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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, 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, H$_2$O$_2$ 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 Undem, 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 hypersensitive 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 Chandell, 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-a (Corda 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 During 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) (Nesuashvili et al., 2013). It is not yet known...
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 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 Knall, 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\(_2\)PO\(_4\), 1.2 MgSO\(_4\), 1.9 CaCl\(_2\), 25.0 NaHCO\(_3\), and 11.1 g-glucose, and equilibrated with 95% O\(_2\) and 5% CO\(_2\) at 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, multi-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 (DTS1002B; Tektronix, Beaverton, OR). The scaled output from the amplifier was captured and analyzed by a Macintosh computer using NerveOff 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 (54; 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 (Kollari 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) × 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\(^{-/-}\) mice, nociceptive status was assessed solely by polymerease 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 (Nesanashvili et al., 2013). Isolated neurons were plated onto poly
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 μM) evoked significant action potential discharge in TRPA1-expressing bronchopulmonary fibers via a ROS-dependent TRPA1 activation—this mechanism is described in our previous study (Nesuashvili 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 μM) 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 P2X2/3 agonist α,β-mATP and that this response is reproducible (Kollarik et al., 2003; Nassenstein et al., 2010). Consistent with these reports, 30 μM α,β-mATP activated >90% of bronchopulmonary C-fibers regardless of their conduction velocity or TRP channel expression. To confirm the reproducibility of P2X2/3 receptor responses, we determined the action potential discharge to repeated treatment of 30 μM 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 μM antimycin A, the response to 30 μM 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).
The action potential discharge to $\alpha,\beta$-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 $\alpha,\beta$-mATP treatment (i.e., after antimycin A) as a percentage of each individual fiber's response to the first $\alpha,\beta$-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 $\alpha,\beta$-mATP. There was a trend toward a greater increase in excitability to $\alpha,\beta$-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 indo-RES (1 $\mu$M, $n = 8$) (Wahl et al., 2001), we found that antimycin A–induced sensitization to $\alpha,\beta$-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 $\alpha,\beta$-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 $\alpha,\beta$-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 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; Gyulkhandanyan and Pennefather, 2004; Trettter 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 $\mu$M) treatment, there was no significant change in action potential discharge in response to $\alpha,\beta$-mATP ($n = 8$; Fig. 4A). Myxothiazol is a selective
inhibitor of the Qo site on complex III. Inhibition of Qo causes mitochondrial membrane potential depolarization and a decrease in ATP production but only evokes very mild superoxide production (Turrens et al., 1985; Gyulkhandanyan and Pennefather, 2004; Tretter et al., 2007). Ten minutes following myxothiazol (500 nM) treatment, there was also no change in action potential discharge to \(\alpha,\beta\)-mATP \((n = 7); \text{Fig. } 4A\). These data suggest that antimycin A–induced nociceptor hyperexcitability to \(\alpha,\beta\)-mATP is not specifically due to a decrease in ATP production.

**Involvement of ROS and PKC in Antimycin A–Induced Hyperexcitability to \(\alpha,\beta\)-mATP.** We hypothesized that antimycin A–induced nociceptor hyperexcitability to \(\alpha,\beta\)-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 \(N\)-acetyl-cysteine (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 \(\mu\)M) treatment. Both DTT \((n = 4)\) and NAC \((n = 8)\) completely abolished the antimycin A–induced increase in excitability to \(\alpha,\beta\)-mATP \((P < 0.01\) compared with vehicle (no antimycin), \(P = 0.17\) compared with antimycin A alone) \((\text{Fig. } 4B)\). 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 \(\alpha,\beta\)-mATP.

Selective inhibition of Qo using myxothiazol is known to substantially reduce subsequent superoxide production.
Myxothiazol prevented the development of antimycin A-induced nociceptor hyperexcitability to \( \alpha,\beta\)-mATP (Fig. 4B), as follows: for antimycin alone the response to the second \( \alpha,\beta\)-mATP was 276 ± 54% (\( n = 24 \)); with myxothiazol pretreatment of antimycin A the response to the second \( \alpha,\beta\)-mATP was 128 ± 41% (\( n = 9, P < 0.05 \)). These findings suggest that ROS evoked from mitochondrial complex III are required for antimycin A–induced hyperexcitability to \( \alpha,\beta\)-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\(_2\)O\(_2\)) 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 \( \alpha,\beta\)-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 \( \mu\)M) for 10 minutes prior to and during antimycin A (20 \( \mu\)M) treatment, there was no increase in nociceptor hyperexcitability, as follows: the response to the second \( \alpha,\beta\)-mATP was only 136 ± 16% (\( n = 14, P < 0.01 \) compared with antimycin A alone) (Fig. 4B). However, pretreatment with the inactive analog BIM V (1 \( \mu\)M) for 10 minutes prior to and during antimycin A (20 \( \mu\)M) treatment failed to prevent antimycin A–induced nociceptor hyperexcitability, as follows: the response to the second \( \alpha,\beta\)-mATP was 231 ± 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 \( \alpha,\beta\)-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. In previous studies expressed TRPA1 (as determined by

evoked by inhibition of the Q\(_1\) site (antimycin A) (Turrens et al., 1985; Gyulkhandanyan 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 (Nesuashvili et al., 2013). In this study, we pretreated the lungs with myxothiazol (500 nM) 3 minutes prior to antimycin A (20 \( \mu\)M) treatment. Myxothiazol prevented the development of antimycin A–induced nociceptor hyperexcitability to \( \alpha,\beta\)-mATP (Fig. 4B), as follows: the response to the second \( \alpha,\beta\)-mATP was 267 ± 111% (\( n = 11, P < 0.05 \) compared with vehicle) (Fig. 5). Using a similar protocol, we found that exogenously applied H\(_2\)O\(_2\) also induced airway nociceptor hyperexcitability to \( \alpha,\beta\)-mATP, as follows: 2 minutes after H\(_2\)O\(_2\) (1 \( \mu\)M), the response to the second \( \alpha,\beta\)-mATP was 207 ± 35% (\( n = 15, P < 0.01 \) compared with vehicle) (Fig. 5). Consistent with the mechanism of antimycin A–induced hyperexcitability to \( \alpha,\beta\)-mATP, H\(_2\)O\(_2\)-induced hyperexcitability to \( \alpha,\beta\)-mATP was prevented by pretreatment with the selective PKC inhibitor BIM I (1 \( \mu\)M), as follows: the response to the second \( \alpha,\beta\)-mATP was only 89 ± 8% (\( n = 5, P < 0.005 \) compared with H\(_2\)O\(_2\) alone) (Fig. 5).

Some of the airway C-fibers (8 of 16) used in the H\(_2\)O\(_2\) hyperexcitability studies expressed TRPA1 (as determined by

**Fig. 3.** Antimycin A–induced nociceptor hyperexcitability to \( \alpha,\beta\)-mATP is independent of TRPA1 and TRPV1. (A) Mean ± S.E.M. response to second application of \( \alpha,\beta\)-mATP (30 \( \mu\)M) normalized to response to first application of \( \alpha,\beta\)-mATP prior to antimycin A (20 \( \mu\)M). Bronchopulmonary C-fibers were characterized by conduction velocity and sensitivity to TRPV1 agonist (capsaicin, 1 \( \mu\)M) and TRPA1 agonist (ATC, 300 \( \mu\)M) (Kollarik et al., 2003; Nassenstein et al., 2008). Significant increase in second \( \alpha,\beta\)-mATP–induced responses after antimycin A, *\( P < 0.05 \). (B) Mean ± S.E.M. response to second application of \( \alpha,\beta\)-mATP (30 \( \mu\)M) normalized to response to first application of \( \alpha,\beta\)-mATP prior to either vehicle (white bar) or antimycin A (20 \( \mu\)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 \( \mu\)M) or by using C-fibers from TRPV1−/− mice. Significant increase in second \( \alpha,\beta\)-mATP–induced responses after antimycin A compared with vehicle, *\( P < 0.05 \), CV, conduction velocity.
Antimycin A–induced nociceptor hyperexcitability to α,β-mATP is dependent on mitochondrial ROS and PKC. (A) Mean ± S.E.M. response to second application of α,β-mATP (30 μM) normalized to response to first application of α,β-mATP prior to either vehicle (white bar), antimycin A (20 μM, black bar), oligomycin (10 μM, 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 ± S.E.M. response to second application of α,β-mATP (30 μM) normalized to response to first application of α,β-mATP prior to either vehicle (white bar) or antimycin A (20 μM, 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 μM), or BIM V (1 μM). 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.

Mitochondrial ROS induces hyperexcitability to both mechanical and chemical inputs. Antimycin A increases α,β-mATP–induced responses in nociceptive C-fibers via PKC. Mean ± S.E.M. response to second application of α,β-mATP (30 μM) normalized to response to first application of α,β-mATP prior to either vehicle (white bar), PMA (3 μM, gray bar), or H2O2 (1 mM, black bars) in nociceptive C-fibers. The roles of PKC in H2O2–induced hyperexcitability to α,β-mATP were determined using pretreatment with BIM I (1 μM). Significant increase in second α,β-mATP–induced responses compared with vehicle, *P < 0.05. Significant reduction in H2O2–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 During 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 chemical inputs.
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 nocifensive 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$_{1}$ 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 $\alpha,\beta$-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 $\alpha,\beta$-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 $\alpha,\beta$-mATP. We briefly investigated the effect of 1 mM H$_{2}$O$_{2}$ 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 H$_{2}$O$_{2}$ suggest that, along with TRPA1, P2X channels also contribute to H$_{2}$O$_{2}$–induced C-fiber activation (Lin et al., 2013).

Our mechanistic studies suggest that antimycin A evokes airway nociceptor hyperexcitability to $\alpha,\beta$-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$_{1}$ 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$_{1}$ 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$_{1}$ site (antimycin A), and complex III Q$_{1}$ 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$_2$O$_2$) 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$_2$O$_2$ caused nociceptor hyperexcitability to $\alpha$-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 $\mu$M 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 (Toulec 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$_2$O$_2$. 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$_2$O$_2$ 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 ($\alpha$, $\beta$, and $\gamma$) require both Ca$^{2+}$ and DAG for activation; novel isoforms (delta, $\epsilon$, $\eta$, and $\theta$) require DAG but not Ca$^{2+}$ for activation; and atypical isoforms ($\delta$, $\beta$, $\alpha$, and $\zeta$) 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, $\beta_1$, $\beta_2$, $\delta$, $\epsilon$, and $\zeta$ are expressed in complex combinatorial patterns (Cosere 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 (Cosere 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 (Nesuashvili 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; Aguiler-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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