The Mitochondrial Barriers Segregate Agonist-induced Calcium-dependent Functions in Human Airway Epithelia

CARLA M. PEDROSA RIBEIRO, ANTHONY M. PARADISO, ALESSANDRA LIVRAGHI, and RICHARD C. BOUCHER

Cystic Fibrosis/Pulmonary Research and Treatment Center and Department of Medicine, The University of North Carolina at Chapel Hill, NC 27599

ABSTRACT In airway epithelia, purinergic receptor (P2Y$_R$) stimulation of intracellular calcium (Ca$^{2+}$)–regulated ion transport is restricted to the membrane domain ipsilateral to receptor activation, implying compartmentalization of Ca$^{2+}$ signaling. Because mitochondria can spatially restrict cellular Ca$^{2+}$ signals, immunocytochemical, electron microscopic, and fluorescent studies of mitochondria localization were performed in human airway epithelia. Although concentrated at the apical domain, mitochondria were found distributed at both the apical and the basolateral poles and in close association with the endoplasmic reticulum. The role of mitochondria in locally restricting P2Y$_R$-induced Ca$^{2+}$ signals was investigated by measuring changes in mitochondrial Ca$^{2+}$ (Ca$^{2+}_{mito}$) in human airway epithelial monolayers. P2Y$_R$ activation induced Ca$^{2+}_{mito}$ accumulation in mitochondria confined to the domain ipsilateral to P2Y$_R$ stimulation, which was blocked by mitochondrial uncoupling with 1 mM CCCP and 2.5 µg/ml oligomycin. The role of mitochondria in restricting the cellular cross-talk between basolateral P2Y$_R$-dependent Ca$^{2+}$ mobilization and apical membrane Ca$^{2+}$-activated Cl$^-$ secretion was investigated in studies simultaneously measuring Ca$^{2+}_{mito}$ and Cl$^-$ secretion in cystic fibrosis human airway epithelial monolayers. Activation of basolateral P2Y$_R$s produced similar increases in Ca$^{2+}$, in monolayers without and with pretreatment with uncouplers, whereas Ca$^{2+}$-activated Cl$^-$ secretion was only efficiently triggered in mitochondria-uncoupled conditions. We conclude that (a) mitochondria function as a Ca$^{2+}$-buffering system in airway epithelia, compartmentalizing Ca$^{2+}$-dependent functions to the membrane ipsilateral to receptor stimulation; and (b) the mitochondria provide structural barriers that protect the airway epithelia against nonspecific activation of Ca$^{2+}_{mito}$-modulated functions associated with Ca$^{2+}$ signals emanating from the apical or the basolateral membrane domains.

KEY WORDS: calcium signaling • mitochondria • endoplasmic reticulum • chloride secretion • purinergic receptors

INTRODUCTION

Epithelial cells are exposed to distinct physical and chemical stimuli in their mucosal and serosal environments. Epithelia can adapt to these different environments by confining functional responses selectively to their apical or basolateral epithelial membranes. In airway epithelia, membrane-specific intracellular calcium (Ca$^{2+}_{mito}$)-dependent responses can be demonstrated by activation of apical or basolateral purinergic receptors (P2Y$_R$s) coupled to phospholipase C stimulation and inositol 1,4,5-trisphosphate (IP$_3$) formation (Paradiso et al., 1995; Ribeiro et al., 2001). Ca$^{2+}_{mito}$ signals resulting from activation of P2Y$_R$s are restricted to the membrane domain ipsilateral to receptor activation (Paradiso et al., 1995).

The confinement of Ca$^{2+}_{mito}$ signals to the apical or the basolateral cellular poles in airway epithelia has functional correlates. For example, apical P2Y$_R$-R activation results in Ca$^{2+}$ mobilization that efficiently couples to apical Cl$^-$ secretion through Ca$^{2+}$-activated Cl$^-$ channels (CaCC) in monolayers of cystic fibrosis (CF) human airway epithelia (Paradiso et al., 2001). In contrast, an effective coupling between P2Y$_R$-R activation and CaCC-mediated Cl$^-$ secretion is not present after activation of basolateral P2Y$_R$s in CF airway epithelia (Clarke and Boucher, 1992; Paradiso et al., 1995, 2001). These data have led to the speculation that Ca$^{2+}_{mito}$ mobilized by apical P2Y$_R$-R activation locally activates CaCC, whereas Ca$^{2+}$ signals triggered by basolateral P2Y$_R$-R activation do not transit the cell to activate CaCC in the apical membrane.

A number of studies suggest that mitochondria, if properly organized, play a role in restricting Ca$^{2+}$ permeation within polarized cells by buffering Ca$^{2+}$ signals generated by activation of plasma membrane receptors (Rizzuto et al., 1994; Simpson and Russell, 1996; Babcock et al., 1997; Landolfi et al., 1998; Boitier et al., 1999; Hajnoczky et al., 1999; Tinell et al., 1999).

Address correspondence to Carla M. Pedrosa Ribeiro, Cystic Fibrosis/Pulmonary Research and Treatment Center and Department of Medicine, The University of North Carolina at Chapel Hill, NC 27599-7248. Fax: (919) 966-5178; email: carla_ribeiro@med.unc.edu

Abbreviations used in this paper: CaCC, Ca$^{2+}$-activated Cl$^-$ channels; P2Y$_R$s, purinergic receptors; TG, thapsigargin.

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Agonist-induced ER Ca\(^{2+}\) store release can be buffered by mitochondrial Ca\(^{2+}\) uptake through a Ca\(^{2+}\) uniporter whose activity is dependent on the mitochondrial membrane potential (Gunter et al., 2000). After the return of cytoplasmic Ca\(^{2+}\) toward basal levels, Ca\(^{2+}\) can exit the mitochondria through three possible mechanisms that have different kinetics and inhibitor susceptibilities: A Na\(^{+}\)-dependent efflux (3 Na\(^{+}\) to 1 Ca\(^{2+}\) or 2 Na\(^{+}\) to 1 Ca\(^{2+}\)), a Na\(^{+}\)-independent efflux (2 H\(^{+}\) for 1 Ca\(^{2+}\)), and, in some cases, through the mitochondrial permeability transition (MPT) pore (Gunter et al., 2000). As evidence of the role for mitochondria in Ca\(^{2+}\) signaling, Tinel et al. (1999) have shown that mitochondria localized at the apical pole of polarized pancreatic acinar cells limited Ca\(^{2+}\) diffusion from the apical to the basolateral membrane. After mitochondrial inhibition, this mitochondrial “barrier” was lost and apical Ca\(^{2+}\) signals were able to spread to the basolateral pole (Tinel et al., 1999).

The Tinel et al. (1999) studies suggest that segregation of Ca\(^{2+}\), signals in human airway epithelia may result from the spatial distribution of ER Ca\(^{2+}\) stores and, possibly, mitochondria. Therefore, we investigated the role mitochondria play in compartmentalizing P2Y\(_2\)-R-dependent Ca\(^{2+}\) signals to the membrane ipsilateral to receptor activation. For these studies, analyses of mitochondria distribution and mitochondrial Ca\(^{2+}\) uptake upon P2Y\(_2\)-R activation were performed in human airway epithelia. We also simultaneously measured \(\Delta\text{Ca}^{2+}\) and anion (Cl\(^{-}\)) secretion to test the functional role of mitochondria in preventing Ca\(^{2+}\) signals generated at one pole of the cell from reaching the opposite pole.

**MATERIALS AND METHODS**

**Tissues**

Human bronchial airway epithelia were freshly isolated at the time of transplantation and obtained from the University of North Carolina Cystic Fibrosis Center Tissue Culture Core under the auspices of protocols approved by the Institutional Committee on the Protection of the Rights of Human Subjects.

**Cell Culture**

Human bronchial epithelial cells from main stem or lobar bronchi, harvested from excess tissues from excised CF and recipient lungs, were obtained from the University of North Carolina Cystic Fibrosis Center Tissue Culture Core at the time of lung transplantation. Disaggregated airway epithelial cells were seeded on 1-cm\(^2\) Transwell Col (T-Col) filters (pore diameter = 0.45 \(\mu\)m) at a density of 0.25 \(\times\) 10\(^6\)/cm\(^2\) in Ham’s F12-based medium supplemented with 10 \(\mu\)g/ml insulin, 5 \(\mu\)g/ml transferrin, 1 \(\mu\)M hydrocortisone, 30 nM triiodothyronine, 25 ng/ml epidermal growth factor, and 3.75 \(\mu\)g/ml endothelial cell growth substance. All cell preparations were maintained at an air–liquid surface interface, and polarized monolayer cultures were studied 6–11 d later.

**Confocal Immunofluorescence Microscopy of Mitochondrial Distribution in Airway Epithelia**

Bronchial epithelia from transplant lungs were fixed in 4% paraformaldehyde, embedded in paraffin blocks, 10-\(\mu\)m sections were obtained and deparaffinized, and immunocytochemistry performed according to a previous method (Ribeiro et al., 1997). Sections were washed with PBS (120 mM NaCl, 2.6 mM KCl, 8.1 mM Na\(_2\)HPO\(_4\), 1.5 KH\(_2\)PO\(_4\), pH 7.4; all incubations and rinses were performed with PBS), permeabilized with 1% Triton X-100 for 10 min at 25\(^\circ\)C, rinsed three times, blocked overnight with 3% bovine serum albumin at 4\(^\circ\)C, and rinsed three times. To stain mitochondria, sections were incubated with a mouse monoclonal anti-mitochondrial heat shock protein 70 antibody (Affinity Bioreagents) at 1:50 dilution for 60 min at 37\(^\circ\)C, washed three times, incubated for 30 min at 25\(^\circ\)C with a Texas red–labeled affinity-purified goat anti-mouse antibody (1:200 dilution; Jackson Immunoresearch Laboratories), and rinsed three times. Mitochondria were localized by laser confocal microscopy (TCS 4D; PL APO 63\(\times\)/1.20 mm water lens; Leica). As a control for the mitochondria immunostain, the primary antibody was omitted and only the secondary antibody was used. No stain was observed under these conditions.

**Fluorescent Labeling of Mitochondria with MitoTracker**

Primary culture monolayers of airway epithelia were bilaterally incubated with F12 medium containing 100 nM of MitoTracker Red CMX Ros (Molecular Probes) for 30 min at 37\(^\circ\)C. Cultures were subsequently washed in a HEPES-buffered saline solution and mitochondrial distribution visualized by laser confocal microscopy (SP2-AOBS; PL APO 63\(\times\)/1.20 mm water lens; Leica) in the XZ-scanning mode.

**Electron Microscopy (EM) Studies of Mitochondrial Distribution in Airway Epithelia**

Bronchial airway epithelia from transplant lungs were fixed in 2% gluteraldehyde + 2% paraformaldehyde + 0.25% tannic acid, postfixed in 1% OsO\(_4\), and processed for EM as described previously (Robinson and Gray, 1996). For EM studies of the physical interaction between mitochondria and ER, monolayers of primary cultures were fixed and processed for EM in the same manner as freshly excised bronchial epithelia.

**Confocal Microscopic Studies of Mitochondrial Calcium (Ca\(^{2+}\)\(_m\))**

For confocal Ca\(^{2+}\)\(_m\) measurements, monolayers were loaded with rhod2 according to a modification of a previous method (Trolinger et al., 1997) by incubation with 5 \(\mu\)M rhod2/AM at 4\(^\circ\)C for 18 h in a HEPES-buffered saline solution, followed by incubation with 5 \(\mu\)M rhod2/AM in F12 medium at 37\(^\circ\)C for 1 h. Ca\(^{2+}\)\(_m\) mobilization (changes in rhod2 fluorescence) was studied by laser confocal microscopy (TCS 4D; PL APO 63\(\times\)/1.20 mm water lens; Leica) on the XY or XZ scanning mode. The fluorescence intensity of rhod2 from XY or XZ confocal scans was measured with the MetaMorph software (Universal Imaging). Regions of interest were designated for the apical or the basolateral domains, depending on the protocol, and the same region was quantified at each time point. The same acquisition parameters (e.g., laser power, contrast, brightness, and pinhole value) were used throughout the time course. The fluorescence intensity values (in arbitrary units) from the designated regions were expressed as percentage of the fluorescence intensity from baseline (\(t = 0\)) in every experiment.
To uncouple the mitochondria, the mitochondrial membrane potential was dissipated by exposing the airway epithelia to 1 μM carbonyl cyanide m-chlorophenylhydrazone (CCCP) in conjunction with 2.5 μg/ml oligomycin (to block the activity of the F_{0}F_{1} ATP synthase) before or after P_{2}Y_{2}-R activation.

**Perfusion Chamber and Bioelectric Measurements**

For simultaneous measurements of Cl⁻ secretion (I_{eq}) and Ca^{2+}, CF culture monolayers on T-Col filters were mounted in a miniature Ussing chamber over an objective of a microscope coupled to a microfluorimeter as described previously (Paradiso et al., 2001). For passing current, two circular Ag/AgCl electrodes were placed in the two half-chambers to generate a uniform density of current through the preparation. For measurements of transepithelial electrical potential difference (V_{t}), polyethylene bridges containing 2 M KCl in 3% agar were positioned in the two half-chambers and connected to calomel electrodes. Briefly, V_{t} was measured by a voltage-clamp/pulse generator (model VCC600; Physiologic Instruments) and recorded on a two-channel recorder (Linses model L200S). To calculate changes in Cl⁻ secretory current (ΔI_{eq}), a defined 1-s current pulse was delivered across the monolayer every 10 s. The airway epithelia were converted from their native Na⁺ absorptive state to a Cl⁻ secretory state by exposing the monolayers to a basolateral medium of Krebs bicarbonate Ringer solution (KBR; in mM, 125 NaCl, 2.5 K_{2}HPO_{4}, 1.3 CaCl_{2}, 1.3 MgCl_{2}, 25 NaHCO_{3} and 5 d-glucose) equilibrated with 5% CO_{2}/95% O_{2}, and to an apical medium of KBR containing 0 Na⁺/low Cl⁻ (N-methyl-D-glucamine substituting for Na⁺) as reported previously (Paradiso et al., 2001). Polarized monolayers of CF airway epithelia were loaded with Fura-2/AM (5 μM at 37°C for 25 min), as described earlier (Paradiso et al., 2001), before being mounted in a miniature Ussing chamber over an objective (ZEISS LD Achromat 40, NA 0.6; working distance 1.8 mm) of a ZEISS Axiovert 35 microscope.

**Statistical Analyses**

Data in bar graphs represent the mean ± SEM from at least three experiments from individual donors (tissue codes). Where appropriate, data were analyzed by unpaired t test or two-way analysis of variance (ANOVA) with the GraphPad InStat software and statistical significance was defined with P < 0.05.

**RESULTS**

**Mitochondrial Distribution in Polarized Human Airway Epithelia**

The immunocytochemical localization of mitochondrial heat shock protein 70 in freshly isolated bronchial airway epithelia demonstrated that mitochondria are present throughout the epithelial cells, but distributed predominantly toward the apical domain (Fig. 1 A). This localization was confirmed in studies of freshly isolated human bronchial airway epithelia subjected to transmission electron microscopic analysis (Fig. 1 B). The micrograph reveals a concentration of mitochondria, i.e., a “barrier”, at the apical cellular pole, although, to a lesser extent, mitochondria were also localized at the basolateral domain around and below the nucleus. Fig. 1 C illustrates that the mitochondrial distribution observed in Fig. 1, A and B, was reproduced.
Mitochondria and Ca\(^{2+}\)/H\(^{11001}\) Signals in Airway Epithelia

in primary culture monolayers of human bronchial airway epithelia labeled with the mitochondrial fluorescent dye MitoTracker Red CMX Ros.

**P2Y\(_{2}\)-R Activation Induces Mitochondrial Ca\(^{2+}\) Uptake in the Cellular Pole Ipsilateral to Receptor Stimulation in Human Airway Epithelia**

We next addressed whether the mitochondria are involved in compartmentalization of Ca\(^{2+}\) signals in airway epithelia. If mitochondria restrict Ca\(^{2+}\) movements via an uptake mechanism, changes in mitochondrial Ca\(^{2+}\) (Ca\(^{2+}\)-m) should be detectable after P2Y\(_{2}\)-R activation. To measure Ca\(^{2+}\)-m, mitochondria were loaded with the fluorescent Ca\(^{2+}\) indicator rhod-2, and Ca\(^{2+}\)-m uptake was studied by laser confocal–scanning microscopy. Fig. 2 A illustrates a time series of XY confocal images from the apical domain of CF human airway epithelial monolayers loaded with rhod-2. Mucosal application of UTP (100 \(\mu\)M), which activates apical P2Y\(_{2}\)-Rs and triggers a rise in Ca\(^{2+}\) (Clarke and Boucher, 1992; Paradiso et al., 2001), rapidly induced large elevations of Ca\(^{2+}\)-m (Fig. 2 A, top). To test whether the changes in rhod-2 fluorescence resulted from P2Y\(_{2}\)-R activation-dependent Ca\(^{2+}\)-m uptake, the same monolayers were subsequently treated with 0.1 \(\mu\)M carbonyl cyanide m-chlorophenylhydrazone (CCCP) and 2.5 \(\mu\)g/ml oligomycin to depolarize the mitochondria and abolish the driving force for Ca\(^{2+}\)-m uptake (Tinel et al., 1999). Mitochondrial uncoupling abolished the UTP-induced rise in mitochondrial rhod-2 fluorescence (Fig. 2 A, bottom), in agreement with previous studies (Hajnoczky et al., 2000). Fig. 2 B summarizes the time series studies illustrated in Fig. 2 A. Conversely, mitochondrial uncoupling before mucosal application of UTP prevented a subsequent P2Y\(_{2}\)-R activation-induced Ca\(^{2+}\)-m uptake.
Ribeiro et al. (unpublished data). These studies strongly suggest that Ca\(^{2+}\), released from IP\(_3\)-sensitive ER Ca\(^{2+}\) stores after P2Y\(_2\)-R stimulation, accumulates in functioning mitochondria in airway epithelia.

Since mitochondrial Ca\(^{2+}\) uptake after P2Y\(_2\)-R activation may require an intimate association between mitochondria and IP\(_3\)-sensitive ER sites (Simpson and Russell, 1996; Boitier et al., 1999; Hajnoczky et al., 1999; Khodorov et al., 1999; Tinel et al., 1999; Duchen, 2000), the spatial distribution of these organelles was studied in electron micrographs from primary cultures of human airway epithelia. Fig. 3 illustrates that the mitochondrial and the ER networks are intimately associated in airway epithelia, similar to previous findings (Rizzuto et al., 1993, 1998; Landolfi et al., 1998; Tinel et al., 1999). These data suggest that the association of mitochondria and ER may provide an efficient system for the mitochondrial role in restricting the spread of Ca\(^{2+}\) signals resulting from P2Y\(_2\)-R activation in airway epithelia.

To address the Ca\(^{2+}\)\(_m\) uptake under conditions where Ca\(^{2+}\) signals are of lower magnitude compared with those elicited by P2Y\(_2\)-R activation, additional studies were performed with the ER Ca\(^{2+}\)-ATPase inhibitor thapsigargin (TG). Since the kinetics of ER Ca\(^{2+}\) release are slower in TG-treated cells, Ca\(^{2+}\) levels do not rise as quickly and do not reach the same magnitude compared with UTP-dependent Ca\(^{2+}\) signals resulting from IP\(_3\)-mediated ER Ca\(^{2+}\) release (Paradiso et al., 1995). Fig. 4 A depicts a time series of XY confocal images from the apical domain of CF human airway epithelial monolayers loaded with rhod-2. Mucosal application of TG (1 \(\mu\)M) induced Ca\(^{2+}\)\(_m\) uptake, which was inhibited by mitochondrial uncouplers, but exhibited slower kinetics and lower magnitude when compared with the Ca\(^{2+}\)\(_m\) uptake elicited by P2Y\(_2\)-R activation (Fig. 2). Fig. 4 B summarizes the time series studies exemplified in Fig. 4 A. These findings suggest that, in airway epithelia, the magnitude of the Ca\(^{2+}\)\(_m\) uptake is a function of the magnitude of the Ca\(^{2+}\) signal that reaches the mitochondria after ER Ca\(^{2+}\) store depletion.

The role of mitochondria in the compartmentalization of Ca\(^{2+}\) signals was further studied in rhod2-
loaded CF monolayers with XZ confocal scans to simultaneously visualize the apical and the basolateral domains. Apical P2Y2-R activation induced a rapid and sustained apical \( \text{Ca}^{2+} \) accumulation (Fig. 5 A). The rise in \( \text{Ca}^{2+} \) was restricted to the mitochondria localized at the apical pole of the cells, with no changes in \( \text{Ca}^{2+} \) detected at the contralateral, basolateral domain. The compiled data from these studies are depicted in Fig. 5 B.

Conversely, basolateral P2Y2-R activation promoted \( \text{Ca}^{2+} \) accumulation in the mitochondria localized at the basolateral domain, without affecting \( \text{Ca}^{2+} \) in the...
mitochondria localized at the apical cellular pole (Fig. 6 A). The average data from these studies are shown in Fig. 6 B. Collectively, these findings suggest that apically or basolaterally distributed mitochondria exert functional barriers against the spread of Ca$^{2+}$/H$^{+}$ signals toward the domain contralateral to the membrane of P2Y$_2$-R activation.

Basolateral UTP-dependent Ca$^{2+}$/H$^{+}$ Mobilization and Apical Cl$^{-}$/H$^{+}$ Secretion under Coupled and Uncoupled Mitochondrial Conditions in Human Airway Epithelia

We next addressed whether mitochondria play a functional role in restricting the spatial range of P2Y$_2$-R-promoted Ca$^{2+}$/H$^{+}$ mobilization by using Ca$^{2+}$-dependent Cl$^{-}$ secretion as a read-out of transcellular Ca$^{2+}$/H$^{+}$ permeation. These studies were performed in monolayers of polarized CF human airway epithelia which, due to the absence of functional CFTR, utilize CaCC as the sole pathway for apical Cl$^{-}$ secretion (Paradiso et al., 2001).

We simultaneously measured Ca$^{2+}$/H$^{+}$ mobilization and CaCC-mediated Cl$^{-}$ secretion (equivalent current; $I_{eq}$) in a miniature Ussing chamber that permits the independent perfusion of the serosal and the mucosal compartments.

Fig. 7 A depicts representative simultaneous recordings of Ca$^{2+}$ levels and $I_{eq}$ (transepithelial electrical potential difference) from CF monolayers with intact mitochondrial function. Basolateral addition of forskolin (10 μM) failed to induce any changes in $I_{eq}$, consistent with the absence of cAMP-induced Cl$^{-}$ secretion through CFTR in CF cultures. The subsequent maximal activation of basolateral P2Y$_2$-Rs by addition of 100 μM UTP (Paradiso et al., 2001) elicited Ca$^{2+}$/H$^{+}$ mobilization. However, this response was only coupled to small changes in $I_{eq}$ as reported previously (Clarke and Boucher, 1992; Paradiso et al., 1995, 2001).

Studies were then performed after pretreatment with mitochondrial uncouplers to inhibit mitochondrial function. Fig. 7 B illustrates representative simultaneous recordings of Ca$^{2+}$ mobilization and $I_{eq}$ from CF monolayers subjected to the same protocol described in Fig. 7 A except that, after basolateral forskolin addition, the mitochondria were uncoupled by bilateral treatment with 1 μM CCCP and 2.5 μg/ml oligomycin. Before UTP, CCCP and oligomycin promoted a small Ca$^{2+}$/H$^{+}$ rise, suggesting that mitochondrial uncoupling released Ca$^{2+}$ from the mitochondria (Rizzuto et al., 1994; Simpson and Russell, 1996; Babcock et al., 1997; Hajnoczky et al., 1999). More importantly, this Ca$^{2+}$/H$^{+}$ mobilization correlated with a small increase in $I_{eq}$, suggesting that this small Ca$^{2+}$/H$^{+}$ rise was effective in stimulating apical CaCC activity under mitochondria-uncoupled conditions. Since most mitochondria are distributed at the apical pole of airway epithelial cells (Fig. 1),
these findings suggest that the close proximity of mitochondria to CaCC sites may account for the moderately efficient stimulation of apical Cl\(^{-}\) secretion after Ca\(^{2+}\)\(_{m}\) release. After mitochondrial inhibition, basolateral P2Y\(_{2}\)-R activation with 100 \(\mu\)M UTP increased Ca\(^{2+}\)\(_{i}\) to the same degree as to mitochondria-coupled cultures (Fig. 7 B). However, under mitochondria-uncoupled conditions, basolateral P2Y\(_{2}\)-R activation much more efficiently triggered CaCC-mediated Cl\(^{-}\) secretion. Fig. 7, C and D, depict the summary \(\Delta\)Ca\(^{2+}\)\(_{i}\) (peak – baseline Ca\(^{2+}\)\(_{i}\) levels) and \(\Delta I_{eq}\) (derived from the \(V_{i}\) values) data, respectively, from the experiments shown in Fig. 7, A and B.

**DISCUSSION**

Polarized airway epithelial cells express specific Ca\(^{2+}\)\(_{i}\)-dependent functions, e.g., Ca\(^{2+}\)\(_{i}\)-regulated ion channels, confined to the apical or the basolateral domains (Paradiso et al., 1995). Although both apical or basolateral P2Y\(_{2}\)-R activation couple to PLC stimulation–dependent Ca\(^{2+}\)\(_{i}\) mobilization, Ca\(^{2+}\)\(_{i}\)-mediated channel regulation at the apical or the basolateral membrane can only be elicited by ipsilateral receptor activation (Clarke and Boucher, 1992; Paradiso et al., 1995, 2001). Therefore, P2Y\(_{2}\)-R activation can be used as a tool to study mechanisms by which Ca\(^{2+}\)\(_{i}\)-dependent responses are compartmentalized within airway epithelia.

This study demonstrates that mitochondria efficiently function as a Ca\(^{2+}\)\(_{i}\)-buffering system in airway epithelia. The studies with mitochondria-compartmentalized rhod-2 revealed that Ca\(^{2+}\)\(_{m}\) accumulated upon apical (Figs. 2 and 5) or basolateral (Fig. 6) P2Y\(_{2}\)-R activation selectively in the mitochondria localized at the pole ipsilateral to receptor stimulation. Therefore, by virtue of their Ca\(^{2+}\)\(_{i}\)-buffering activity, mitochondria appear functionally capable of restricting the distance that Ca\(^{2+}\) signals generated by P2Y\(_{2}\)-R activation can travel across the airway epithelia.

This notion was tested functionally in experiments that demonstrated that, under mitochondria-coupled conditions (Fig. 7 A), basolateral P2Y\(_{2}\)-R activation did not efficiently couple to Ca\(^{2+}\)\(_{i}\)-regulated apical Cl\(^{-}\) secretion, despite a robust Ca\(^{2+}\)\(_{i}\) mobilization. However, after mitochondrial uncoupling (Fig. 7 B), basolateral P2Y\(_{2}\)-R activation increased Ca\(^{2+}\)\(_{i}\) to the same level compared with cultures with coupled mitochondria, but promoted efficient Ca\(^{2+}\)\(_{i}\)-dependent apical secretion (Fig. 7, C and D).

The present data offer a mechanism to account for previous observations of membrane-restricted Ca\(^{2+}\)\(_{i}\) signaling events in airway epithelia (Clarke and Boucher, 1992; Paradiso et al., 1995, 2001). Specifically, the mitochondria localized at the apical or the basolateral poles serve as barriers to prevent global cell Ca\(^{2+}\)\(_{i}\) signaling upon plasma membrane receptor activation. Moreover,
Our findings are consistent with a previous study addressing the mitochondrial participation in \( \text{Ca}^{2+} \) signaling in polarized pancreatic acinar cells. In this study, the mitochondria were shown to localize around the apical pole, the site of IP\(_3\)-sensitive \( \text{Ca}^{2+} \) stores, to limit \( \text{Ca}^{2+} \) diffusion from the apical to the basolateral domain (Tinel et al., 1999). After mitochondrial inhibition, this mitochondrial barrier was lost and \( \text{Ca}^{2+} \) signals could spread toward the basolateral pole (Tinel et al., 1999).

The present observation of a close association between mitochondria and ER in airway epithelia (Fig. 3) is consistent with reports describing a role for mitochondria in \( \text{Ca}^{2+} \) homeostasis in other systems. For example, mitochondria have been shown to localize in close proximity to ER \( \text{Ca}^{2+} \)-releasing sites (Rizzuto et al., 1993, 1998; Landolfi et al., 1998; Tinel et al., 1999), where microdomains of high \( \text{Ca}^{2+} \) result from IP\(_3\)-dependent \( \text{Ca}^{2+} \) release trigger fast mitochondrial uptake of large amounts of \( \text{Ca}^{2+} \). The physical interaction between mitochondria and IP\(_3\)-dependent \( \text{Ca}^{2+} \)-releasing sites in the ER, together with the rapid mitochondrial \( \text{Ca}^{2+} \) uptake, not only allow for the fast modulation of mitochondrial metabolism, but may also limit the amplitude as well as the spatiotemporal aspects of agonist-generated \( \text{Ca}^{2+} \) signals (Simpson and Russell, 1996; Boitier et al., 1999; Hajnoczky et al., 1999; Khodorov et al., 1999; Tinel et al., 1999; Duchen, 2000).

In conclusion, our findings have important implications for airway epithelial biology. A model illustrating the role of the apical and the basolateral mitochondrial barriers in confining \( \text{Ca}^{2+} \) signals in human airway epithelia is depicted in Fig. 8. The strategic apical polarization of the mitochondria and their association with ER \( \text{Ca}^{2+} \) stores provides for an optimal \( \text{Ca}^{2+} \) uptake when \( \text{Ca}^{2+} \) levels rise as a result of autocrine/paracrine regulation of apical P2Y\(_7\)-Rs by, e.g., apical nucleotide release. The large mitochondrial barrier at the apical cellular pole, by acting as a buffer for \( \text{Ca}^{2+} \) signals resulting from luminal airway stress (e.g., cough-induced shear stress), may provide a functional barrier between the apical domain and the rest of the cell to prevent global \( \text{Ca}^{2+} \) waves, thereby protecting the nuclear and basolateral cytoplasmic compartments from \( \text{Ca}^{2+} \)-modulated functions in response to luminal stress. Conversely, the \( \text{Ca}^{2+} \)-buffering activity of the basolateral mitochondria serves to restrict \( \text{Ca}^{2+} \) signals generated by serosal agonists to the basolateral domain of airway epithelia, resulting in efficient \( \text{Ca}^{2+} \)-dependent regulation of basolateral \( \text{K}^+ \) channels, but not apical CaCC.

The mitochondrial compartmentalization of \( \text{Ca}^{2+} \) signals resulting from basolateral P2Y\(_7\)-R activation has profound consequences for CF as compared with normal airway epithelia. In normal airway epithelia, CFTR-
mediated Cl\(^{-}\) secretion can be triggered by basolateral P2Y\(_{2}\)-R activation coupled to induction of two pathways (Paradiso et al., 2001): (a) membrane hyperpolarization due to Ca\(^{2+}\)-activated basolateral K\(^{+}\) channels, which increases the driving force for CFTR-mediated Cl\(^{-}\) secretion and (b) activation of a Ca\(^{2+}\)-independent protein kinase C, which, directly or indirectly, activates CFTR at the apical membrane. On the other hand, in CF airway epithelia lacking functional CFTR, basolateral P2Y\(_{2}\)-R activation does not induce Cl\(^{-}\) secretion since the only Cl\(^{-}\) secretory pathway available at the apical membrane, CaCC (Paradiso et al., 2001), cannot be activated by the resultant Ca\(^{2+}\) mobilization by virtue of the Ca\(^{2+}\)-buffering activity of basolateral mitochondria.

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