Cellular localization of mitochondria contributes to K<sub>v</sub> channel-mediated regulation of cellular excitability in pulmonary but not mesenteric circulation

Amy L. Firth,¹⁎ Dmitri V. Gordienko,²,³⁎ Kathryn H. Yuill,¹⁎ and Sergey V. Smirnov¹

¹Department of Pharmacy and Pharmacology, University of Bath, Bath; and ²Division of Basic Medical Sciences, Ion Channels and Cell Signalling Centre, St George’s University of London, London, United Kingdom; and ³Laboratory of Molecular Pharmacology and Biophysics of Cell Signaling, Bogomolets Institute of Physiology, Kiev, Ukraine

Submitted 11 June 2008; accepted in final form 16 December 2008

Firth AL, Gordienko DV, Yuill KH, Smirnov SV. Cellular localization of mitochondria contributes to K<sub>v</sub> channel-mediated regulation of cellular excitability in pulmonary but not mesenteric circulation. Am J Physiol Lung Cell Mol Physiol 296: L347–L360, 2009. First published December 19, 2008; doi:10.1152/ajplung.90341.2008.—Mitochondria are proposed to be a major oxygen sensor in hypoxic pulmonary vasoconstriction (HPV), a unique response of the pulmonary circulation to low oxygen tension. Mitochondrial factors including reactive oxygen species, cytochrome c, ATP, and magnesium are potent modulators of voltage-gated K<sup>+</sup> (K<sub>v</sub>) channels in the plasmalemmal membrane of pulmonary arterial (PA) smooth muscle cells (PASMCs). Mitochondria have also been found close to the plasmalemmal membrane in rabbit main PA smooth muscle sections. Therefore, we hypothesized that differences in mitochondrial localization in rat PASMCs and systemic mesenteric arterial smooth muscle cells (MASMCs) may contribute to the divergent oxygen sensitivity in the two different circulations. Cellular localization of mitochondria was compared with immunofluorescent labeling, and differences in functional coupling between mitochondria and K<sub>v</sub> channels was evaluated with the patch-clamp technique and specific mitochondrial inhibitors antimycin A (acting at complex III of the mitochondrial electron transport chain) and oligomycin A (which inhibits the ATP synthase). It was found that mitochondria were located significantly closer to the plasmalemmal membrane in PASMCs compared with MASMCs. Consistent with these findings, the effects of the mitochondrial inhibitors on K<sub>v</sub> current (I<sub>Kv</sub>) were significantly more potent in PASMCs than in MASMCs. The cytoskeletal disruptor cytochalasin B (10 μM) also altered mitochondrial distribution in PASMCs and significantly attenuated the effect of antimycin A on the voltage-dependent parameters of I<sub>Kv</sub>. These findings suggest a greater structural and functional coupling between mitochondria and K<sub>v</sub> channels specifically in PASMCs, which could contribute to the regulation of PA excitability in HPV.

THE PULMONARY CIRCULATION represents a vascular bed with unique properties. Under physiological conditions this vasculature is a highly oxygenated, low-pressure system. When oxygen tension drops during, for example, chronic obstructive pulmonary disease, a response known as hypoxic pulmonary vasoconstriction (HPV) occurs, diverting the blood flow to the higher oxygenated areas to match ventilation to perfusion. The mechanisms involved in this response are complex and currently incompletely understood (14, 48, 52). Such responses do not occur in the systemic circulation, which dilates when oxygen tension is reduced to maximize blood supply to all parts of the body.

Smooth muscle cell contraction is critical to vasoconstriction; however, to date no definitive mechanism can account for how these cells sense changes in oxygen tension or how increased contractility occurs. The response is considered to be a complex, multifactorial phenomenon ultimately contributing to elevated intracellular Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]i), essential for formation of the calcium-calmodulin complex and activation of the myosin light chain kinase. Hypoxic inhibition of voltage-gated K<sup>+</sup> (K<sub>v</sub>) channels, which results in membrane depolarization and voltage-dependent Ca<sup>2+</sup> influx in pulmonary arterial (PA) smooth muscle cells (PASMCs), was originally suggested as one of the important factors leading to increased [Ca<sup>2+</sup>]i, during HPV (3, 36, 47, 51). Currently, mitochondria, the main consumers of cellular oxygen during respiration, are considered to be one of the putative oxygen sensors in pulmonary smooth muscle cells (18, 22, 50, 53), and it has been postulated that K<sub>v</sub> channels may be regulated by an effector of mitochondrial origin (3, 22). This idea is supported by the observations that 1) inhibitors of the mitochondrial electron transport chain (mETC) such as rotenone and antimycin A, acting at complexes I and II, respectively, and the mitochondrial uncoupler FCCP mimic the effect of hypoxia on K<sub>v</sub> current (I<sub>Kv</sub>) in PASMCs (3, 57) and ductus arteriosus (23) and 2) mice lacking the active gp91<sup>phox</sup> subunit of the membrane-bound NADPH oxidase preserve the effect of hypoxia on I<sub>Kv</sub> in PASMCs (5). Numerous products of mitochondrial metabolism have also been shown to activate K<sub>v</sub> channels, including cytochrome c (34) and hydrogen peroxide in pulmonary and coronary arterial smooth muscle cells (39, 40), while reducing agents inhibit K<sub>v</sub> channels (27, 28, 38). In addition, we recently reported (9) that that the voltage-dependent characteristics of I<sub>Kv</sub> in PASMCs are potently modulated via a mitochondrion-mediated Mg<sup>2+</sup>-dependent mechanism.

Although products of mitochondrial metabolism are currently established to have effects on K<sub>v</sub> channels, the exact reasons why these mediators and channels existing in all circulatory beds should behave differently in the pulmonary circulation in response to hypoxia remain unknown. One possibility could be functional differences of the mitochondria present in different vascular beds (22, 37). Another possibility could be that the intracellular locality of mitochondria in...
relation to other organelles and the cellular membrane may vary in the different vascular beds. Using electron microscopy imaging, Vallières et al. (46) demonstrated the presence of mitochondria close to the plasmalemmal membrane in rabbit main PA smooth muscle sections. Such close proximity between mitochondria and the plasmalemmal membrane, if it specifically exists in PASMCs, could be responsible for the opportunistic detection of a mitochondrial-dependent mediator by $K_v$ channels.

No systematic comparison of mitochondrial distribution in smooth muscle cells (SMCs) isolated from resistance PAs and systemic arteries has yet been performed. Therefore, the main aim of this study was to investigate the differences in cellular localization of mitochondria in PASMCs and mesenteric arterial SMCs (MASMCs, as an example of systemic circulation) with fluorescence confocal imaging. The functional differences between the inhibition of mitochondria and $I_{K_v}$ characteristics in both cell types were investigated with the patch-clamp technique using the inhibitor of complex III of the mETC antimycin A and the inhibitor of the mitochondrial Fo/FT ATP synthase oligomycin. These inhibitors were chosen because they have a most pronounced effect on $I_{K_v}$ in rat PASMCs (9).

**MATERIALS AND METHODS**

**Materials.** All standard chemicals were purchased from BDH Merck or Fisher. Collagenase type XI and type P were obtained from Sigma and Roche, respectively. Papain, dithiothreitol, mETC inhibitor antimycin A (a mixture of antimycins from Streptomyces species), and ATP synthase inhibitor oligomycin were all obtained from Sigma; cytochalasin B was purchased from Calbiochem. Brefeldin A BODIPY 558/568 [absorbance (Abs)/emission (Em) 559 nm/568 nm], which was previously demonstrated to stain the SR and mitochondria was analyzed only in PASMCs or MASMCs, could be responsible for the mitochondrial-dependent mediators caused by waveguide dispersion of light, which occurs when the wave propagates through any inhomogeneous structure such as a cell.

**Electrophysiology.** Cells were placed in a chamber with a volume of 100–200 μl and continually superfused (~1 ml/min) with a physiological saline solution (PSS) or a test solution via a five-barrel pipette at room temperature. PSS contained (mM) 140 NaCl, 5 KCl, 1.5 CaCl$_2$, 1.2 MgCl$_2$, 10 HEPES, and 10 glucose, pH 7.2. Control pipette solution contained (mM) 140 KCl, 0.5 MgCl$_2$, 10 HEPES, 10 EGTA, and 0.5 CaCl$_2$, pH 7.2, and was used for recording unless otherwise stated. Cells were dialyzed with pipette solution for 5 min before currents were recorded. The effects of inhibitors were studied by 1 min after addition. PaXilin (1 μM) and glibenclamide (10 μM) to block Ca$^{2+}$-activated and ATP-sensitive K$^+$ currents, respectively, were present in all extracellular recording solutions, isolating the voltage-dependent K$^+$ channels from other K$^+$ conductances as previously described (43). To evaluate cell size, whole cell capacitative transient currents were measured with a 10-nA hyperpolarizing step after stable whole cell access was achieved in each cell. Cell membrane capacitance ($C_m$) was then calculated as an area under a capacitative transient and expressed in picofarads.

$I_{K_v}$ amplitude was measured with a 200-ms voltage step applied from the holding potential of −80 mV to membrane potentials between −100 and +50 mV in 10-mV increments with a frequency of 0.1 Hz. After membrane depolarization cells were repolarized to −20 (PASMCs) or 0 (MASMCs) mV for 160 ms to measure tail currents, which were used to assess changes in the steady-state activation of $I_{K_v}$. A more positive step potential in MASMCs was required to increase the size of tail currents because of the significantly smaller current amplitude in these cells. Current-voltage (I-V) curves were constructed from the tail current measures 2–3 ms after repolarization and fitted with the following equation:
where $V_a$ and $k_0$ are the half-activation potential and the e-fold steepness of the activation dependence (the slope factor of activation), respectively, and $V_m$ is membrane potential. $I_{K_v}$ block was calculated as a percentage of the current at $+50$ mV in control conditions from the same cell.

$I_{K_v}$ inactivation was measured with a two-pulse protocol consisting of a 10-s conditioning pulse stepping in 10-mV increments from $-100$ to $+40$ mV, followed by a test potential to $+60$ mV for 200 ms. Current at the end of the test pulse was measured, normalized to the maximal current recorded, and plotted versus conditioning potential. The resulting $I$-$V$ curve was fitted to the following equation:

$$I_{K_v} = \frac{1}{1 + \exp[(V_n - V_a)/k_0]} + A$$

where $V_n$ and $k_0$ are the half inactivation potential and the e-fold steepness of the inactivation dependence (the slope factor of inactivation), respectively, and $A$ denotes the noninactivating component of the current.

Data analysis and statistics. Data were analyzed and presented with pCLAMP 8 (Axon Instruments), Microsoft Excel, and Microcal Origin 6.0 software. Data are expressed as means ± SE. Statistical comparisons were performed with a paired or unpaired two-tailed t-test, with $P < 0.05$ deemed significant unless stated otherwise. When normality test failed (Shapiro-Wilk test) the Wilcoxon signed rank test and the Mann-Whitney test were used for comparison of paired and unpaired data, respectively. Paired statistical tests were used to compare the difference in parameters obtained in the control condition and in the presence of mitochondrial inhibitor in the same cell, whereas unpaired tests were used to compare differences between two groups of cells (e.g., PASMCs vs. MASMCs).

RESULTS

Differences in spatial distribution of mitochondria in PASMCs and MASMCs. The distribution of mitochondria [stained with MitoTracker Green FM (24)] in relation to the SR [stained with brefeldin A BODIPY 558/568 (54)] was compared in SMCs isolated from rat small PAs and MAs. The association between mitochondria and SR elements was assessed with the confocal z-sectioning protocol described in MATERIALS AND METHODS. This approach revealed that in PASMCs, on movement from the cell bottom toward the middle of the myocyte depth (Fig. 1A), the fluorescent signal from MitoTracker Green appeared in the x-y confocal optical slices (Fig. 1C) before that of brefeldin A BODIPY (Fig. 1B). In PASMCs, in individual x-z and y-z optical cross sections of the myocyte MitoTracker Green fluorescence (mitochondria) was detected distinctly closer to the cell periphery than that of brefeldin A BODIPY (SR) (Fig. 1, D and E). In MASMCs, however, brefeldin A BODIPY fluorescence staining the SR appeared before or simultaneously with MitoTracker Green fluorescence (Fig. 2B). Such opposing juxtaposition between the SR and mitochondria in MASMCs compared with PASMCs becomes more evident from the comparison of individual x-z and y-z optical cross sections of the myocyte Mitoc-
The area with MitoTracker Green fluorescence, while in MASMCs a similar area is predominantly occupied with the SR (proportional to the area with brefeldin A BODIPY fluorescence). Figure 3 also shows diagrammatic representations of these areas, comparing PASMCs with MASMCs.

The existence of the subplasmalemmal pool of mitochondria in PASMCs, but not in MASMCs, was further studied by double staining of both types of cells with MitoTracker Green and di-8-ANEPPS, selective fluorescent probes for mitochondria and the plasmalemmal membrane, respectively (Fig. 4). Figure 4A shows x-y confocal images taken though the middle of a representative PASMC. As can be seen clearly in Fig. 4A, MitoTracker Green fluorescence often appeared to be “fused” (within ~200 nm, the limit of lateral resolution of our confocal microscope) with di-8-ANEPPS fluorescence shown in red (plasmalemma). Conversely, in MASMCs, a clear gap between the two fluorescent signals was observed (Fig. 4B). To quantitatively compare relative distances between mitochondria and the plasma membrane in the two cell types, the spatial profiles of fluorescent signals recorded for di-8-ANEPPS (red) and MitoTracker Green (green) were constructed across the shortest distance between the center of each mitochondrion and the plasma membrane (shown by yellow lines in Fig. 4, C and D, for PASMC and MASMC, respectively). Histograms of distribution of mitochondria according to their distance from the plasma membrane measured in six PASMCs and six MASMCs are compared in Fig. 4E. The histogram bin size was set to 0.5 μm, which is 2.5-fold greater than the lateral optical resolution of our imaging system determined with 0.2-μm fluorescent beads. This analysis clearly shows that in PASMCs the majority of peripheral mitochondria are localized between 0.5 and 1.5 μm from the plasma membrane, compared with ~1.5–3.5 μm in MASMCs. On average, the mean distance between peripheral mitochondria and the plasma membrane calculated as described above was twofold greater in MASMCs (2.4 ± 0.05 μm, n = 405) than in PASMCs (1.2 ± 0.05 μm, n = 235) (P < 0.0001; Fig. 4F).

Comparison of I_{Kv} characteristics in PASMCs and MASMCs. In view of the locality of the mitochondria to the plasma membrane demonstrated above, it could be postulated that the mitochondrion-mediated effects on K_{v} channels that occur in PASMCs may exist in MASMCs but would be less effective. Consequently, I_{Kv} was isolated pharmacologically (as described in MATERIALS AND METHODS), and its electrophysiological properties were compared in PASMCs and MASMCs under identical experimental conditions.

With the I_{Kv} activation protocol featured in Fig. 5A, whole cell I_{Kv} was recorded in both PASMCs and MASMCs. The amplitude of the current recorded from a representative MASMC (Fig. 5C) was considerably smaller than in a PASMC (Fig. 5B). On average, the differences in I_{Kv} amplitude were significantly different between −40 and +50 mV. For example, at 0 and +50 mV, the mean amplitude of I_{Kv} was equal to 69 ± 5 and 270 ± 17 pA (n = 60), respectively, in MASMCs and 446 ± 20 and 1,369 ± 59 pA (n = 184), respectively, in PASMCs (P < 0.0001). On the other hand, the size of MASMCs, calculated from C_m, was significantly larger than that of PASMCs [16.3 ± 0.6 pF (n = 60) vs. 11.1 ± 0.3 pF (n = 184); P < 0.0001]. The smaller I_{Kv} and the larger cell size in MASMCs result in significant differences in the whole cell current density in the two cell types (Fig. 5D), which was 10-

### Figure 2
Spatial organization of mitochondria relative to SR in mesenteric arterial smooth muscle cells (MASMCs). A: schematic diagram indicating sectional imaging from bottom to center of representative MASMC. For statistical analysis, the 1st optical slice (from the bottom of the cell) that revealed the fluorescence of either of the dyes was referred to as slice 1. B: gallery of the sequential x-y optical slices from slice 1 to slice 5 (from bottom to center of cell depth) shows images of MitoTracker Green fluorescence, brefeldin A BODIPY fluorescence, and their overlay (as indicated). C: 3 individual optical slices: x-y image (blue box) was taken at z position depicted by blue line in x-z and y-z images, x-z image (green box) was taken at y position depicted by green line in x-y and y-z images, and y-z image (red box) was taken at z position depicted by red line in x-y and x-z images. Note that in x-z image cell bottom is toward bottom of image, while in y-z image cell bottom is toward right of image. D: 3D images of mitochondria (green), SR (red), and their overlay (reconstructed from the 1st 10 optical slices and presented after rotation by 90° around x-axis). Note that in 3D images cell bottom is at bottom of image.

Tracker Green (mitochondria) and brefeldin A BODIPY (SR) fluorescent signals (Fig. 2, C and D).

To quantify these differences, the total area occupied by pixels showing MitoTracker Green and brefeldin A BODIPY fluorescence was analyzed in four PASMC and MASMCs and plotted against the optical slice number in Fig. 3. This analysis clearly shows the existence of an area close to the surface of PASMCs that is enriched with mitochondria (proportional to...
to 18-fold greater between -30 and 0 mV and 8- to 9-fold greater between 0 and +50 mV in PASMCs compared with MASMCs.

Comparison of the steady-state activation of \( I_{Kv} \) measured from the normalized I-V curves (which were derived from the \( I_{Kv} \) tail current measurements) from the representative PASMC and MASMC shown in Fig. 5, B and C, indicates that \( I_{Kv} \) activation in MASMC was shifted by ~6 mV to more positive membrane voltages and was steeper than in PASMC (Fig. 5E). These differences in the activation parameters were significant (Table 1).

Comparison of \( I_{Kv} \) inactivation measured with the voltage protocol shown in Fig. 6A from the representative PASMC (Fig. 6B) and MASMC (Fig. 6C) demonstrates that \( I_{Kv} \) inactivation in PASMCs is relatively slow and incomplete compared with that in MASMCs. The normalized \( I_{Kv} \), recorded during the test pulse was plotted against the prepulse potential, and resulting dependencies were fitted to the equation described in MATERIALS AND METHODS, yielding the parameters of \( I_{Kv} \) inactivation, the half-inactivation potential \( V_h \), the slope factor \( k_h \), and the noninactivating component \( A \) (Fig. 6D).

Statistical comparison of these parameters showed that \( I_{Kv} \) in MASMCs is inactivated at more negative membrane potentials, its dependence on the conditional prepulse potential is steeper, and it has less noninactivated current than \( I_{Kv} \) in PASMCs; these differences in inactivation characteristics were significant (Table 1).

Therefore, direct comparison of the steady-state activation and inactivation of \( I_{Kv} \) measured under identical experimental conditions in both cell types clearly demonstrates that PASMCs have significantly greater current amplitude and more available and less inactivated \( K_v \) channels in the negative range of membrane potentials compared with MASMCs.

Mitochondrial inhibition regulates \( I_{Kv} \) more potently in PASMCs. Inhibition of the mETC was previously shown to inhibit \( I_{Kv} \) in PASMCs (3, 23, 57). We recently showed (9) that steady-state activation and \( I_{Kv} \) block were significantly affected by mitochondrial inhibition in PASMCs, with the most pronounced effects observed for antimycin A (inhibiting the mETC at complex III) and oligomycin (blocking the ATP synthase). These two mETC inhibitors therefore were chosen to verify whether functional coupling between mitochondria and \( K_v \) channels in PASMCs is greater than in MASMCs, as would be predicted based on the difference in the distribution of mitochondria in the two cell types as demonstrated in Figs. 1–4.

The different sites of action of antimycin A and oligomycin in mitochondria are schematically depicted in Fig. 7A. The differences in the mean parameters for \( I_{Kv} \) activation and inactivation due to mitochondrial inhibition with antimycin A and oligomycin in PASMCs and MASMCs are comprehensively analyzed in Table 2, while Fig. 7B–D, compares the relative changes in \( I_{Kv} \) activation, inactivation, and block in PASMCs to those in MASMCs. Although both inhibitors caused significant negative shifts in \( I_{Kv} \) inactivation (Fig. 7C) and significant decreases in \( I_{Kv} \) amplitude at ±50 mV (Fig. 7D) in both cell types, all these effects were significantly smaller in MASMCs than in PASMCs (Fig. 7, B–D). Similarly, antimycin
A had a significantly greater effect in PASMCs than in MASMCs (Fig. 7B). Oligomycin, on the other hand, caused significant differences in IKv activation only in PASMCs and not in MASMCs (Fig. 7B and Table 2). Furthermore, inhibitor-induced changes in other parameters, such as \( k_a, k_h, \) and \( A \), were less pronounced and overall not significantly different in MASMCs from those in PASMCs (Table 2). Thus, differences in potency of the effects of the two different mitochondrial inhibitors in two cell types correlate with the difference in mitochondrial distribution described above.

Disruption of cytoskeleton with cytochalasin B alters both peripheral mitochondria distribution and antimycin A-dependent modulation of IKv. A link between mitochondria and the cytoskeleton has been demonstrated previously in cardiomyocytes (2), neurons (25), human coronary arterioles (19), and pulmonary endothelial cells (1) but not in PASMCs. If the cytoskeleton is involved in juxtaposition of mitochondria and the plasma membrane in PASMCs, then disruption of the cytoskeleton should alter the association between mitochondria and the plasma membrane and at the same time attenuate the effect of the mitochondrial inhibitors on IKv. The effect of treatment of PASMCs with 10 \( \mu \)M cytochalasin B, a disrupter of F-actin microfilaments (1, 17, 19), on distribution of mitochondria was visualized with MitoTracker Green and confocal imaging. Figure 8, A and B, compare distribution of mitochondria (shown in green) in relation to the plasma membrane (stained with di-8-ANEPPS and shown in red) in a single PASMC before and 10 min after incubation with cytochalasin B, respectively. To reduce possible errors resulting from changes in the cell shape due to a disruption of the cytoskeleton, partially contracted PASMCs were used in these experiments. As can be seen in Fig. 8, cytochalasin B did not significantly change the cell shape; however, the pattern of green fluorescence across the whole cell was altered, suggesting that the mitochondria are associated with F-actin filaments in PASMCs. Importantly, the distribution of mitochondria at the submembrane regions was altered, as can be seen at an increased magnification in Fig. 8, A and B, bottom. Similar effects were observed in the other eight PASMCs studied.

The above results suggest that if Kc channels in the plasma membrane and peripheral mitochondria are functionally coupled in PASMCs as the electrophysiological evidence indicates (Fig. 7; Ref. 9), then the cell treatment with cytochalasin B should also modulate the effect of the mitochondrial inhibitors
on $I_{Kv}$ characteristics. To test this prediction, the effect of 10 μM cytochalasin B on antimycin A-induced changes in $I_{Kv}$ activation, inactivation, and block was assessed. Cytochalasin B caused a decrease in $I_{Kv}$ amplitude by 35.7 ± 6.1% ($n = 5$, $P < 0.01$) but itself did not have any significant effect on the steady-state activation or inactivation parameters of $I_{Kv}$ (Table 3). Importantly, the effect of antimycin A on $I_{Kv}$ activation was completely abolished (Fig. 8C) and antimycin A-induced changes in $I_{Kv}$ inactivation were significantly attenuated (Fig. 8D) in the presence of cytochalasin B. Thus the ability of cytochalasin B to inhibit the antimycin A-induced changes in the voltage-dependent $I_{Kv}$ characteristics correlates well with cytochalasin B-mediated changes in mitochondrial distribution in the vicinity of the plasma membrane, providing direct evidence for close functional coupling between $K_v$ channels and peripheral mitochondria in PASMCs. Pretreatment of cells with the cytoskeletal disruptor did not, however, significantly change the antimycin A-mediated inhibition of $I_{Kv}$ (Fig. 8E), suggesting that other factors are likely to contribute to the $I_{Kv}$ block caused by the mitochondrial inhibitors.

Intracellular Mg$^{2+}$ is involved in antimycin A-mediated changes in $I_{Kv}$ inactivation. We recently demonstrated (9) that dialysis of PASMCs with Na$_2$ATP and EDTA, which are more potent chelators of Mg$^{2+}$ than Ca$^{2+}$, compared with EGTA

Table 1. Comparison of steady-state $I_{Kv}$ parameters in MASMCs and PASMCs in control conditions

| Cell Type         | $V_{a,m}$ mV | $k_a$ mV | $V_{n,m}$ mV | $k_n$ mV | $A$ mV | $n$ |
|-------------------|--------------|----------|--------------|----------|--------|-----|
| Pulmonary         | $-13.2 ± 0.6$| $10.6 ± 0.3$| $-24.3 ± 1.1$| $11.4 ± 0.4$| $0.28 ± 0.01$| 64  |
| Mesenteric        | $-9.4 ± 1.1$ | $9.2 ± 0.3$ | $-37.0 ± 1.4$| $8.0 ± 0.3$ | $0.16 ± 0.01$| 51  |
| Significance      | $P < 0.001$  | $P < 0.05$ | $P < 0.0001$ | $P < 0.0001$ | $P < 0.0001$ |

Values are means ± SE for $n$ cells. Comparison was performed by using all pooled data for voltage-gated K$^+$ channel currents ($I_{Kv}$) recorded in pulmonary arterial smooth muscle cells (PASMCs) or mesenteric arterial smooth muscle cells (MASMCs) bathed in physiological saline solution containing 10 μM glibenclamide and 1 μM paxilline. $V_a$, half-activation potential; $k_a$, $e$-fold steepness of activation dependence; $V_n$, half-inactivation potential; $k_n$, $e$-fold steepness of inactivation dependence; $A$, noninactivating current component. Student’s unpaired $t$-test or nonparametric Mann-Whitney test was used as described in MATERIALS AND METHODS.
present in the pipette solution, significantly attenuated the effects of the mitochondrial inhibitors on \(I_{Kv}\) activation and block. Considering that the effect of the mitochondrial inhibitors on the steady-state inactivation of \(I_{Kv}\) has not been studied previously, it is important to demonstrate whether changes in \(I_{Kv}\) inactivation induced by the mitochondrial inhibitors are also mediated by intracellular Mg\(^{2+}\). To test this possibility, the effect of antimycin A and oligomycin was studied in cells dialyzed with pipette solutions containing 5 mM EDTA or 5 mM Na\(_2\)ATP, respectively. Figure 9, A and B, clearly demonstrate that the relative changes in \(V_h\) caused by both inhibitors were significantly reduced under these conditions.

We previously found (9) that cell dialysis with increased Mg\(^{2+}\) concentration also mimicked the effects of the mitochondrial inhibitors on \(I_{Kv}\) activation and block. When the steady-state inactivation of \(I_{Kv}\) was measured in cells dialyzed with 5 mM instead of 0.5 mM MgCl\(_2\), the pipette solution but in the absence of mitochondrial inhibitors, a significant leftward shift by \(\sim 7\) mV in the inactivation dependence was observed. The mean \(V_h\) was changed from \(-23.9 \pm 1.1\) mV for 0.5 mM MgCl\(_2\) \((n = 73)\) to \(-30.7 \pm 2.8\) mV for 5 mM MgCl\(_2\) \((n = 12, P < 0.026)\). The noninactivating component \(A\) of \(I_{Kv}\) was also decreased under this condition, from \(0.28 \pm 0.01\) (0.5 mM) to \(0.21 \pm 0.02\) (5 mM) \((P < 0.03)\). No significant changes in \(k_h\) were observed \([11.2 \pm 0.4 (n = 72)\) in 0.5 mM MgCl\(_2\) vs. \(10.4 \pm 0.5\) mV \((n = 12)\) in 5 mM MgCl\(_2\)]. Furthermore, antimycin A-induced changes in \(V_h\) were significantly attenuated (Fig. 9C), whereas changes in \(k_h\) and \(A\) remained similar (compare Tables 2 and 4) under these conditions.

**DISCUSSION**

**Subplasmalemmal localization of mitochondria provides structural basis for functional interaction with \(K_v\) channels in PASMCs.** Although the amount of intracellular diffusible mediators in close vicinity to the \(K_v\) channel cannot be precisely quantified, the overall local cellular changes could be substantial, providing that diffusion processes in the vicinity of the \(K_v\) channels are limited. The existence of a submembrane compartment formed by the SR and called the “superficial barrier” has been previously proposed in systemic vascular SMCs [recently reviewed by Poburko et al. (35)]. Indeed, a tight juxtaposition between the plasmalemmal membrane and the SR has been directly visualized in vascular (11) and visceral (12) SMCs. In myocytes isolated from intralobar rat PAs, we previously demonstrated (42) the presence of a restricted diffusion space by measuring the changes in the K\(^+\) gradient during the development of \(I_{Kv}\). This phenomenon has not been reported in other vascular SMCs. Comparison of the relative distribution of mitochondria and the SR in PASMCs and MASMCs clearly demonstrates the presence of a distinctive population of mitochondria close to the plasmalemmal membrane and relatively separate from the SR in PASMCs but not in MASMCs, where mitochondria are more closely associated with the SR (Figs. 1–4). This novel and important observation demonstrates the presence of a subplasmalemmal pool of mitochondria in PASMCs that are potentially functionally associated with the \(K_v\) channels, as our electrophysiological evidence shows. Such compartmentalization, similar to that formed by the SR in other types of vascular SMCs (35), could
be primarily responsible for the lack of sensitivity of \( I_{Kv} \) to hypoxia in mesenteric arteries previously reported by others (31, 56, 58). Notably, close association between the plasma membrane and mitochondrion has previously been shown by electron microscopy (46). Although the full physiological significance of the existence of the distinctive subplasmalemmal population of mitochondria in PASMCs remains to be established, it is possible that a close juxtaposition of the plasma membrane and mitochondrion in PASMCs might be important in polarization of mitochondrion-mediated signals [e.g., reactive oxygen species (ROS) release] in a similar manner to the “mitochondrial belt” involvement in cytosolic Ca\(^{2+}\) signal polarization in pancreatic acinar cells (45).

The ability of the F-actin filament disrupter cytochalasin B to alter mitochondrial distribution in PASMCs suggests that the cytoskeleton contributes to cellular organization of mitochondria in PASMCs. Whether mitochondria are similarly associated with the cytoskeleton in MASMCs remains to be established. Interestingly, inhibition of F-actin filament polymerization with cytochalasin suppressed mitochondrial ROS production induced by stretch in bovine pulmonary endothelial cells (1) and by flow in human coronary arterioles (19). Whether the cytoskeleton is involved in the generation of ROS in PASMCs has not been yet investigated. Our data clearly suggest that in PASMCs mitochondria are spatially arranged in the distinctive layer separate from the SR, forming a compartment with re-

### Table 2. Comparison of changes in steady-state \( I_{Kv} \) characteristic kinetics due to mitochondrial inhibitors antimycin A and oligomycin

| Cell Type   | Condition | Changes in Steady-State \( I_{Kv} \), Activation | Changes in Steady-State \( I_{Kv} \), Inactivation |
|------------|-----------|-----------------------------------------------|-----------------------------------------------|
| Pulmonary  | Control   | \( V_n = -10.4 \pm 1.1 \), \( k_a = 11.7 \pm 1.0 \) | \( V_n = -16.1 \pm 1.7 \), \( k_a = 12.6 \pm 0.8 \) |
| Significance | Antimycin A | \( P < 0.0001 \)                        | \( P < 0.001 \)                          |
| Mesenteric | Control   | \( V_n = -24.2 \pm 1.9 \), \( k_a = 8.2 \pm 0.7 \) | \( V_n = -32.0 \pm 2.6 \), \( k_a = 8.0 \pm 0.8 \) |
| Significance | Antimycin A | \( P < 0.0001 \)                        | \( P < 0.001 \)                          |
| Pulmonary  | Control   | \( V_n = -9.8 \pm 2.8 \), \( k_a = 10.4 \pm 0.6 \) | \( V_n = -43.2 \pm 3.6 \), \( k_a = 7.3 \pm 0.5 \) |
| Significance | Antimycin A | \( P < 0.05 \)                        | \( P < 0.001 \)                          |
| Pulmonary  | Control   | \( V_n = -16.4 \pm 3.4 \), \( k_a = 10.1 \pm 0.9 \) | \( V_n = -47.8 \pm 3.6 \), \( k_a = 6.1 \pm 0.5 \) |
| Significance | Antimycin A | \( P < 0.05 \)                        | \( P < 0.001 \)                          |
| Pulmonary  | Control   | \( V_n = -14.7 \pm 1.5 \), \( k_a = 9.9 \pm 0.5 \) | \( V_n = -25.2 \pm 3.5 \), \( k_a = 12.2 \pm 1.3 \) |
| Significance | Oligomycin | \( P < 0.0001 \)                        | \( P < 0.01 \)                          |
| Mesenteric | Control   | \( V_n = 26.1 \pm 1.9 \), \( k_a = 8.8 \pm 0.6 \) | \( V_n = -40.1 \pm 3.4 \), \( k_a = 9.0 \pm 0.7 \) |
| Significance | Oligomycin | \( P < 0.0001 \)                        | \( P < 0.001 \)                          |

Values are means ± SE for \( n \) cells. Data compare mean \( I_{Kv} \) parameters measured in control condition and in presence of inhibitor (1 \( \mu \)M) in 2 cell types. Student’s paired \( t \)-test or Wilcoxon test was used as defined in MATERIALS AND METHODS. NS, not significant.

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stricted diffusion in the subplasmalemmal space, thus providing a structural basis for a better functional coupling between mitochondria and \( I_{Kv} \) channels in PASMCs than in systemic arterial myocytes. The correlation between the disruption of the distribution of peripheral mitochondria by cytochalasin B and significant attenuation of the effects of antimycin A on \( I_{Kv} \) activation and inactivation directly supports this conclusion.

Table 3. Effect of cytoskeletal disrupter cytochalasin B on antimycin A-induced changes in steady-state \( I_{Kv} \) characteristic kinetics in PASMCs

| Condition             | Changes in Steady-State \( I_{Kv} \) Activation | Changes in Steady-State \( I_{Kv} \) Inactivation |
|-----------------------|-----------------------------------------------|-----------------------------------------------|
|                       | \( V_a, \text{mV} \)  | \( k_a, \text{mV} \)  | \( n \) | \( V_a, \text{mV} \)  | \( k_a, \text{mV} \)  | \( A \) | \( n \) |
| Control               | \(-16.2\pm3.6\)  | \(9.6\pm0.7\)  | 5 | \(-27.2\pm3.1\)  | \(10.1\pm1.0\)  | \(0.36\pm0.02\)  | 7 |
| Cytochalasin B        | \(-17.5\pm3.9\)  | \(11.2\pm1.9\)  | 5 | \(-30.2\pm1.7\)  | \(9.9\pm1.1\)  | \(0.47\pm0.04\)  | 7 |
| Significance          | \(\text{NS}^*\)  | \(\text{NS}^*\)  | 7 | \(\text{NS}^*\)  | \(\text{NS}^*\)  | \(\text{NS}^*\)  | 7 |
| Cytochalasin + Antimycin A | \(-16.4\pm4.3\)  | \(13.6\pm2.8\)  | 5 | \(-39.3\pm1.8\)  | \(8.1\pm0.8\)  | \(0.12\pm0.01\)  | 7 |
| Significance          | \(\text{NS}^{*+}\)  | \(\text{NS}^{*+}\)  | \(\text{NS}^{*+}\)  | \(\text{NS}^{*+}\)  | \(\text{NS}^{*+}\)  | \(\text{NS}^{*+}\)  | 7 |

Values are means \(\pm\) SE for \( n \) cells. *Data compare mean \( I_{Kv} \) parameters measured in control condition and in presence of either cytoskeletal disrupter cytochalasin B (10 \(\mu\)M) alone or cytochalasin B + mitochondrial inhibitor antimycin A (1 \(\mu\)M) measured in the same PASMCs. †Data compare mean \( I_{Kv} \) parameters measured in presence of cytochalasin B alone and after addition of mitochondrial inhibitor antimycin A (1 \(\mu\)M). Student’s paired \( t \)-test or Wilcoxon test was used as described in MATERIALS AND METHODS.
Mechanisms involved in $I_{K_v}$ channel regulation by mitochondria in PASMCs. The electrophysiological evidence presented in Fig. 9, demonstrating that addition of Mg$^{2+}$-chelating agents into the pipette solution inhibits changes in $V_h$ caused by antimycin A and oligomycin, clearly suggests that an increase in intracellular concentration of Mg$^{2+}$ plays a key role in the modulation of $I_{K_v}$ inactivation by mitochondrial inhibitors. The ability of the increased pipette Mg$^{2+}$ concentration first to mimic and then to attenuate the effect of antimycin A (Fig. 9C) also supports this notion. We previously demonstrated (9) that similar pipette interventions significantly attenuated changes in $V_h$ of mitochondrion-mediated changes in intracellular Mg$^{2+}$ concentration represent one of the major mechanisms controlling the functional activity of $K_v$ channels. On the other hand, the failure of cytochalasin B to attenuate $I_{K_v}$ block, while inhibiting changes in $I_{K_v}$ activation and inactivation (Fig. 8, C–E), suggests the presence of other factors (e.g., ROS) that may influence the ability of Mg$^{2+}$ to interact with $K_v$ channels. The identification of these factors and the establishment of their importance in mitochondrion-mediated and Mg$^{2+}$-dependent modulation of $I_{K_v}$ in PASMCs in normoxic and hypoxic conditions will entail further experimental work.

Stronger functional coupling exists between mitochondria and $K_v$ currents in PASMCs than in MASMCs. To analyze the differences in functional coupling between mitochondria and $K_v$ channels in PASMCs and MASMCs, the inhibitors of complex III (antimycin A) and of the ATP synthase (oligomycin) were used because, as we previously reported (9), their effects on $I_{K_v}$ activation and $I_{K_v}$ block caused by antimycin A and oligomycin in PASMCs, thus suggesting that changes in mitochondrial-mediated changes in intracellular Mg$^{2+}$ concentration represent one of the major mechanisms controlling the

Table 4. Role of intracellular Mg$^{2+}$ in antimycin A- and oligomycin-induced changes in $I_{K_v}$ inactivation in PASMCs

| Condition | Changes in Steady-State $I_{K_v}$ Inactivation | $V_h$, mV | $I_w$, mV | $A$, n |
|-----------|---------------------------------------------|----------|----------|--------|
| 5 mM EDTA | Control                                     | $-19.6\pm2.1$ | $12.2\pm1.3$ | $0.31\pm0.03$ | 8 |
|          | Antimycin A                                | $-24.0\pm1.9$ | $7.3\pm1.1$ | $0.08\pm0.02$ | 8 |
|          | Significance                               | NS       | P < 0.05 | P < 0.0001 |
| 5 mM Na$_2$ATP | Control                        | $-12.0\pm2.9$ | $11.3\pm2.9$ | $0.38\pm0.04$ | 6 |
|          | Oligomycin                                 | $-13.8\pm2.6$ | $9.4\pm1.3$ | $0.28\pm0.01$ | 6 |
|          | Significance                               | NS       | NS       | P < 0.05 |
| 5 mM MgCl$_2$ | Control                          | $-31.5\pm4.5$ | $10.7\pm0.8$ | $0.22\pm0.04$ | 6 |
|          | Antimycin A                                | $-39.7\pm3.6$ | $7.4\pm0.7$ | $0.06\pm0.02$ | 6 |
|          | Significance                               | P < 0.05 | P < 0.05 | P < 0.01 |

Values are means ± SE for n cells. Data compare mean $I_{K_v}$ inactivation parameters measured in absence (control) and in presence of mitochondrial inhibitor antimycin A or oligomycin (both at 1 μM) measured in the same PASMCs in different pipette solutions. In EDTA-containing solution, 5 mM EGTA was replaced with 5 mM EDTA. Student’s paired t-test or Wilcoxon test was used as described in MATERIALS AND METHODS.
Our experimental conditions and pharmacological agents used we report in the present study (Table 1), suggesting that those are comparable with the 9.4 and 37 mV in MASMCs compared with PASMCs. Also, the midpoint for $I_{Kv}$ amplitude, cell size, and steady-state activation and inactivation parameters alone between the two cell types could only predict a 2.6- to 3-fold increase in the steady-state open probability of $I_{Kv}$ channels available in the same voltage range (calculated with the mean values for $I_{Kv}$ activation and inactivation parameters listed in Table 1 and the theoretical model described in Ref. 26). It is therefore likely that the number of functional $K_v$ channels expressed per unit of the cell membrane area should also be greater in PASMCs than in MASMCs.

In conclusion, our findings demonstrate the presence of a distinctive layer of mitochondria at the cell periphery in PASMCs, forming a restricted diffusion cytosolic space between the mitochondria and plasmalemmal membrane and thus providing a structural basis for a close functional interaction between the $K_v$ channels and mitochondria as supported by the electrophysiological evidence. The smaller density of $K_v$ channels in MASMCs alongside the greater diffusion space between the mitochondria and the cell membrane may provide one plausible explanation for the differences in cellular responses during hypoxia.

ACKNOWLEDGMENTS

Present address of A. L. Firth: Div. of Pulmonary and Critical Care Medicine, Dept. of Medicine, University of California, San Diego, La Jolla, CA 92039.

GRANTS

This work was supported by the British Heart Foundation (Grants PG03/059 and PG04/069 supporting K. H. Yuill and PG/08/062/25382 awarded to...
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