Caspofungin induces the release of Ca\(^{2+}\) ions from internal stores by activating ryanodine receptor-dependent pathways in human tracheal epithelial cells

Sabrina Müller\(^1,4\), Christian Koch\(^1,4\), Sebastian Weiterer\(^2\), Markus A. Weigand\(^2\), Michael Sander\(^1\) & Michael Henrich\(^3\)*

The antifungal drug caspofungin is known to alter the cell function of cardiomyocytes and the cilia-bearing cells of the tracheal epithelium. The objective of this study was to investigate the homeostasis of intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) after exposure to caspofungin in isolated human tracheal epithelial cells. The [Ca\(^{2+}\)]\(_i\) was measured using the ratiometric fluorophore FURA-2 AM. We recorded two groups of epithelial cells with distinct responses to caspofungin exposure, which demonstrated either a rapid transient rise in [Ca\(^{2+}\)]\(_i\) or a sustained elevation of [Ca\(^{2+}\)]\(_i\). Both patterns of Ca\(^{2+}\) kinetics were still observed when an influx of transmembrane Ca\(^{2+}\) ions was pharmacologically inhibited. Furthermore, in extracellular buffer solutions without Ca\(^{2+}\) ions, caspofungin exposure still evoked this characteristic rise in [Ca\(^{2+}\)]\(_i\). To shed light on the origin of the Ca\(^{2+}\) ions responsible for the elevation in [Ca\(^{2+}\)]\(_i\), we investigated the possible intracellular storage of Ca\(^{2+}\) ions. The depletion of mitochondrial Ca\(^{2+}\) stores using 25 µM 2,4-dinitrophenol (DNP) did not prevent the caspofungin-induced rise in [Ca\(^{2+}\)]\(_i\), which was rapid and transient. However, the application of caffeine (30 mM) to discharge Ca\(^{2+}\) ions that were presumably stored in the endoplasmic reticulum (ER) prior to caspofungin exposure completely inhibited the caspofungin-induced changes in [Ca\(^{2+}\)]\(_i\). In summary, caspofungin has been shown to trigger an increase in [Ca\(^{2+}\)]\(_i\), independent from extracellular Ca\(^{2+}\) ions by liberating the Ca\(^{2+}\) ions stored in the ER, mainly via a RyR pathway.

The mucociliary clearance of lower airways contributes to the removal of debris and pathogens that are trapped in the upper mucus layer from the lower airways. This complex mechanism is driven by cilia-bearing cells of the bronchiolar and tracheal epithelium. The kinocilia of these cells bear over 200 motor proteins as a propelling apparatus, including dynein, the ATP hydrolyzing enzyme\(^1,2\). Under basal conditions, cilia beat continuously without external stimulation but can beat faster when necessary. This elevated beating frequency depends on several interdependent signal transduction cascades, including changes in intracellular concentrations of Ca\(^{2+}\) ions ([Ca\(^{2+}\)]\(_i\))\(^3-6\). However, most signal cascades in these cells depend on at least a transient [Ca\(^{2+}\)]\(_i\), to be activated in order to allow the cilia to beat faster for prolonged periods\(^7,8\). Thus, the regulation of [Ca\(^{2+}\)]\(_i\), is a cornerstone for many cellular signal cascades and cell functions. In epithelial cells in the airway, different canonical Ca\(^{2+}\) stores or Ca\(^{2+}\) influx pathways are involved in the regulation of [Ca\(^{2+}\)]\(_i\). The known intracellular stores are the

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\(^1\)Department of Anesthesiology and Intensive Care Medicine, University Hospital of Giessen and Marburg, Giessen, Germany. \(^2\)Department of Anesthesiology and Intensive Care Medicine, Heidelberg University Hospital, Heidelberg, Germany. \(^3\)Department of Anesthesiology and Intensive Care Medicine,VIDIA Clinic Karlsruhe St. Vincentius gAG, Karlsruhe, Germany. \(^4\)These authors contributed equally: Sabrina Müller and Christian Koch. \(^*\)email: michael.henrich@vincentius-ka.de
endoplasmic reticulum (ER) and the mitochondria. The ER can reduce \([\text{Ca}^{2+}]\), via activation of the sarcoplasmic ATPase (SERCA)-carrying \([\text{Ca}^{2+}]\) ions into the ER. The release of \([\text{Ca}^{2+}]\) ions from the ER is mainly orchestrated by activating the ryanodine receptors (RyR) or IP3 receptors to enhance \([\text{Ca}^{2+}]\). There may also be alternative pathways that lead to \([\text{Ca}^{2+}]\) leakage from the ER; however, these are still unknown. Mitochondria can buffer \([\text{Ca}^{2+}]\) ions when \([\text{Ca}^{2+}]\) exceeds a threshold of 500 nM and they slowly release \([\text{Ca}^{2+}]\) into the cytosol when \([\text{Ca}^{2+}]\) falls below the aforementioned threshold. It is still not known whether further intracellular \([\text{Ca}^{2+}]\) stores such as lysosomes contribute to the regulation of \([\text{Ca}^{2+}]\).

Different plasma membrane-bound \([\text{Ca}^{2+}]\) channels are known as \([\text{Ca}^{2+}]\) influx pathways, which include transient receptor potential (TRP) channels, store-operated calcium (SOC) channels or voltage-dependent calcium channels that can all enhance \([\text{Ca}^{2+}]\). Consequently, any disturbance or rise in \([\text{Ca}^{2+}]\), in epithelial airway cells can be achieved by the activation of different \([\text{Ca}^{2+}]\) pathways or by liberating them from internal stores. Elevated \([\text{Ca}^{2+}]\), directly contribute to altered cell function, e.g., mucociliary clearance or mucus secretion, which can either lead to colonization of the lower airways by pathogens or can lead to improved clearance rates. When treating mycopic infections in critically ill patients, echinocandines represent the established first-line therapy of antymycotic substances including caspofungin. Caspofungin is used under clinical conditions, with a high capability to treat systemic or local \(Candida\) spp. infections. In healthy men, a mean maximum serum concentration Cmax = 9.1–11 µM was achieved after a loading dose of 70 mg caspofungin.

In order to successfully treat mycotic infections in different organs, caspofungin has to reach therapeutic concentrations in many tissues or regions including the liver and the lower airways of the lungs that exceed plasma concentrations. This distribution pattern depends on the physiology of specific organs. In critically ill patients and in perfused isolated rat hearts, we have previously reported that caspofungin has severe effects on cardio-circulatory function. Caused by impaired contractility of cardiomyocytes as a result of changes in \([\text{Ca}^{2+}]\) levels. It is therefore of interest to determine whether caspofungin also changes the \([\text{Ca}^{2+}]\), in isolated human tracheal epithelial cells (HTEpC) and tried to elucidate the underlying regulatory mechanism.

We measured \([\text{Ca}^{2+}]\), using the fluoroprobe FURA-2 AM. To determine the amount of \([\text{Ca}^{2+}]\) ions in the ER, we used Mag-Fluo-4 AM. Pharmacological approaches were used to identify signal pathways that are involved in the regulation of \([\text{Ca}^{2+}]\). The data gathered by this study should expand our knowledge of the interactions of this antifungal drug in terms of the regulation of \([\text{Ca}^{2+}]\), in airway epithelial cells, which can be considered to be part of the immune system.

Materials and methods

Calcium imaging in isolated epithelial cells. For calcium imaging, we used the human tracheal epithelial cell line (HTEpC, C12644, PromoCell, Heidelberg, Germany). Cells were cultivated in an Airway Epithelial Cell Growth Medium Kit (C-21160) containing the Airway Epithelial Cell Growth Medium Supplement Pack (C-39160, PromoCell, Heidelberg, Germany). The airway epithelial cells were kept in a humidified chamber at 37 °C with air containing 5% CO₂. For the \([\text{Ca}^{2+}]\) imaging, cells were seeded onto laminin-coated coverslips. Dye loading with 2.5 µM FURA-2 AM (Biotium, Fremont, CA, USA) in darkness was performed in a 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer for 30 min at 37 °C. As single wavelength indicators are difficult to calibrate, we used normalized Mag-Fluo-4 signals (F/F₀) to express changes in the regulation of \([\text{Ca}^{2+}]\). The excitation light was provided by a 50 W xenon lamp. The microscope was equipped with a dichromatic excitation longpass mirror (400 nm).

The ratiometric dye, FURA-2 AM, was excited at 340 nm and 380 nm (±8 nm) when equipped with bandpass excitation filters. The emitted fluorescence was directed through a dichromatic shortpass filter of 560 nm to a bandpass filter of 510 nm.

Imaging of luminal \([\text{Ca}^{2+}]\) ions in the ER of tracheal epithelial cells. For calcium imaging in the ER, HTE cells were seeded on laminin-coated coverslips. To assess the intraluminal \([\text{Ca}^{2+}]\) concentration ([Ca⁡²⁺]ER) within the ER, the cells were loaded with 5 µM of the low-affinity calcium indicator Mag-Fluo-4 AM (Kd = 22 µM, Invitrogen, Paisley, UK) in darkness at 37 °C for 60 min. Afterward, the cells were rinsed in fresh HEPES buffer and were transferred to the recording chamber of an upright fluorescence microscope equipped with 20 x immersion lenses (BX50 WI, Olympus, Hamburg, Germany), which contains 2 ml HEPES. The excitation light was provided by a 50 W xenon lamp. The microscope was equipped with a dichromatic excitation longpass mirror (400 nm).

Fluorometric ROS measurements in tracheal epithelial cells under exposure to caspofungin. As an indicator of reactive oxygen species (ROS), we measured hydrogen peroxide (H₂O₂) in collected cell culture supernatants by using the fluorometric Hydrogen Peroxide Assay Kit (PromoCell, Heidelberg, DE). HTE cells were seeded on laminin-coated coverslips and were incubated with 60, 90 or 120 µM caspofungin (CAS) in 1 ml HEPES solution for 30, 60 and 90 min. Additionally, we incubated cells without adding CAS as a control. The cell supernatant (250 µl) was collected and centrifuged at 1,200 G for 15 min to remove all cell parti-
lar Ca²⁺ stores, e.g., DNP (25 µM) was used in Ca²⁺-free buffer solutions to deplete the mitochondrial Ca²⁺ stores. n = 30) than the peak transients observed in the Ca²⁺-containing buffer solutions (2.27 ± 0.17, n = 30, p < 0.01; potential eliciting an efflux of Ca²⁺ ions from these organelles. This uncoupling of the respiratory chain induces a complete depolarization of the mitochondrial membrane in [Ca²⁺]i which did not return to the baseline during the remaining exposure period (Fig. 2A). Increasing the caspofungin concentration to 120 µM resulted in prolonged elevation of [Ca²⁺]i that remained above the baseline (2.47 ± 0.18, n = 30, p = 0.093, n = 30, Fig. 2B, E).

**Statistical analysis.** A Mann–Whitney U test was used to compare equivalent measuring points from different experiments, and a Wilcoxon rank-sum test was used to compare dependent variables. Statistical data evaluation and testing were performed using the GraphPad PRISM (Version 5.04) software (GraphPad Software, La Jolla, CA, USA).

**Results**

**Changes in [Ca²⁺] in tracheal epithelial cells.** The [Ca²⁺]i was expressed as changes in the FURA-2-fluorescence ratio. For the controls, isolated tracheal epithelial cells were rested in Ca²⁺-containing or in Ca²⁺-free buffer solutions for more than 800 s. In the cells resting in Ca²⁺-containing buffer, the FURA-2-fluorescence ratio did not change significantly during this period (1.09 ± 0.08, p = 0.12, compared to initial FURA-2-fluorescence ratio). At the end of all the experiments, we applied a brief pulse of KCl (200 mM), and only the cells that responded to this vitality test by showing a significant increase in the FURA-2-fluorescence ratio were included for further statistical evaluation (Fig. 1). Cells resting in the Ca²⁺-free buffer medium also showed no changes in the FURA-2-fluorescence ratio throughout the entire exposure period (0.89 ± 0.03, p = 0.09, Wilcoxon rank-sum test, Fig. 1C, D). However, these cells also responded to a brief KCl pulse with an increase in the FURA-2-fluorescence ratio.

When the tracheal epithelial cells were exposed to caspofungin (60 µM) in Ca²⁺-containing buffer, almost all of the investigated cells immediately showed an elevation in [Ca²⁺]i. We observed a majority of cells responding with two subsequent Ca²⁺-transients with a maximum of 2.46 ± 0.29 (FURA-2-fluorescence ratio, n = 30). The first rapid increasing Ca²⁺ transient returned to the baseline, while the second one showed a rapid increase in [Ca²⁺], which did not return to the baseline during the remaining exposure period (Fig. 2A). Increasing the caspofungin concentration to 120 µM resulted in prolonged elevation of [Ca²⁺], that remained above the baseline at the end of the exposure period. There was a caspofungin dose-dependency of maximum amplitudes of initial Ca²⁺ transients (Fig. 2B). The application of NiCl₂ was used to inhibit Ca²⁺ entry via the plasma-membrane-bound Ca²⁺ channels. However, it did not prevent the peak rise in [Ca²⁺], induced by caspofungin (60 µM, 2.47 ± 0.18, p = 0.093, n = 30, Fig. 2B, E).

**Caspofungin induces a rise in [Ca²⁺], independent of external Ca²⁺ ions.** To further elucidate the caspofungin-induced rise in [Ca²⁺], we applied caspofungin to tracheal epithelial cells in Ca²⁺-free solutions. Exposure to caspofungin in the Ca²⁺-free solution evoked a rise in [Ca²⁺], in all of the investigated cells (n = 30) which was biphasic, and which remained above the baseline level until the end of the experimental observation period (Fig. 3A). The peak rise in [Ca²⁺]i, was significantly higher in the Ca²⁺-free buffer solutions (2.95 ± 0.72, n = 30) than the peak transients observed in the Ca²⁺-containing buffer solutions (2.27 ± 0.17, n = 30, p < 0.01; Fig. 3B).

**Caspofungin liberates Ca²⁺ from internal stores.** In order to determine the source of the Ca²⁺ ions that increase the levels of [Ca²⁺], during caspofungin exposure, we pharmacologically depleted the known intracellular Ca²⁺ stores, e.g., DNP (25 µM) was used in Ca²⁺-free buffer solutions to deplete the mitochondrial Ca²⁺ stores. This uncoupling of the respiratory chain induces a complete depolarization of the mitochondrial membrane potential eliciting an efflux of Ca²⁺ ions from these organelles.
The Ca²⁺ efflux from the mitochondria was visible by brief Ca²⁺ transients (increase in FURA-2 ratio) that almost immediately returned to baseline levels (Fig. 4A). The following exposure to caspofungin (60 µM), still in the presence of DNP, evoked transient elevation in [Ca²⁺]ᵢ that always returned to the baseline. The transient increases in Ca²⁺ ions, when exposed to caspofungin in the presence of DNP, were significantly less than the transient increases in Ca²⁺ ions when the mitochondrial stores were not depleted (2.41 ± 0.07, n = 11, p < 0.01, Fig. 4B).

Ca²⁺ stores in the ER were identified and depleted by cyclopiazonic acid (CPA, 10 µM), a reversible inhibitor of SERCA. In a Ca²⁺-free buffer solution, CPA induced a prolonged increase in [Ca²⁺]ᵢ that slowly returned to baseline levels (Fig. 5A). Cell vitality was verified by exposure to a depolarizing concentration of KCl (200 mM, Fig. 5A). The depletion of ER Ca²⁺-stores by caffeine (30 mM) led to a rapid but transient increase in [Ca²⁺]ᵢ. The subsequent application of CPA (10 µM) had no further effect, demonstrating that caffeine had already depleted the ER Ca²⁺ stores, which are identical to CPA-sensitive stores (Fig. 5B).

In further experiments, exposing the cells to caffeine led to a brief Ca²⁺ transients of the FURA-2 fluorescence ratio that almost immediately returned to the baseline. The subsequent application of caspofungin (60 µM or 120 µM) had no effect on any tracheal epithelial cells investigated.

Figure 1. Prolonged incubation of tracheal epithelial cells in buffer solutions did not change [Ca²⁺]ᵢ. (A) The [Ca²⁺]ᵢ was indicated by a FURA-2 ratio of 340 nm/380 nm. As a control, isolated tracheal epithelial cells were kept under long-term incubation in Ca²⁺-containing 2.5 mM buffer. During this prolonged incubation period, the basal [Ca²⁺]ᵢ did not change. The vitality test at the end of each experiment was carried out by applying KCl (200 mM), inducing a rapid Ca²⁺-influx and an increase in [Ca²⁺]ᵢ. (B) The initial levels of [Ca²⁺]ᵢ had not changed when they were briefly measured at the end of the exposure period before the application of KCl. (C) The prolonged incubation of tracheal epithelial cells in Ca²⁺-free buffer solution did not change the [Ca²⁺]ᵢ. The application of KCl (200 mM, containing 2.5 mM CaCl₂) precipitated an increase in [Ca²⁺]ᵢ. (D) In the Ca²⁺-free buffer solution, the initial [Ca²⁺]ᵢ also did not change when it was briefly measured at the end of the exposure period before the KCl application (n = number of individual experiments, ns = not significant, Wilcoxon rank-sum test).
Figure 2. In tracheal epithelial cells, caspofungin triggers an increase in $[\text{Ca}^{2+}]_{i}$. (A) In the $\text{Ca}^{2+}$-containing buffer solution (2.5 mM), the application of caspofungin (60 µM) induced a sudden rise in the FURA-2 ratio displaying $[\text{Ca}^{2+}]_{i}$. In the majority of the investigated cells ($n = 21$) we observed two transient prolonged peaks in $[\text{Ca}^{2+}]_{i}$, whereas the remaining cells ($n = 4$) showed a single transient rise of $[\text{Ca}^{2+}]_{i}$. (B) The amplitude of the initial $\text{Ca}^{2+}$ increase induced by the caspofungin in the tracheal epithelial cells is concentration-dependent, with an $EC_{50}$ of 55 µM. The dose–response curve fits a Hill equation. (C) Fluorescence images of the FURA-2 ratio converted into false colors that were taken at different time points from a prolonged recording of caspofungin application. I: Early response of cells to caspofungin application (50 s after caspofungin application); II: Further response of cells to caspofungin application with an increase in FURA-2 ratio (100 s after caspofungin application); III: Prolonged elevation of FURA-2 ratio (200 s after caspofungin application); IV: Prolonged elevated FURA-2 ratio (approx. 600 s after caspofungin application and just before KCl administration). (D) $\text{Ni}^{2+}$ was applied in order to inhibit the influx of $\text{Ca}^{2+}$ ions into the tracheal epithelial cells. The subsequent application of caspofungin still evoked a transient rise in $[\text{Ca}^{2+}]_{i}$, that was independent of the $\text{Ca}^{2+}$ influx. (E) A significant increase in $[\text{Ca}^{2+}]_{i}$, was triggered when exposed to caspofungin (60 µM). Inhibition of membrane-bound $\text{Ca}^{2+}$ channels by application of $\text{Ni}^{2+}$ ions did not reduce the amplitudes of a transient rise in $[\text{Ca}^{2+}]_{i}$. (scale bar in C: 20 µm. n = number of individual cells, ns = not significant, *** $p < 0.001$, Mann–Whitney U test).
We observed no increase in \([\text{Ca}^{2+}]_i\) in the presence of caspofungin (1.32 ± 0.02, n = 30, p = 0.1 when compared to the FURA-2 fluorescence ratio after the addition of caffeine but before caspofungin application). However, \(\text{Ca}^{2+}\) transients were significantly reduced vs. \(\text{Ca}^{2+}\) transients induced by caspofungin in \(\text{Ca}^{2+}\)-free buffer solutions (p < 0.001, Fig. 5C, D).

caspofungin enhances \([\text{Ca}^{2+}]_i\), via the activation of IP3 and ryanodine receptors. To evaluate the route by which \(\text{Ca}^{2+}\) ions are liberated by caspofungin from the ER's \(\text{Ca}^{2+}\) stores, we pharmacologically inhibited the known \(\text{Ca}^{2+}\) pathways. 2-APB (40 µM) was applied to \(\text{Ca}^{2+}\)-free extracellular solutions in order to inhibit inositol-1,4,5-triphosphate (IP3) receptors prior to caspofungin application. 2-APB had no effect on the FURA-2 fluorescence ratio indicating transients of \([\text{Ca}^{2+}]_i\) (1.04 ± 0.003, n = 30, p = 0.21, Fig. 6A, B).
subsequent application of caspofungin still induced single $\text{Ca}^{2+}$ transients with a significantly reduced peak to $2.04 \pm 0.08$ ($n = 30$, $p < 0.001$) compared to $\text{Ca}^{2+}$ transients induced by caspofungin alone ($2.95 \pm 0.72$, Fig. 6B). However, we observed no prolonged elevation in the FURA-2 fluorescence ratio, which we observed when the $\text{Ca}^{2+}$-free buffer solution was exposed to caspofungin (Fig. 3A). In a further series of experiments, we inhibited the ryanodine receptors using 40 $\mu$M ryanodine. This concentration of ryanodine did not induce an efflux of $\text{Ca}^{2+}$ from ER stores, as demonstrated by the unaltered FURA-2 fluorescence ratio (Fig. 6C, D).

The application of caspofungin in the presence of ryanodine did not evoke any increase in the FURA-2 fluorescence ratio, and no $\text{Ca}^{2+}$ transients in any of the investigated cells were observed (Fig. 6C, D). The $[\text{Ca}^{2+}]_i$ was no different to the values obtained prior to caspofungin application ($1.1 \pm 0.01$, $n = 30$, $p = 0.52$). At the end of each experiment, $\text{Ca}^{2+}$ transients were still elicited by applying KCl, demonstrating the vitality of the cells (Fig. 6C).

Figure 5. ER $\text{Ca}^{2+}$ stores contribute to caspofungin-induced increases in $[\text{Ca}^{2+}]_i$. (A) The ER $\text{Ca}^{2+}$ stores are identified by their sensitivity to the SERCA inhibitor CPA (10 $\mu$M). Exposure to CPA led to a slow increase in $[\text{Ca}^{2+}]_i$, that returned to baseline levels while still in the presence of CPA. (B) Caffeine (30 mM) also depleted the ER $\text{Ca}^{2+}$ stores leading to a rapid increase in $[\text{Ca}^{2+}]_i$, that soon returned to baseline levels. The subsequent application of CPA did not lead to a further rise in $[\text{Ca}^{2+}]_i$, demonstrating that the caffeine had already depleted ER $\text{Ca}^{2+}$ stores that are identical to CPA-sensitive $\text{Ca}^{2+}$ stores. (C) Applying 30 mM of caffeine was enough to deplete the ER $\text{Ca}^{2+}$ stores. The consecutive application of caspofungin while in the presence of caffeine did not change the $[\text{Ca}^{2+}]_i$. Under these conditions, no $\text{Ca}^{2+}$ transients were observed. (D) The $[\text{Ca}^{2+}]_i$, measured under caffeine application and subsequent caspofungin application was significantly lower than the rise in $[\text{Ca}^{2+}]_i$, measured when the ER's $\text{Ca}^{2+}$ stores were not depleted (the horizontal bars in the experimental recordings depict the exposure periods of defined pharmacological agents. $n =$ number of individual investigated cells, $^{**}p < 0.01$, $^{***}p < 0.001$, Mann–Whitney U test).
Caspofungin depletes ER Ca\(^{2+}\) stores in tracheal epithelial cells. For the controls, HTEpC-c cells loaded with Mag-Fluo-4 AM were rested in Ca\(^{2+}\)-containing buffer (Fig. 7A), Ca\(^{2+}\)-free HEPES buffer (Fig. 7B), and Ca\(^{2+}\)-free HEPES buffer after prior loading with BAPTA-AM (Fig. 7C). None of these conditions reduced the Mag-Fluo-4 fluorescence signal displaying [Ca\(^{2+}\)]\(_{\text{ER}}\), demonstrating that the ER Ca\(^{2+}\) stores were stable under resting conditions without significant leakage of Ca\(^{2+}\) ions into the cytosol (Fig. 7A). However, exposure to caspofungin rapidly altered the Mag-Fluo-4 fluorescence. In all the cells that were investigated, we observed a rapid decrease in fluorescence within a few seconds of exposure (Fig. 7D, E) that immediately recovered, before a slower and more prolonged decline in the fluorescence showed the [Ca\(^{2+}\)]\(_{\text{ER}}\). The amplitude of the initial downward spike of Mag-Fluo-4 fluorescence and the degree of the prolonged reduction was concentration-dependent (Fig. 8A, B). A final exposure to the Ca\(^{2+}\)-containing buffer solution with a depolarizing KCl concentration
demonstrated a recovery of the Mag-Fluo-4 signal equivalent to the replenishment of Ca\(^{2+}\) stores within the ER (Fig. 7D).

The initial sharp decrease in Mag-Fluo-4 fluorescence completely disappeared when the cells were loaded with BAPTA prior to caspofungin exposure; only the slow, prolonged phase of the declining Mag-Fluo-4 was still visible. In addition, replenishing the ER Ca\(^{2+}\) stores by Ca\(^{2+}\)-containing buffer and depolarizing the cells with KCl only had a limited effect, assuming that the cytosolic Ca\(^{2+}\) ions were instantly buffered by BAPTA (Fig. 7F).

The total depletion of ER Ca\(^{2+}\) stores was achieved by the application of caffeine (30 mM). The stores were replenished after subsequent exposure to a buffer solution containing depolarizing KCl (200 mM) and Ca\(^{2+}\) (2.5 mM), inducing a maximum rise in Mag-Fluo-4 fluorescence (Fig. 9A). In the presence of caffeine, exposure to caspofungin had no further effect. Neither was the initial downward Ca\(^{2+}\) spike visible nor was the prolonged reduction of Mag-Fluo-4 fluorescence further diminished (Fig. 9B, C).

**ROS generation under caspofungin exposure in the tracheal epithelium.** Here, we were able to determine whether the observed effects of caspofungin on intracellular Ca\(^{2+}\) stores were caused by cellular ROS generation. In freshly isolated mice tracheae, we measured ROS generation under exposure to caspofungin (120 μM) using a fluorescence dye. The results showed no significant ROS generation in the control experiment or when the tracheae were exposed to caspofungin for 30 min (Fig. 9D).
Discussion

In this study, which looked at isolated HTEpCs, we found evidence that caspofungin triggers a rise in $[\text{Ca}^{2+}]_i$ by releasing $\text{Ca}^{2+}$ ions from caffeine-sensitive ER stores, primarily via a RyR pathway and via an IP3 regulated cascade to a lesser extent. This increase in $[\text{Ca}^{2+}]_i$ and the releasing of $\text{Ca}^{2+}$ ions from the ER was slightly dependent on extracellular $\text{Ca}^{2+}$ concentrations or transmembraneous $\text{Ca}^{2+}$ influxes, even though we could not prove it by blocking transmembraneous $\text{Ca}^{2+}$ channels by using NiCl2. It is possible that NiCl2 is not able to block all the transmembraneous $\text{Ca}^{2+}$ channels of tracheal epithelial cells or that we used an insufficient concentration. However, the cells showed a reaction directly after the application of 500 µM NiCl2, implying that the dosage was sufficient. In most of the cells investigated, the rise in $[\text{Ca}^{2+}]_i$ was biphasic, whereby an initial transient rise in $[\text{Ca}^{2+}]_i$ was followed by a second prolonged rise in $[\text{Ca}^{2+}]_i$. This data is supported by our experiments using Mag-Fluo-4-fluorescence, which showed a rapid biphasic depletion of ER $\text{Ca}^{2+}$ stores after exposure to caspofungin. These present findings are similar to the effect of acetylcholine on the tracheal ciliary beating rate, inducing a transient rise in $[\text{Ca}^{2+}]_i$ that is followed by a prolonged increase in the beating rate. We found no evidence that caspofungin induces ROS generation since ROS are known to interfere with calcium-signaling pathways.

The stimulation of cilia-bearing cells in order to enhance their beating rate is the main task to rapidly convey heavy loads of particles and pathogens. This mechanism depends on the liberation of $\text{Ca}^{2+}$ ions from internal stores. It is also in line with reports that evidenced the activation of the ciliary beat frequency following the kinetics of $[\text{Ca}^{2+}]_i$. We were also able to demonstrate that caspofungin activates intracellular signal transduction cascades that lead to the release of $\text{Ca}^{2+}$ ions from ER stores via RyR. We also demonstrated that caspofungin depleted the $\text{Ca}^{2+}$ stores in the ER.

However, although we still cannot conclude that caspofungin directly affects RyR, we have excluded any interaction with membrane-bound receptors or with their downstream reaction cascades. Therefore, with the present data, we can assume that caspofungin can penetrate tracheal epithelial cells and to activate specifically RyR. This is similar to cardiomyocytes, in which caspofungin also triggers the liberation of $\text{Ca}^{2+}$ ions from the ER via RyR.

The permeability of caspofungin and its diffusion into mammalian tissues and cells. Caspofungin inhibits the synthesis of 1,3-β-D-glucan, which is an essential component of the fungal cell wall. While caspofungin underlies very low excretion kinetics that result in high sustained plasma levels, it attains therapeutic concentrations in various tissues. It is also characterized by its ability to diffuse into many organs and tissues in order to reach the concentrations necessary to treat invasive candidiasis; thus, its penetration into the central nervous system (CNS) is poor. Even when the meninges are inflamed, the concentrations of caspofungin in the CNS are far below serum concentrations. In contrast, caspofungin concentrations are highest in the liver, lungs, kidney, and spleen, where caspofungin can deploy its antifungal properties. In the lungs, the concentration of caspofungin leads to favorable response rates of up to 62% in neutropenic patients suffering from pulmonary invasive fungal disease.
In mammals, caspofungin mainly acts extracellularly against most *Candida* species. However, caspofungin, a membrane-anchored cyclic lipopeptide, is also able to penetrate mammalian cells and can reach high concentrations within the tracheal epithelium allowing it to act upon cilia-bearing cells. We can also conclude that caspofungin concentrations are also high in the epithelial lining fluid, and it may act from the luminal side onto epithelial cells.

Since caspofungin is used to treat pulmonary infections caused by *Candida spp.*, it is able to diffuse via the epithelium into the airway lumen so that it can reach these therapeutic concentrations. We found evidence that caspofungin can reach intracellular concentrations in isolated lower airway cells that are high enough to liberate Ca²⁺ ions from cell organelles. So far, the effects of caspofungin on intracellular organelles have only been described for a few cell types. In stimulated mammalian macrophages, caspofungin has been shown to reach concentrations that will have an effect on trapped *Candida glabrata*²⁶. Recently, we described a change in the contractility of isolated cardiomyocytes via the liberation of Ca²⁺ ions from internal Ca²⁺ stores¹⁵,¹⁷. These data

![Figure 9](https://www.nature.com/scientificreports/)
support our present findings and lead us to postulate that the diffusion of caspofungin into mammalian cells is not restricted to particular cell types; it seems rather a general characteristic of this antymycotic substance.

**The effects of caspofungin on intracellular Ca²⁺ stores.** In our present study, we identified that caspofungin could trigger the release of Ca²⁺ ions from Ca²⁺ stores in the ER, mainly via a caffeine/ryanodine-sensitive pathway. We found no evidence for the liberation of Ca²⁺ ions stored in mitochondria, although these organelles do store Ca²⁺ ions under resting conditions (see Fig. 4). However, they primarily buffer Ca²⁺ ions when [Ca²⁺]r exceeds a threshold of approximately 500 nM. In liver cells and cardiomyocytes, caspofungin is known to disturb the electron transport within the mitochondrial respiratory chain by inhibiting complex I and III. The effect on complex III is probably caused by its interference with cytochrome C²⁸. However, it is not known whether this mechanism also depolarizes mitochondrial membrane potential, which is essential for Ca²⁺ buffering in these organelles⁹. Here we measured no effect of caspofungin onto mitochondrial Ca²⁺ stores.

When the mitochondrial Ca²⁺ stores were depleted by DNP, exposure to caspofungin had little effect (see Fig. 4). This small decrease in caspofungin response can be explained by the inhibition of ATP synthesis due to the application of DNP application²⁹. ATP usually powers the sarco-endoplasmic calcium ATPase (SERCA) that refills the ER’s Ca²⁺ stores. Therefore, the interrupted mitochondrial ATP synthesis after exposure to DNP reduces SERCA activity, which then leads to a slow Ca²⁺ leakage from the ER stores via different pathways⁹,³⁰. Eventually, this results in a slightly reduced Ca²⁺ response during subsequent caspofungin application, which was what was observed here. Our observations provide evidence that the mitochondria in tracheal epithelial cells store Ca²⁺ ions under resting conditions but are not affected by caspofungin.

Under steady-state conditions, the influx of Ca²⁺ ions into the cytosol from the ER stores is balanced on the one hand by RyR, IP₃, or alternative leaks that drive the efflux from the ER, and on the other hand by SERCA, which regulates Ca²⁺ influx into the ER resulting in a zero net flux of Ca²⁺ ions into the cytosol³²,³³. Ca²⁺ sparks are transient elevations of the [Ca²⁺], that are caused by a net efflux from the ER. This efflux is caused by an increased open probability of the RyR, which cannot immediately be offset by the SERCA-driven influx. Using caffeine to open the RyR pathway leads to a rapid and transient Ca²⁺ efflux that depletes the ER’s stores. The following balance of efflux and influx of Ca²⁺ ions to pre-caffeine levels while still in the presence of caffeine is caused by clearing the elevated [Ca²⁺], by pumping Ca²⁺ ions out of the cells or into alternative stores while the RyR is still activated and open³³. The refilling of the ER stores cannot be achieved under these conditions, which is the reason why we observed no further Ca²⁺ transients when caspofungin was applied after the ER stores were depleted by the addition of caffeine. When caspofungin is applied to cells that have not had their ER stores depleted by caffeine, we observed Ca²⁺ transients in most of the cells.

In our experiments using Mag-Fluo-4 as an indicator for ER luminal Ca²⁺ contents, we observed a rapid decline in Mag-Fluo-4 fluorescence followed by a partial recovery, which eventually led to a prolonged decline in Mag-Fluo-4 fluorescence. The partial recovery is most probably a response to the SERCA balancing the influx and efflux of Ca²⁺ ions from the ER, as is generally assumed for many cell types³⁴. A similar mechanism of Ca²⁺ depletion from the ER has been described previously for smooth muscle cells derived from different organs in rats³⁵,³⁶. Here, the prolonged decline of Mag-Fluo-4 fluorescence was caused by the continuous presence of caspofungin activating RyR.

Under these conditions, SERCA was unable to balance the influx and efflux of Ca²⁺ ions from the ER stores. Most probably, this net efflux is provoked by caspofungin triggering the increase in open state probability of RyR, since the kinetics of Ca²⁺ transients are similar. The clearance of cytosolic Ca²⁺ transients is caused by alternative clearance pathways, e.g., the transmembrane efflux, buffering, or by an uptake in alternative Ca²⁺ stores like mitochondria. We interpret these findings such that caspofungin specifically activates ER-bound RyR and depletes these Ca²⁺ stores. Caspofungin now provokes an imbalance in favor of a net Ca²⁺ efflux that results in an immediate increase in [Ca²⁺], which is either transient or sustained. The rapid amplitude in [Ca²⁺]r is caused by the steep function of Ca²⁺ content in the ER as has previously been described in cardiomyocytes³⁷,³⁸. This Ca²⁺ content contributes to the regulation of ciliary beat activity by the activation of different signal cascades⁸.

Alternatively, caspofungin may promote the dissociation of Ca²⁺ ions from cytosolic buffers as an alternative source for increasing [Ca²⁺]. In many cell types, cytosolic Ca²⁺ is strongly buffered, and for every free cytosolic Ca²⁺ ion approximately 100 to 200 Ca²⁺ ions are bound to buffers³⁹,⁴⁰. In cardiomyocytes, the major buffers are troponin and SERCA, whereas in airway epithelial cells the Ca²⁺ is supposed to be buffered by calmodulin³⁴. However, in this case, the kinetics of increased [Ca²⁺]r would be different.

We have not observed biphasic Ca²⁺ transients or sustained elevations of [Ca²⁺]. This is because Ca²⁺ pumps on the plasma membrane, the ER and mitochondrial Ca²⁺ uptake would still be fully active and would be sufficient to immediately reduce elevated [Ca²⁺]r. Since the initial binding of Ca²⁺ ions to SERCA contributes significantly to Ca²⁺ buffering, it may be that caspofungin contributes to the dissociation of Ca²⁺ ions from these binding sites⁴¹. Nevertheless, in this case, we would observe Ca²⁺ transients while RyR was inhibited by ryanodine, or when the ER stores were depleted by caffeine. However, we did not observe these Ca²⁺ kinetics. These data argue against the assumption that caspofungin drives Ca²⁺ ions out of the buffering sites of the SERCA. Furthermore, the inhibition of SERCA by caspofungin would lead to a slow, sustained increase in [Ca²⁺], which then returns to the baseline, as we observed for CPA (Fig. 5)³⁹,⁴⁰. In contrast, we did not observe a slow increase in [Ca²⁺], under caspofungin exposure; the kinetics was always steep and rapid. In contrast, in many of the airway cells observed, the decay in the elevated [Ca²⁺]r levels was slow. This prolonged kinetic may be caused either by reduced ATP content and subsequently reduced activity of ATP-driven ion pumps or by the inhibition of ion transporters. However, it is questionable whether caspofungin reduces ATP generation or inhibits ion transport and this needs to be investigated further.
In our experiments, we noted a reduced response to caspofungin while using 2-APB, which binds to the IP₃ receptor. Since phospholipase-C activation and liberation of Ca²⁺ ions from Ca²⁺ stores via the IP₃ receptor contribute to Ca²⁺ wave propagation in airway epithelia, we investigated whether caspofungin interferes with this signal cascade. The reduced response to caspofungin exposure when 2-APB was applied may be caused by the activation of these receptors by caspofungin in competition with 2-APB. This assumption is supported by the fact that the response to caspofungin was delayed in the presence of 2-APB. Therefore, 2-APB might only be effective when caspofungin concentration is high enough to prevent 2-APB from binding. Further findings revealed that ryanodine abolished the calcium signals elicited by Caspofungin. Controversially, IP₃-mediated calcium release seems not to occur in parallel to RyR-mediated calcium release. Nevertheless, we observed a reduced response to caspofungin in the presence of 2-APB. 2-APB is also known to modulate TRP channel, gap junctions, STIM ORAI channel conductance and Iᵣₑₐ in higher concentrations. Owing to this lack of specificity for the IP₃ receptor, 2-APB could also impair other calcium pathways and not particularly the activity of the IP₃ receptor. On the other hand, our experiments were performed in calcium free buffer solution, so store-operated calcium entry (SOCE) could not occur. Further experiments are necessary to explore whether the IP3 receptor pathway is affected by using a more specific IP₃ receptor blocker such as Xestospongin C or to examine SOCE pathways, which is more complicated due to diverse interactions and a fine tuned orchestration of multiple targets. Regardless, we conclude that RyR is the principal target of caspofungin since we measured the main effects of caspofungin via this Ca²⁺ efflux pathway.

The observed sustained elevation of Ca²⁺ ions under caspofungin exposure may be due to several reasons, as a similar effect has been described when these cells are exposed to histamine or acetylcholine. Most probably, this prolonged kinetic is caused by an imbalance between the influx and efflux of Ca²⁺ ions from the cytosol due to a slow clearance rate when reaching baseline levels of [Ca²⁺], during the observation period. These findings imply that caspofungin has effects on other cell organelles. It may also interfere with mitochondrial ATP synthesis, as has been described for mammalian mitochondria. Under these circumstances, the remaining ATP supply from mitochondria, glycolysis or phosphocreatine stores is too low to fully activate Ca²⁺ ATPase in the plasma membrane. This mechanism prevents a rapid return of [Ca²⁺], to baseline levels. Beyond plasma membrane-bound, sodium-calcium exchangers are also activated when [Ca²⁺] is high and may blunt the peak of observed the Ca²⁺ transients.

We also found no evidence that caspofungin depolarizes mitochondrial membrane potential, which is the driving force behind Ca²⁺ storage in these organelles. This is supported by our findings that the number of Ca²⁺ transients was only slightly altered when mitochondria were depolarized using DNP. Therefore, in many tracheal epithelial cells, caspofungin may have additional effects on other cellular structures that cause a prolonged elevation of [Ca²⁺], with the slow return to the baseline level due to an energetic restriction.

HTE cells, which are isolated from the human tracheal epithelium, are suitable for investigating the different effects and signal pathways under stable conditions, whereas freshly isolated human tracheal cells should only be used in further studies to underline these observations. Additionally, a contribution of alternative Ca²⁺ influx pathways, e.g. store operated calcium entry (SOCE) or IP₃ receptors in the intact tracheal epithelium, should also be considered.

**Conclusion**

Caspofungin liberates Ca²⁺ ions from internal ER stores and enhances [Ca²⁺] either transiently or in a sustained manner. This mechanism likely occurs due to the activation of a RyR pathway. A sustained elevation in [Ca²⁺] levels may be supported via energetic imbalances during caspofungin application. Further studies should focus on RyR activation by caspofungin, detecting its binding site to these receptors, and determining how cellular ATP synthesis is compromised by caspofungin.

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Competing interests
The authors declare no competing interests.

Additional information
Correspondence and requests for materials should be addressed to M.H.

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