to +20 mV and plotted as a function of conditioning pulse voltage. The plots of normalized current versus voltage of the command step or conditioning pulse were best fit with single Boltzmann functions (Fig. 5). Values for half-maximal potential (\( V_{0.5} \)) and slope for activation and inactivation curves from 15 and 9 myocytes, respectively, were averaged and are given in Table 1. Similar values for these parameters were also obtained in two smooth muscle cells (e.g., 0.14 and 1.1 s at +5 mV for canine tracheal myocytes; see Ref. 7).

Comparison of \( I_{K(dr)} \) in bronchial myocytes from control and sensitized dogs. Whether the properties of \( I_{K(dr)} \) were altered in myocytes obtained from ragweed pollen-sensitized dogs was determined by applying similar voltage-clamp protocols under identical recording conditions to myocytes from control dogs. The properties of \( I_{K(dr)} \) in these myocytes were similar to those in myocytes obtained from control dogs (Fig. 5A, 5B), and the data for these myocytes were averaged and are given in Table 1. The voltage dependence of \( I_{K(dr)} \) activation and inactivation was studied. Figure 5 illustrates representative examples of 1) tail currents recorded at −40 mV after steps to between −80 and +30 mV (Fig. 5A and 2) whole cell currents evoked by a double-pulse protocol that employed 4-s conditioning pulses to between −110 and +20 mV followed by command steps of 200-ms duration to +20 mV (Fig. 5B). Figure 5C shows the average values for normalized peak tail current versus voltage and normalized \( I_{K(dr)} \) availability as a function of command and conditioning step voltages, respectively. Peak tail current amplitude at −40 mV after command steps to between −80 and +30 mV was measured (i.e., peak minus sustained amplitude after 200 ms), normalized to the maximal amplitude, and plotted as a function of command step voltage. Availability after a 4-s conditioning pulse was determined from the amplitude of current present at the end of 200-ms pulses to +20 mV and plotted as a function of conditioning pulse voltage. The plots of normalized current versus voltage of the command step or conditioning pulse were best fit with single Boltzmann functions (Fig. 5). Values for half-maximal potential (\( V_{0.5} \)) and slope for activation and inactivation curves from 15 and 9 myocytes, respectively, were averaged and are given in Table 1. Similar values for these parameters were also obtained in two smooth muscle cells (e.g., 0.14 and 1.1 s at +5 mV for canine tracheal myocytes; see Ref. 7).

Comparison of \( I_{K(dr)} \) in bronchial myocytes from control and sensitized dogs. Whether the properties of \( I_{K(dr)} \) were altered in myocytes obtained from ragweed pollen-sensitized dogs was determined by applying similar voltage-clamp protocols under identical recording conditions to myocytes from control dogs.
the physiological range of membrane potential between
that the voltage dependence of availability of
I
\(_\text{K}\text{(dr)}\) was altered in cells from ragweed pollen-sensitized dogs.

In contrast to the lack of change in density, voltage
dependence of activation, and kinetics of I
\(_\text{K}\text{(dr)}\), we found that the voltage dependence of availability of I
\(_\text{K}\text{(dr)}\) was altered in cells from ragweed pollen-sensitized dogs. The availability of I
\(_\text{K}\text{(dr)}\) was significantly reduced over the physiological range of membrane potential between
\(-20\) and \(-40\) mV due to a leftward shift in the voltage
dependence of inactivation (Fig. 5). Table 1 shows that the average value for V0.5 in the myocytes from sensi-
tized animals was \(-4\) mV more negative compared
with that of control dogs. No change in the slope factor
for the voltage dependence of inactivation between the
two groups was apparent.

Inhibition of I
\(_\text{K}\text{(dr)}\) 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 pathogen-
esis of asthma and bronchospasm of hyperresponsive
airways of sensitized animals (4, 17, 35). Accordingly,
we determined the effects on I
\(_\text{K}\text{(dr)}\) of direct PKC activa-
tion with an analog of DAG, 1,2-diC8, and the phorbol
ester PDBu. Eleven myocytes from control and sensi-
tized dogs were used (6 and 5 myocytes, respectively),
and a similar change in I
\(_\text{K}\text{(dr)}\) during PKC activation
was observed in all cells tested. Figure 7 shows the
effects of the inactive DAG analog 1,3-diC8 followed by
1,2-diC8 on I
\(_\text{K}\text{(dr)}\) amplitude. To monitor the time course
of effects of the DAG analogs on I
\(_\text{K}\text{(dr)}\) amplitude,
depolarizing steps to +10 mV followed by a brief step to
240 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 IₑK(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 IₑK(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 IₑK(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 IₑK(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 IₑK(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 Kₑ and average changes in IₑK(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 IₑK(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 IₑK(dr) in bronchial myocytes is different from that in the trachea, 2) inactivation is shifted to more negative poten-
nal current in canine bronchial smooth muscle.

Two K⁺ channels, BKCa and Kdr, were shown to contribute to macroscopic currents of canine bronchial myocytes on the basis of pharmacology, Ca²⁺ sensitivity, and kinetic behavior. Noisy macroscopic current carried by BKCa was present positive to −20 mV with minimal intracellular Ca²⁺ chelation and was selectively inhibited with 0.1 mM TEA⁺. In contrast, macroscopic current due to Kdr was insensitive to low TEA⁺ and high levels of intracellular Ca²⁺ 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). Idr of canine bronchial myocytes was studied using 10 mM BAPTA in the pipette to provide for substantial chelation of intracellular Ca²⁺ and minimal contamination of whole cell currents by Ca²⁺-activated conductances, such as BKCa, Ca²⁺-activated Cl⁻, and Ca²⁺-dependent nonselective cation currents. Additionally, depolarizing voltage-clamp steps were limited to potentials of +30 mV or less to avoid voltage-dependent activation of BKCa. The ability of 4-AP to substantially inhibit tail currents produced during deactivation of Kdr at −40 mV as well as end-pulse current amplitude is consistent with minimal contamination by non-Kdr channel activity. The presence of Idr 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 Idr 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-diC₈ resulted from the change in Idr; see Refs. 2 and 10) indicating its absence or complete inactivation at the holding potential (~60 mV) employed. A dual-exponential fit of Idr 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 Idr of canine bronchial myocytes is activated at voltages positive 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 Kdr window current may be present between approximately −45 and 0 mV, with peak outward Idr at approximately −25 mV. These properties are similar to those previously reported for Idr of ferret tracheal myocytes; Kdr 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 Idr activation, but not inactivation, of Kdr 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 Idr 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 Kdr channel.

Fig. 7. Time course of effect of diacylglycerol analogs on Idr.

A: representative changes in end-pulse current amplitude (I) evoked by 250-ms pulses from −60 to +10 mV and normalized to the amplitude under control conditions at time 0 (I₀). Pulses were applied at 15-s intervals under control conditions and during sequential exposure to 10 µM 1,3-diC₈ and 1 and 10 µM 1,2-diC₈. Insets: representative traces of current recordings in the absence of drug and in the presence of either 1,3-diC₈ or 1,2-diC₈ at 1 and 10 µM as indicated by arrows. B: superimposed, 1,2-diC₈-sensitive difference currents obtained by digital subtraction of macroscopic current evoked by a similar protocol as in A applied at 30-s intervals during exposure to 1,2-diC₈ (10 µM) from current recorded in the absence of drug. Open and closed circles indicate difference currents for pulses applied immediately after switching to bath solution containing diacylglycerol analog and during peak, sustained effect of the drug, respectively.

Although inactivation of Idr properties are similar to those previously reported for Idr of ferret tracheal myocytes; Kdr 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 Idr activation, but not inactivation, of Idr 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 Idr 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 Kdr 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 $I_{K(\text{dr})}$ of

Fig. 8. Protein kinase C (PKC) activation with diacylglycerol analog reversibly inhibits bronchial smooth muscle $I_{K(\text{dr})}$. 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-diC8, 10 µM 1,2-diC8, 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-diC8 (open circle; n = 5) or 1,2-diC8 (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.

Fig. 9. PKC activation with the phorbol ester 4β-phorbol 12,13-dibutyrate (PDBu; 100 nM) inhibits bronchial smooth muscle $I_{K(\text{dr})}$. 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,(dr)} 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,(dr)} 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,(dr)} (6).

We also found that the availability of I_{K,(dr)} 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,(dr)} 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,(dr)} 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 heterotetramers 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,(dr)} in myocytes from trachea and bronchi, as well as the negative shift in availability of I_{K,(dr)} 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,(dr)} of bronchial smooth muscle. The active DAG analog 1,2-diC_{8} and the phorbol ester PDBu were found to cause a decrease in the amplitude of I_{K,(dr)}, but the inactive analog 1,3-diC_{8} was without effect. This indicates that nonspecific effects of 1,2-diC_{8}, 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-diC_{8} 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,(dr)} 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-diC_{8} and PDBu on I_{K,(dr)} of bronchial myocytes can be attributed to PKCd, ε, or δ for the following reasons. PKCb1, 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,(dr)} in our experiments, and the Ca^{2+}-independent isoform PKCζ is insensitive to DAG and phorbol esters (9). In rabbit portal vein, the effect of PKC on I_{K,(dr)} was attributed to PKCe (10). Inhibition of I_{K,(dr)} 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,(dr)}, 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 $I_{K_{dr}}$ in situ that would not be observed in the isolated myocytes in the absence of antigen, mast cells, and/or histamine.

The changes in $I_{K_{dr}}$ 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 $I_{K_{dr}}$ 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). $I_{K_{dr}}$ 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 $I_{K_{dr}}$ 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 $I_{K_{dr}}$ 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 $I_{K_{dr}}$ 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 $I_{K_{dr}}$ 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 $I_{K_{dr}}$ 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.

We thank Fabrice Chomienne for expert assistance in the isolation of single myocytes.

This study was supported by grants from the Medical Research Council of Canada (MT-13506 to W. C. Cole), Respiratory Health National Centres of Excellence (N. L. Stephens), and Icelandic University and Science Foundations (S. B. Sigurdsson). G. J. Waldron is a Fellow of the Canadian Hypertension Society and Medical Research Council of Canada. S. B. Sigurdsson and E. A. Aiello were Alberta Heritage Foundation for Medical Research Visiting Scientists. W. C. Cole is a Senior Scholar of the Alberta Heritage Foundation for Medical Research.

Address for reprint requests: W. C. Cole, Smooth Muscle Research Group, Univ. of Calgary, 3330 Hospital Dr., NW, Calgary, Alberta, Canada T2N 4N1.

Received 23 December 1997; accepted in final form 7 April 1998.

REFERENCES


