Sensory Nerve Terminal Mitochondrial Dysfunction Induces Hyperexcitability in Airway Nociceptors via Protein Kinase C

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ABSTRACT

Airway sensory nerve excitability is a key determinant of respiratory disease-associated reflexes and sensations such as cough and dyspnea. Inflammatory signaling modulates mitochondrial function and produces reactive oxygen species (ROS). Peripheral terminals of sensory nerves are densely packed with mitochondria; thus, we hypothesized that mitochondrial modulation would alter neuronal excitability. We recorded action potential firing from the terminals of individual bronchopulmonary C-fibers using a mouse *ex vivo* lung-vagal ganglia preparation. C-fibers were characterized as nociceptors or non-nociceptors based upon conduction velocity and response to transient receptor potential (TRP) channel agonists. Antimycin A (mitochondrial complex III Q_i site inhibitor) had no effect on the excitability of non-nociceptors. However, antimycin A increased excitability in nociceptive C-fibers, decreasing the mechanical threshold by 50% and increasing

the action potential firing elicited by a P2X_{2/3} agonist to 270% of control. Antimycin A-induced nociceptor hyperexcitability was independent of TRP ankyrin 1 or TRP vanilloid 1 channels. Blocking mitochondrial ATP production with oligomycin or myxothiazol had no effect on excitability. Antimycin A-induced hyperexcitability was dependent on mitochondrial ROS and was blocked by intracellular antioxidants. ROS are known to activate protein kinase C (PKC). Antimycin A-induced hyperexcitability was inhibited by the PKC inhibitor bisindolylmaleimide (BIM) I, but not by its inactive analog BIM V. In dissociated vagal neurons, antimycin A caused ROS-dependent PKC translocation to the membrane. Finally, H₂O₂ also induced PKC-dependent nociceptive C-fiber hyperexcitability and PKC translocation. In conclusion, ROS evoked by mitochondrial dysfunction caused nociceptor hyperexcitability via the translocation and activation of PKC.

Introduction

A large subset of sensory nerves in the somatosensory and visceral systems is nociceptive; it is adapted to detect noxious and potential noxious stimuli and then trigger sensations, behaviors, and reflexes that are intended to diminish future noxious threats. Nevertheless, nociceptor excitability is not static and, in damaged and inflamed tissues, nociceptors can become hyperexcitable such that subthreshold stimuli inappropriately evoke nociceptive responses or superthreshold stimuli evoke inappropriately large responses. The bronchopulmonary airways are densely innervated by nociceptive vagal C-fibers, whose activation leads to the initiation of reflexes (cough, hypersecretion, bronchospasm) and sensations (dyspnea, itch, urge to cough) (Carr and Udem, 2003). Airway nociceptive reflexes are exacerbated in inflammatory and infectious airway disease (Riccio et al., 1996; Carr and Lee, 2006; Sabogal et al., 2005; Nassenstein et al., 2007), most clearly observed clinically as an excessive and hyperresponsive cough in respiratory disease (Jesenak et al., 2009; Morice, 2010). As such, there is considerable interest in

understanding the mechanisms through which inflammation and other noxious stimuli modulate nociceptor excitability.

Increasing evidence suggests that mitochondrial dysfunction contributes to inflammation in multiple tissues via the actions of mitochondrially evoked reactive oxygen species (ROS) (Reddy, 2011; Sena and Chandel, 2012). DNA mutations leading to aberrant mitochondrial protein function/expression have been known for many years to dramatically impact ATP production and ROS signaling. Recently, it was shown that numerous signaling events can also increase mitochondrial ROS production, for example, hypoxia (Bell et al., 2007), tumor necrosis factor- α (Corda et al., 2001), neurotrophins via p75NTR (Pehar et al., 2007), transforming growth factor- β (Michaeloudes et al., 2011), and Toll-like receptors (West et al., 2011). This is likely of particular importance for the peripheral terminals of sensory nerves, which contain a high density of mitochondria (Hung et al., 1973; von Düring and Andres, 1988). We have previously shown an interaction between nerve terminal mitochondria and nerve terminal function in a study that demonstrated that mitochondrial ROS can cause overt bronchopulmonary C-fiber activation (action potential discharge) via the selective gating of transient receptor potential (TRP) ankyrin 1 (TRPA1) (Nesushvili et al., 2013). It is not yet known

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ABBREVIATIONS: AITC, allyl isothiocyanate; BIM, bisindolylmaleimide; DAG, diacylglycerol; DRG, dorsal root ganglion; DTT, dithiothreitol; GSH, glutathione; α,β -mATP, α,β -methylene ATP; MnTMPyP, manganese(III) tetrakis(1-methyl-4-pyridyl)porphyrin; NAC, *N*-acetylcysteine; PBST, phosphate-buffered saline with 0.0025% Tween 20; PKC, protein kinase C; PMA, phorbol-12-myristate-13-acetate; ROS, reactive oxygen species; TRP, transient receptor potential; TRPA1, TRP ankyrin 1; TRPV1, TRP vanilloid 1.

whether mitochondrial ROS can also modulate neuronal excitability.

To study the effect of mitochondrial dysfunction and ROS on bronchopulmonary C-fiber excitability, we have used antimycin A, a selective inhibitor of the Q_i site on complex III in the mitochondrial electron transport chain. This selective inhibitor stimulates mitochondrial superoxide formation and the subsequent production of numerous ROS (Turrens et al., 1985; Gyulkhandanyan and Pennefather, 2004; Tretter et al., 2007; Stowe and Camara, 2009). Cellular oxidation can modulate the function of numerous proteins, including ion channels and enzymes (Thannickal and Fanburg, 2000; Stowe and Camara, 2009). In particular, protein kinase C (PKC) can be activated by ROS independently of Ca^{2+} or diacylglycerol (DAG) (Gopalakrishna and Anderson, 1989; Knapp and Klann, 2000). PKC activation is known to induce nociceptor hyperexcitability in somatosensory neurons via its phosphorylation of voltage- and ligand-gated ion channels (Cesare et al., 1999; Amadesi et al., 2004; Baker, 2005; Wu et al., 2012). In this study, we found that mitochondrial ROS induced hyperexcitability of nociceptive bronchopulmonary C-fibers via oxidative activation and translocation of PKC.

Materials and Methods

All experiments were approved by the University of South Florida Institutional Animal Care and Use Committee.

Bronchopulmonary C-Fiber Extracellular Recordings

Male C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME) were killed by CO_2 asphyxiation, followed by exsanguination. The innervated isolated lung preparation was prepared, as previously described (Nassenstein et al., 2008; Nesuashvili et al., 2013). Briefly, the airways and lungs with their intact extrinsic innervation (vagus nerve including vagal ganglia) were taken and placed in a dissecting dish containing Krebs bicarbonate buffer solution composed of the following (mM): 118 NaCl, 5.4 KCl, 1.0 NaH_2PO_4 , 1.2 $MgSO_4$, 1.9 $CaCl_2$, 25.0 $NaHCO_3$, and 11.1 D-glucose, and equilibrated with 95% O_2 and 5% CO_2 (pH 7.3–7.4) and containing 3 μM indomethacin. Connective tissue was trimmed away, leaving the trachea and lungs with their intact nerves. The airways were then pinned to the larger compartment of a custom-built two-compartment recording chamber that was lined with silicone elastomer. A vagal ganglion was pulled into the adjacent compartment of the chamber through a small hole and pinned. Both compartments were separately superfused with Krebs bicarbonate buffer (37°C). A sharp glass electrode was pulled by a Flaming Brown micropipette puller (P-87; Sutter Instruments, Novato, CA) and filled with 3 M NaCl solution. The electrode was inserted and placed near the cell bodies of vagal ganglion. The recorded action potentials were amplified (Microelectrode AC amplifier 1800; A-M Systems, Everett, WA), filtered (0.3 kHz low cutoff and 1 kHz high cutoff), and monitored on an oscilloscope (TDS1002B; Tektronix, Beaverton, OR). The scaled output from the amplifier was captured and analyzed by a Macintosh computer using NerveOfft software (Phocis, Baltimore, MD). Action potential discharge was quantified off-line and recorded in 1-second bins. The background activity was usually either absent or less than 2 Hz.

Nerve Fiber Characterization. To measure conduction velocity, an electrical stimulation (S44; Grass Instruments, Quincy, MA) was applied to the center of the receptive field. The conduction velocity of the individual bronchopulmonary afferent was calculated by dividing the distance along the nerve pathway by the time delay between the shock artifact and the action potential evoked by electrical stimulation. Mechanical stimulation was determined using manual application of calibrated von Frey fibers to the center of the receptive field.

C-fibers were also characterized for their expression of TRPA1 and TRP vanilloid 1 (TRPV1) channels by their response to the selective TRPA1 agonist allyl isothiocyanate (AITC; 300 μM), and to the selective TRPV1 agonist capsaicin (1 μM). Chemical stimuli were intratracheally applied as a 1-ml bolus over 10 seconds. A response to either mechanical or chemical stimuli was considered positive if the number of action potentials in any 1-second bin was twice the average background response. All excitatory chemical treatments were separated by at least a 15-minute wash. Due to potential heterologous desensitization, capsaicin was only given at the end of each experiment. Only those bronchopulmonary fibers that had a positive response to both mechanical and chemical stimuli were included in the study. Due to a lack of TRPV1 channels in bronchopulmonary C-fibers from TRPV1^{-/-} mice, nociceptive status was assessed solely by conduction velocity and the response to TRPA1 agonists (Kollarik et al., 2003; Nassenstein et al., 2008).

Determination of Hyperexcitability via Changes in Mechanical Threshold. The threshold for mechanical pressure required to evoke action potential discharge was determined by progressively decreasing the weight of von Frey fiber applied to the lung receptive field until no response was evoked. The least force required to evoke mechanically induced action potential firing was then repeated once more to confirm threshold reproducibility (Riccio et al., 1996). The lungs were then treated with antimycin A (20 μM). Ten minutes later, von Frey fibers were used to determine the mechanical threshold again following mitochondrial modulation. Hyperexcitability was quantified by comparing the mechanical threshold before and after mitochondrial modulation (paired analysis). Due to the irreversible inhibition of complex III by antimycin (Turrens et al., 1985; Stowe and Camara, 2009), only one antimycin A treatment was used per preparation.

Determination of Hyperexcitability via Changes in Response to Chemical Stimulus. We determined the action potential discharge response to α,β -methylene ATP (α,β -mATP; P2X_{2/3} agonist, 30 μM) intratracheally applied as a 1-ml bolus over 10 seconds (Nassenstein et al., 2010). This submaximal dose is sufficient to activate all P2X_{2/3} expressing bronchopulmonary C-fibers (Nassenstein et al., 2010). The lungs were then treated with antimycin A (20 μM) or other mitochondrial modulators. Ten minutes later, the action potential discharge response to α,β -mATP (30 μM) was again determined. Hyperexcitability to α,β -mATP treatment was quantified in raw form in two ways, as follows: either by comparing the total number of action potentials discharged within 120 seconds or comparing the peak response (maximum number of action potentials that occurred within any 1-second bin) before and after mitochondrial modulation (both paired analyses). Due to variations in responsiveness to α,β -mATP between bronchopulmonary C-fibers, we also normalized each fiber's second response to α,β -mATP to its first α,β -mATP response, as follows: (total number of action potentials discharged in second response/total number of action potentials discharged in first response) $\times$ 100. This normalized quantification of excitability could then be compared between different treatment groups in our mechanistic studies (unpaired analysis). Due to the irreversible inhibition of complex III by antimycin (Turrens et al., 1985; Stowe and Camara, 2009), only one antimycin A treatment was used per preparation.

Knockout Mice

Male TRPV1^{-/-} (The Jackson Laboratory; B6.129X1-Trpv1tm1Jul/J) were mated with female TRPV1^{-/-}. Genotype of offspring was confirmed by polymerase chain reaction.

Dissociation of Mouse Vagal Ganglia

Male C57BL/6J mice were killed by CO_2 asphyxiation, followed by exsanguination. Vagal ganglia were immediately isolated and enzymatically dissociated using previously described methods (Nesuashvili et al., 2013). Isolated neurons were plated onto poly

(D-lysine)– and laminin-coated coverslips, incubated at 37°C in L-15 (supplemented with 10% fetal bovine serum), and used within 24 hours.

PKC Translocation Immunocytochemistry

Dissociated mouse vagal neurons on glass coverslips were treated with either antimycin A (10 μ M), phorbol-12-myristate-13-acetate (PMA; 300 nM), or H₂O₂ (300 μ M) by direct addition to culture media. Neurons were then fixed 2 minutes later using fresh 4% para-formaldehyde to preserve PKC subcellular location. In some cases, antioxidants were applied to media prior to antimycin A, as follows: 90 minutes prior for tempol (1 mM); 15 minutes prior for MnTMPyP [manganese(III) tetrakis(1-methyl-4-pyridyl)porphyrin; 50 μ M]. Neurons were then permeabilized using phosphate-buffered saline containing 0.1% Triton X-100 for 10 minutes at room temperature and blocked using phosphate-buffered saline with 0.0025% Tween 20 (PBST) containing 2% BSA for 45 minutes at room temperature. Cells were stained using a pan-PKC primary antibody (H-300; Santa Cruz Biotechnology, Santa Cruz, CA). Antibody concentrated to 0.5 mg/ml was diluted 1:50 in blocking buffer and left on cells overnight at 4°C. Cells were washed three times using PBST and then incubated with a CY5-labeled anti-rabbit secondary antibody (Life Technologies, Carlsbad, CA) diluted 1:1000 in blocking buffer and left on cells for 1 hour at room temperature. After three washes with PBST, coverslips were mounted onto glass slides using VECTASHIELD(R) HardSet Mounting Medium with 4',6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA). Imaging was performed using a Leica SP2 confocal microscope with a 63 $\times$ objective, as follows: 4',6-diamidino-2-phenylindole excitation of 405 nm, emission range of 415–480 nm; Cy5 excitation of 633 nm, emission range of 625–735 nm. Analysis of staining intensity was performed using ImageJ. A straight line was drawn across each cell in a region excluding the nucleus, and the Multi-plot function was used to plot the profile of the staining. The ratio of the peak staining at the edge of the cell to the average staining across the center of the cell was generated to give the membrane/cytosolic ratio.

Statistical Analysis

Data were analyzed using GraphPad Prism (GraphPad Software, La Jolla, CA). Where appropriate, paired or unpaired Student's *t* test was used. A *P* value less than 0.05 was taken as significant. All data were expressed as mean $\pm$ S.E.M.

Results

Antimycin A Decreases Mechanical Thresholds in Nociceptive C-Fibers. To study the effect of mitochondrial modulation on the excitability of bronchopulmonary C-fiber terminals, we have used a mouse ex vivo lung-vagal ganglia preparation. In this preparation, the action potential discharge evoked in individual nerves by either mechanical or chemical stimuli to the airways can be quantified. Previous studies have shown that the majority of fibers innervating the lung are slowly conducting and express TRPA1 and TRPV1 (Nassenstein et al., 2008; Nesushvili et al., 2013). This subset of nerves is thought to serve as nociceptors in the mouse airways. In this study, we characterized bronchopulmonary sensory fibers based on their conduction velocity and their responsiveness to the selective TRPA1 and TRPV1 agonists (AITC and capsaicin, respectively). Bronchopulmonary fibers were considered nociceptive if they expressed TRPV1. As described before (Kollarik et al., 2003), TRPV1 expression was largely confined to fibers with conduction velocities <0.7 m/s.

We first studied the effect of antimycin-induced mitochondrial modulation on the mechanical threshold for bronchopulmonary fibers using punctate stimuli of the lung tissue with von Frey fibers. All bronchopulmonary fibers are directly activated by punctate force (Kollarik et al., 2003; Nassenstein et al., 2008) in a P2X-independent manner (Weigand et al., 2012), and in this study a maximum von Frey stimulus (1.4 g) evoked peak action discharge of 16.8 ± 1.7 Hz. In this work, we determined the minimum force required to evoke action potential discharge in each fiber using the rationale that an increase in excitability would decrease the threshold for mechanically induced action potential discharge (Riccio et al., 1996). Each fiber's threshold was stable over the duration of experimentation (Fig. 1A). Antimycin A (20 μ M) evoked significant action potential discharge in TRPA1-expressing bronchopulmonary fibers via a ROS-dependent TRPA1 activation—this mechanism is described in our previous study (Nesushvili et al., 2013). The direct activation of these TRPA1-expressing nerves lasted from 2 to 7 minutes; other fibers were not overtly activated. We tested the mechanical threshold 10 minutes following antimycin A treatment and compared with the mechanical threshold previous to antimycin A treatment. Antimycin A (20 μ M) decreased the threshold for mechanical stimulation by approximately 50% in nociceptive neurons ($P < 0.005$, $n = 13$; Fig. 1, B and C). Seven of these 13 fibers were TRPA1-expressing nociceptors, but there was no correlation of expression of TRPA1 and the antimycin A-induced threshold decrement (data not shown). However, antimycin A had no effect on the threshold for mechanical stimulation for faster conducting, non-nociceptive bronchopulmonary fibers ($P > 0.05$, $n = 6$; Fig. 1C), which do not express TRPA1 or TRPV1.

Antimycin A Increases Excitability to Chemical Stimuli in Nociceptive C-Fibers. The standardization of manually operated von Frey fiber mechanical stimulation of C-fibers can be challenging, particularly in a three-dimensional tissue, such as the lung. We therefore extended our study of mitochondrial modulation-induced hyperexcitability to determine its effects on bronchopulmonary C-fiber responses to chemical stimuli. Previous studies showed that the vast majority of mouse bronchopulmonary sensory nerves are activated by the selective P2X_{2/3} agonist α,β -mATP and that this response is reproducible (Kollarik et al., 2003; Nassenstein et al., 2010). Consistent with these reports, 30 μ M α,β -mATP activated >90% of bronchopulmonary C-fibers regardless of their conduction velocity or TRP channel expression. To confirm the reproducibility of P2X_{2/3} receptor responses, we determined the action potential discharge to repeated treatment of 30 μ M α,β -mATP in 18 nociceptive C-fibers (separated by 15 minutes). There was no significant difference in either the total number of action potentials evoked (first, 64.2 ± 9.5 ; second, 62.7 ± 11.1 ; Fig. 2B) or the peak response (first, 17.7 ± 2.3 Hz; second, 15.9 ± 2.21 Hz; Fig. 2C).

Ten minutes after treatment with 20 μ M antimycin A, the response to 30 μ M α,β -mATP was significantly increased in nociceptive C-fibers ($n = 24$; Fig. 2A), as assessed by the total number of action potentials evoked (first, 105 ± 34.9 ; second, 220 ± 79.4 ; $P < 0.01$) (Fig. 2B) and the peak response (first, 15.4 ± 1.5 Hz; second, 20.5 ± 1.7 Hz; $P < 0.01$) (Fig. 2C).

Fig. 1. Antimycin A decreases the threshold required for mechanical stimulation in nociceptive C-fibers. (A) Representative trace of action potential discharge elicited by von Frey fibers [a = 0.4 g (threshold); b = 0.16 g (subthreshold)] in a nociceptive bronchopulmonary C-fiber [defined by conduction velocity (CV) and sensitivity to capsaicin (data not shown)]. Inserts show magnified timescale of responses. (B) Representative trace of mechanical threshold determination in a nociceptive bronchopulmonary C-fiber before (control) and 10 minutes after treatment with antimycin A (20 μ M). Application of von Frey stimulus denoted by arrows. (C) Mean $\pm$ S.E.M. mechanical threshold for action potential discharge before (control, hatched bars) and 10 minutes after treatment with antimycin A (20 μ M, black bars) in nociceptive and non-nociceptive bronchopulmonary C-fibers, as defined by sensitivity to TRPV1 agonist (capsaicin, 1 μ M) and conduction velocity (Kollarik et al., 2003). Significant difference in mechanical sensitivity after antimycin A, * P < 0.005.

Similar to our mechanical threshold data, antimycin A failed to induce hyperexcitability to α,β -mATP in non-nociceptive bronchopulmonary fibers (n = 9).

The action potential discharge to α,β -mATP varied significantly from nerve to nerve, as follows: of 189 fibers examined in the present study, the total number of action potentials evoked ranged from 16 to 280, and the peak discharge ranged from 3 to 37. To account for the intrinsic variability in neuronal responses, we quantified excitability by the change in the response to the second α,β -mATP treatment (i.e., after antimycin A) as a percentage of each individual fiber's response to the first α,β -mATP treatment. As such, antimycin increased nociceptor excitability to $276 \pm 54\%$ (n = 24) compared with vehicle, which had no effect on excitability ($105 \pm 14\%$, n = 18, P < 0.005) (see Figs. 3B and 4, A and B).

Given that our previous findings had shown that mitochondrial ROS evoked action potential discharge in nociceptors in a TRPA1-dependent manner, we compared the antimycin A-induced excitability in nociceptors that specifically expressed TRPA1 (n = 8) with those nociceptors that did not express TRPA1 (n = 16). Antimycin A increased excitability in both subsets of nociceptive C-fibers (Fig. 3A), indicating that TRPA1 expression was not required for mitochondrial modulation-associated hyperexcitability to α,β -mATP. There was a trend toward a greater increase in excitability to α,β -mATP in TRPA1-negative C-fibers—perhaps due to mild nonspecific tachyphylaxis occurring following substantial TRPA1-mediated depolarization—although this did not reach statistical significance (P = 0.19).

Our data demonstrating that antimycin A evoked hyperexcitability only in nociceptors also led us to test the hypothesis that TRPV1, which is expressed exclusively in almost all nociceptors, was involved. Using a pretreatment of the selective TRPV1 antagonist iodo-resiniferatoxin (1 μ M, n = 8) (Wahl et al., 2001), we found that antimycin A-induced sensitization to α,β -mATP in nociceptors was not reduced (Fig. 3B). In addition, we complemented these studies using ex vivo lung-vagal tissue from TRPV1^{-/-} mice. In this study, genetic ablation of TRPV1 failed to reduce antimycin A-induced nociceptor hyperexcitability to α,β -mATP (n = 14). We therefore conclude that, despite the exclusivity of hyperexcitability in the nociceptive population, TRPV1 is not required for antimycin A-induced hyperexcitability to α,β -mATP.

Comparisons with Other Mitochondrial Modulators. The biochemical response to antimycin A is well characterized in isolated mitochondria and numerous cell types. Antimycin A selectively inhibits the Q_i site on complex III in the electron transport chain in the mitochondria, resulting in superoxide formation and the subsequent production of numerous ROS, mitochondrial membrane depolarization, and a decrease in ATP production (Turrens et al., 1985; Gyulckhandanyan and Pennefather, 2004; Tretter et al., 2007; Stowe and Camara, 2009). Other specific complex inhibitors have also been described, in particular oligomycin and myxothiazol. Oligomycin inhibits complex V (also known as ATP synthase) and thus prevents ATP production by the oxidative phosphorylation machinery (Sipos et al., 2003; Hool and Corry, 2007). Inhibition of complex V also evokes a mild hyperpolarization of the mitochondrial membrane potential but has little effect on superoxide production in most systems. Ten minutes following oligomycin (10 μ M) treatment, there was no significant change in action potential discharge in response to α,β -mATP (n = 8; Fig. 4A). Myxothiazol is a selective

Fig. 2. Antimycin A increases α,β -mATP-induced responses in nociceptive C-fibers. (A) Representative traces of action potential discharge evoked by 10-second challenge with α,β -mATP (P2X_{2/3} agonist, 30 μ M) in a nociceptive bronchopulmonary C-fiber before (control) and 10 minutes after treatment with antimycin A (20 μ M). Quantification of action potential discharge is shown below each trace. (B) Mean $\pm$ S.E.M. total action potential discharge evoked by α,β -mATP (30 μ M) before (control, hatched bars) and 10 minutes after treatment with either vehicle (white bar) or antimycin A (20 μ M, black bars) in nociceptive and non-nociceptive bronchopulmonary C-fibers. Significant difference in second α,β -mATP-induced responses after antimycin A, $*P < 0.01$. (C) Mean $\pm$ S.E.M. peak action potential discharge evoked by α,β -mATP (30 μ M) before (control, hatched bars) and 10 minutes after treatment with either vehicle (white bar) or antimycin A (20 μ M, black bars) in nociceptive and non-nociceptive bronchopulmonary C-fibers. Significant difference in second α,β -mATP-induced responses after antimycin A, $*P < 0.01$. CV, conduction velocity.

inhibitor of the Q_o site on complex III. Inhibition of Q_o causes mitochondrial membrane potential depolarization and a decrease in ATP production but only evokes very mild superoxide production (Turrens et al., 1985; Gyulkhanyan and Pennefather, 2004; Tretter et al., 2007). Ten minutes following myxothiazol (500 nM) treatment, there was also no change in action potential discharge to α,β -mATP ($n = 7$; Fig. 4A). These data suggest that antimycin A-induced nociceptor hyperexcitability to α,β -mATP is not specifically due to a decrease in ATP production.

Involvement of ROS and PKC in Antimycin A-Induced Hyperexcitability to α,β -mATP. We hypothesized that antimycin A-induced nociceptor hyperexcitability to α,β -mATP was mediated by the actions of mitochondrially derived ROS. Many biologic actions of ROS are due to their ability to oxidize protein residues (e.g., cysteine), which can be reversed by a reducing agent such as dithiothreitol (DTT) or by quenching with a cysteine antioxidant, such as *N*-acetylcysteine (NAC) or glutathione (GSH). DTT is membrane permeable. NAC is slowly actively transported across plasma

membranes (Raftos et al., 2007) and has previously been shown to quench intracellular ROS pathways (Hongpaisan et al., 2004; Takahashi et al., 2011). GSH is membrane impermeable and is not subject to active transport as NAC is (Raftos et al., 2007). We tested the contribution of oxidation to antimycin A-induced hyperexcitability by treating the lungs with DTT (1 mM) or NAC (1 mM) or GSH (1 mM) for 10 minutes prior to and during antimycin A (20 μ M) treatment. Both DTT ($n = 4$) and NAC ($n = 8$) completely abolished the antimycin A-induced increase in excitability to α,β -mATP ($P < 0.01$ compared with antimycin A alone), whereas GSH ($n = 4$) failed to inhibit the antimycin A-induced hyperexcitability to α,β -mATP [$P < 0.005$ compared with vehicle (no antimycin), $P = 0.17$ compared with antimycin A alone] (Fig. 4B). Based on the differential distribution of GSH (extracellular) and NAC (extracellular and intracellular), these data suggest that intraneuronal ROS contribute to the antimycin A-induced hyperexcitability to α,β -mATP.

Selective inhibition of Q_o using myxothiazol is known to substantially reduce subsequent superoxide production

Fig. 3. Antimycin A-induced nociceptor hyperexcitability to α,β -mATP is independent of TRPA1 and TRPV1. (A) Mean $\pm$ S.E.M. response to second application of α,β -mATP (30 μ M) normalized to response to first application of α,β -mATP prior to antimycin A (20 μ M). Bronchopulmonary C-fibers were characterized by conduction velocity and sensitivity to TRPV1 agonist (capsaicin, 1 μ M) and TRPA1 agonist (AITC, 300 μ M) (Kollarik et al., 2003; Nassenstein et al., 2008). Significant increase in second α,β -mATP-induced responses after antimycin A, $*P < 0.05$. (B) Mean $\pm$ S.E.M. response to second application of α,β -mATP (30 μ M) normalized to response to first application of α,β -mATP prior to either vehicle (white bar) or antimycin A (20 μ M, black bars) in nociceptive C-fibers. The role of TRPV1 in hyperexcitability was determined using pretreatment with the selective TRPV1 antagonist iodoresiniferatoxin (IRTX; 1 μ M) or by using C-fibers from TRPV1 $^{-/-}$ mice. Significant increase in second α,β -mATP-induced responses after antimycin A compared with vehicle, $*P < 0.05$. CV, conduction velocity.

evoked by inhibition of the Q_i site (antimycin A) (Turens et al., 1985; Gyulhandanyan and Pennefather, 2004; Tretter et al., 2007). We have previously shown that myxothiazol pretreatment prior to antimycin A greatly reduced antimycin A-induced TRPA1 activation (Nesushvili et al., 2013). In this study, we pretreated the lungs with myxothiazol (500 nM) 3 minutes prior to antimycin A (20 μ M) treatment. Myxothiazol prevented the development of antimycin A-induced nociceptor hyperexcitability to α,β -mATP (Fig. 4B),

as follows: for antimycin alone the response to the second α,β -mATP was $276 \pm 54\%$ ($n = 24$); with myxothiazol pretreatment of antimycin A the response to the second α,β -mATP was $128 \pm 41\%$ ($n = 9$, $P < 0.05$). These findings suggest that ROS evoked from mitochondrial complex III are required for antimycin A-induced hyperexcitability to α,β -mATP.

Numerous studies of dorsal root ganglion (DRG) nociceptors involved in somatosensory pain have shown that PKC is a powerful regulator of nociceptor excitability (Cesare et al., 1999; Baker, 2005; Wu et al., 2012). Interestingly, there has been increasing evidence that many PKC isoforms can be activated by cellular oxidants (e.g., superoxide and H_2O_2) independently of either Ca^{2+} or lipid mediators, such as DAG (Gopalakrishna and Anderson, 1989; Knapp and Klann, 2000; Cosentino-Gomes et al., 2012). We hypothesized that mitochondrial ROS induces airway nociceptor hyperexcitability to α,β -mATP via activation of protein kinase C. For these studies, we used a selective PKC inhibitor called bisindolylmaleimide I (BIM I; also known as GF 109203X and Gö 6850) and its inactive analog BIM V (also known as Ro 31-6045). These compounds are membrane permeable and differ in only one side chain. BIM I is a potent PKC inhibitor with an IC_{50} below 500 nM, whereas BIM V has an IC_{50} for PKC $> 100 \mu$ M (Toullec et al., 1991; Davis et al., 1992). When the lungs were pretreated with BIM I (1 μ M) for 10 minutes prior to and during antimycin A (20 μ M) treatment, there was no increase in nociceptor hyperexcitability, as follows: the response to the second α,β -mATP was only $136 \pm 16\%$ ($n = 14$, $P < 0.01$ compared with antimycin A alone) (Fig. 4B). However, pretreatment with the inactive analog BIM V (1 μ M) for 10 minutes prior to and during antimycin A (20 μ M) treatment failed to prevent antimycin A-induced nociceptor hyperexcitability, as follows: the response to the second α,β -mATP was $231 \pm 56\%$ [$n = 20$, $P < 0.05$ compared with vehicle (no antimycin), $P = 0.28$ compared with antimycin A alone] (Fig. 4B). These data suggest that antimycin A-induced nociceptor hyperexcitability to α,β -mATP is dependent on the actions of PKC.

Lastly, we determined whether PKC activation or ROS was sufficient to evoke vagal bronchopulmonary C-fiber hyperexcitability in the absence of mitochondrial dysfunction by antimycin A. PMA is a potent and selective activator of PKC. As before in the antimycin A studies, the nociceptor action potential response to 30 μ M α,β -mATP was determined. Then the lungs were treated with 3 μ M PMA, and the response to α,β -mATP was determined 2 minutes later. As expected, PMA induced nociceptor hyperexcitability to α,β -mATP, as follows: the response to the second α,β -mATP was $267 \pm 111\%$ ($n = 11$, $P < 0.05$ compared with vehicle) (Fig. 5). Using a similar protocol, we found that exogenously applied H_2O_2 also induced airway nociceptor hyperexcitability to α,β -mATP, as follows: 2 minutes after H_2O_2 (1 mM), the response to the second α,β -mATP was $207 \pm 35\%$ ($n = 15$, $P < 0.01$ compared with vehicle) (Fig. 5). Consistent with the mechanism of antimycin A-induced hyperexcitability to α,β -mATP, H_2O_2 -induced hyperexcitability to α,β -mATP was prevented by pretreatment with the selective PKC inhibitor BIM I (1 μ M), as follows: the response to the second α,β -mATP was only $89 \pm 8\%$ ($n = 5$, $P < 0.005$ compared with H_2O_2 alone) (Fig. 5).

Some of the airway C-fibers (8 of 16) used in the H_2O_2 hyperexcitability studies expressed TRPA1 (as determined by

Fig. 4. Antimycin A-induced nociceptor hyperexcitability to α,β -mATP is dependent on mitochondrial ROS and PKC. (A) Mean $\pm$ S.E.M. response to second application of α,β -mATP (30 μ M) normalized to response to first application of α,β -mATP prior to either vehicle (white bar), antimycin A (20 μ M, black bar), oligomycin (10 μ M, dark gray bar), or myxothiazol (500 nM, light gray bar) in nociceptive C-fibers. Significant increase in second α,β -mATP-induced responses compared with vehicle, $*P < 0.01$. (B) Mean $\pm$ S.E.M. response to second application of α,β -mATP (30 μ M) normalized to response to first application of α,β -mATP prior to either vehicle (white bar) or antimycin A (20 μ M, black bars) in nociceptive C-fibers. The roles of ROS and PKC in hyperexcitability to α,β -mATP were determined using pretreatment with DTT (1 mM), NAC (1 mM), GSH (1 mM), myxothiazol (500 nM), BIM I (1 μ M), or BIM V (1 μ M). Significant increase in second α,β -mATP-induced responses after antimycin A compared with vehicle, $*P < 0.05$. Significant reduction in antimycin A-induced hyperexcitability to α,β -mATP, $#P < 0.05$.

sensitivity to AITC). Although not the focus of this study, TRPA1 has been shown to be activated by H_2O_2 in vitro (Andersson et al., 2008). Consistent with those findings, H_2O_2 robustly activated TRPA1-expressing C-fibers (peak discharge of 6.4 ± 1.8 Hz) compared with those C-fibers that did not express TRPA1 (peak discharge of 0.6 ± 0.6 Hz). H_2O_2 -induced C-fiber activation was short-lived, and the response was absent prior to the second α,β -mATP stimulus. As with antimycin A-induced hyperexcitability to α,β -mATP, the H_2O_2 -induced hyperexcitability to α,β -mATP was unaffected by the presence or absence of TRPA1 (data not shown).

Antimycin A Induces PKC Translocation in Vagal Neurons. Critical to PKC activity is its subcellular location (Cosentino-Gomes et al., 2012). Typically, PKC isoforms remain in the cytosol while inactive. Upon activation, PKC translocates to the plasma membrane, in which it engages in phosphorylation of a wide variety of protein targets. Given that previous studies demonstrated multiple isoforms of PKC

heterogeneously expressed throughout the somatosensory DRG neuronal population (Cesare et al., 1999; Hayase et al., 2007; Wu et al., 2012; Zhang et al., 2012) and that PKC isoform expression has not yet been characterized in vagal neurons, we decided in this study to observe global PKC translocation using a pan-PKC antibody that would detect all PKC isoforms. All vagal neurons were immunoreactive for PKC with no fluorescent signal observed in the absence of primary antibody (data not shown). In control conditions, the vast majority of PKC immunoreactivity was located in the cytosol ($n = 225$) (Fig. 6). As expected, there was a significant increase in PKC immunoreactivity at the plasma membrane 2 minutes after PMA (300 nM) treatment ($n = 216$, $P < 0.0001$ compared with control), indicating that activation of PKC causes translocation to the plasma membrane. Consistent with our electrophysiological data at the airway vagal nerve terminal, antimycin A (10 μ M) induced significant PKC translocation to the plasma membrane ($n = 134$, $P < 0.0005$ compared with control). Antimycin A-induced PKC translocation was abolished by the pretreatment of the neurons with a combination of the antioxidants tempol (1 mM) and MnTMPyP (50 μ M) ($n = 41$, $P < 0.05$ compared with antimycin alone), indicating a critical role of mitochondrial ROS in these responses. Finally, we found that exogenously applied H_2O_2 (300 μ M) was sufficient to induce significant PKC translocation to the membrane ($n = 173$, $P < 0.005$ compared with control). Overall, these data indicate that mitochondrial ROS induce PKC translocation, consistent with a role of PKC in mitochondrial ROS-induced airway nociceptor hyperexcitability.

Fig. 5. H_2O_2 increases α,β -mATP-induced responses in nociceptive C-fibers via PKC. Mean $\pm$ S.E.M. response to second application of α,β -mATP (30 μ M) normalized to response to first application of α,β -mATP prior to either vehicle (white bar), PMA (3 μ M, gray bar), or H_2O_2 (1 mM, black bars) in nociceptive C-fibers. The roles of PKC in H_2O_2 -induced hyperexcitability to α,β -mATP were determined using pretreatment with BIM I (1 μ M). Significant increase in second α,β -mATP-induced responses compared with vehicle, $*P < 0.05$. Significant reduction in H_2O_2 -induced hyperexcitability to α,β -mATP, $#P < 0.005$.

Discussion

Inflammation alters mitochondrial function (Sena and Chandel, 2012). Given that peripheral sensory nerve terminals are densely packed with mitochondria (Hung et al., 1973; von Düring and Andres, 1988), we investigated the effect of mitochondrial modulation on sensory nerve terminal excitability. In this study, ROS evoked by mitochondrial dysfunction induced hyperexcitability to both mechanical and

Fig. 6. Antimycin A induces PKC translocation to the plasma membrane via ROS in vagal neurons. (A–E) Representative confocal images of PKC (red) and nuclei (4',6-diamidino-2-phenylindole, blue) in dissociated vagal sensory neurons. Scale denotes 25 μm. In dissociated ganglia preparations, neurons are typically >20 μm in diameter; other non-neuronal cell types are also present that are small with substantial nuclei (Nassenstein et al., 2010). (A) Control conditions. (B) After PMA (300 nM). (C) After antimycin A (10 μM). (D) After antimycin A (10 μM), pretreated with tempol (1 mM) and MnTMPyP (50 μM). (E) After H₂O₂ (300 μM). (F) Mean ± S.E.M. PKC membrane/cytosolic ratio for experiments described in A–E. Significant increase in membrane/cytosolic ratio compared with vehicle, **P* < 0.005. Significant reduction in membrane/cytosolic ratio compared with antimycin A alone, #*P* < 0.05.

chemical stimuli in nociceptive bronchopulmonary C-fibers but had little effect on the excitability of non-nociceptive nerves. Nociceptive nerves protect the innervated organ through the initiation of reflexes and behavioral responses (Carr and Undem, 2003), and hyperexcitability contributes to aberrant signs and symptoms in inflammatory and infectious states (Meyer et al., 1996; Carr and Lee, 2006). Thus, we present evidence of a novel mechanism by which nociceptive airway responses can be inappropriately modulated.

There are two subtypes of mouse vagal bronchopulmonary C-fibers: slowly conducting C-fibers express various nociceptor receptors such as TRPV1 (Kollarik et al., 2003) and are thought to be synonymous with nociceptive afferents in humans that mediate cough, dyspnea, and reflex bronchospasm (Carr and Undem, 2003); faster conducting C-fibers do not express TRPV1 and are considered to be non-nociceptive—perhaps involved in regulation of eupnic breathing. We previously demonstrated that antimycin A, a Q_i site inhibitor of the electron transfer chain complex III that induces production of mitochondrial ROS (Stowe and Camara, 2009), caused the activation of nociceptive C-fibers via the activation of another nociceptive ion channel TRPA1 (Nesuashvili et al., 2013). This activation typically lasted 2–7 minutes. In this study, we found that antimycin A evoked a profound increase in nociceptive C-fiber excitability as determined by decreased threshold for punctate mechanical stimulation and by increased action potential discharge to α,β -mATP, an agonist for P2X_{2/3} ion channels. Antimycin A failed to increase non-nociceptive nerve excitability. Unlike antimycin A-induced C-fiber activation (Nesuashvili et al., 2013), antimycin A-induced hyperexcitability to α,β -mATP was not dependent on the expression of TRPA1. Despite the exclusivity of hyperexcitability in the TRPV1-expressing nociceptive population, TRPV1 was also not required for antimycin A-induced

hyperexcitability to α,β -mATP. We briefly investigated the effect of 1 mM H₂O₂ on nociceptor activation and found that activation correlated with sensitivity to the TRPA1 agonist AITC. This is consistent with other studies using similar doses (Andersson et al., 2008), although studies using 120 mM H₂O₂ suggest that, along with TRPA1, P2X channels also contribute to H₂O₂-induced C-fiber activation (Lin et al., 2013).

Our mechanistic studies suggest that antimycin A evokes airway nociceptor hyperexcitability to α,β -mATP via the actions of ROS and PKC, largely independent of mitochondrial ATP production. Antimycin A, oligomycin (complex V inhibitor), and myxothiazol (complex III Q_o site inhibitor) all decrease ATP production, but only antimycin A causes significant mitochondrial superoxide production (Stowe and Camara, 2009) and only antimycin A evoked significant nociceptor hyperexcitability in this study. Furthermore, myxothiazol, which prevents antimycin A-induced ROS production, prevented antimycin A-induced hyperexcitability, indicating that the hyperexcitability was specifically due to ROS produced from the complex III Q_o site. It should be noted that we have not measured ROS or ATP production from the nerve terminals in this study. The potency of oligomycin, antimycin A, and myxothiazol used in their single concentrations on ROS or ATP production cannot be confirmed, and, thus, caution must be employed in drawing conclusions. Nevertheless, the concentrations were chosen from previous studies showing the selective effects of inhibition of ATP synthase (oligomycin), complex III Q_i site (antimycin A), and complex III Q_o site (myxothiazol) on ROS production, ATP production, and oxygen consumption (Turrens et al., 1985; Sipos et al., 2003; Gyulkhandanyan and Pennefather, 2004; Hool and Corry, 2007; Tretter et al., 2007; Stowe and Camara, 2009). The role of ROS in this pathway was confirmed by the

inhibition of antimycin A–induced hyperexcitability by the antioxidant NAC (detoxifies ROS) and the reducing agent DTT (reverses sulfhydryl group oxidation). NAC is actively transported across the plasma membrane unlike the antioxidant GSH (Raftos et al., 2007). GSH (only detoxifies extracellular ROS) failed to inhibit antimycin A–induced hyperexcitability, indicating that intracellular (i.e., intraneuronal) ROS contributed significantly to this phenomenon. It is not known whether superoxide or other downstream ROS (e.g., H_2O_2) are the end effectors of mitochondrial modulation–induced hyperexcitability, but the inhibition by DTT would argue against a significant role of lipid peroxidation products, which react with proteins in a DTT-insensitive manner (Andersson et al., 2008).

Antimycin A and H_2O_2 caused nociceptor hyperexcitability to α, β -mATP that was sensitive to the selective PKC inhibitor BIM I but not to its inactive analog BIM V. Selectivity is a relative term for kinase inhibitors, but at 1 μM BIM I inhibits PKC without affecting protein kinase A; and the other off-target effects of bisindolylmaleimide compounds are shared by both BIM I and BIM V (Toullec et al., 1991; Davis et al., 1992). That PKC activation could lead to nociceptor hyperexcitability was further demonstrated by PMA, an activator of PKC, which produced hyperexcitability of a similar magnitude to antimycin A and H_2O_2 . PMA and the activation of PKC have been shown previously to induce hyperexcitability in vagal nociceptive neurons (Kagaya et al., 2002; Ikeda et al., 2005; Gu and Lee, 2006, 2009; Matsumoto et al., 2007). PKC translocation from the cytosol to the plasma membrane is an indicator of PKC activation (Cosentino-Gomes et al., 2012). Our electrophysiological studies were supported by confocal imaging in dissociated neurons, indicating that antimycin A and H_2O_2 both induced PKC translocation. Antimycin A–induced PKC translocation was prevented by a combination of tempol (superoxide dismutase mimetic) and MnTMPyP (combined superoxide dismutase and catalase mimetic), indicating that ROS were required for this effect.

There are 15 PKC isoforms, which are divided into three groups based upon sensitivity to Ca^{2+} and DAG, as follows: classic isoforms (α , β , and γ) require both Ca^{2+} and DAG for activation; novel isoforms (δ , ϵ , η , and θ) require DAG but not Ca^{2+} for activation; and atypical isoforms (ι/λ and ζ) require neither Ca^{2+} nor DAG for activation. To date there has not been any characterization of PKC isoform expression in vagal sensory neurons, although PKC expression in somatosensory DRG neurons indicates that, at a minimum, $\beta 1$, $\beta 2$, δ , ϵ , and ζ are expressed in complex combinatorial patterns (Cesare et al., 1999; Wu et al., 2012). Similar to our findings, PKC has repeatedly been shown to play a role in inflammation-induced hyperexcitability in nociceptive DRG (Cesare et al., 1999; Baker, 2005; Wu et al., 2012; Zhang et al., 2012). It is known that PMA binds to the same enzyme subsite as DAG, and thus PMA activates only classic and novel PKC isoforms, suggesting that PKC expression is not limited to atypical isoforms in vagal sensory nerves. Although ROS activate PKC by the oxidation of cysteines in a pair of zinc fingers associated with the DAG binding site (Gopalakrishna and Anderson, 1989; Knapp and Klann, 2000), this does not preclude the contribution of atypical isoforms that are activated by the same mechanism on their single zinc finger site (that does not bind DAG) (Cosentino-Gomes et al., 2012). Further studies are

required to elucidate which PKC isoform is involved in mitochondrial ROS-induced bronchopulmonary C-fiber hyperexcitability.

Another question raised by these studies is how activation of PKC causes hyperexcitability in vagal bronchopulmonary C-fibers. Activated PKC isoforms are capable of modifying neuronal excitability via the phosphorylation of numerous cellular targets, including ion channels and transporters. In particular, PKC has been shown to induce hyperexcitability via tetrodotoxin-resistant voltage-gated Na^+ channels in both vagal (Kagaya et al., 2002; Ikeda et al., 2005; Matsumoto et al., 2007) and DRG neurons (Baker, 2005; Hayase et al., 2007; Wu et al., 2012), indicating possible candidates for mitochondrial ROS-induced bronchopulmonary C-fiber hyperexcitability. However, given that tetrodotoxin-resistant voltage-gated Na^+ channels are expressed in both nociceptive and non-nociceptive neurons (Kwong et al., 2008), further work is needed to elucidate the mechanism highlighted in the present study and its apparent exclusivity to nociceptive C-fibers.

In summary, we have found that antimycin A causes a robust increase in bronchopulmonary C-fiber excitability via the actions of mitochondrial ROS and PKC. Mitochondrial ROS are known to increase excitability in CNS neurons (Nozoe et al., 2008; Yin et al., 2010), to activate vagal nociceptive neurons (Nesushvili et al., 2013), and to regulate Ca^{2+} signaling in DRG nociceptive neurons (Kim and Usachev, 2009). Airway inflammatory disease is associated with mitochondrial dysfunction and mitochondrial ROS production (Mabalirajan et al., 2008; Aguilera-Aguirre et al., 2009; Reddy, 2011), and a wide variety of inflammatory signaling has been shown to increase mitochondrial ROS (Corda et al., 2001; Bell et al., 2007; Pehar et al., 2007; Michaeloudes et al., 2011; West et al., 2011). Given the mitochondrial density within sensory nerve terminals, it is possible that inflammation may induce nociceptor hyperexcitability via the production of mitochondrial ROS. It is now increasingly clear that, in addition to initiating apoptosis, mitochondrial dysfunction and ROS production also contribute to more subtle physiologic signaling (Sena and Chandel, 2012), and it remains to be determined whether chronic mitochondrial ROS in sensory nerve terminals contribute to hyper-reflexia in airway disease.

Authorship Contributions

Participated in research design: Bahia, Taylor-Clark.

Conducted experiments: Hadley, Bahia.

Performed data analysis: Hadley, Bahia, Taylor-Clark.

Wrote or contributed to the writing of the manuscript: Bahia, Taylor-Clark.

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