Review Article

Pulmonary Circulation

Permeability and calcium signaling in lung endothelium: unpack the box. . .

Mary I. Townsley
Department of Physiology & Cell Biology, University of South Alabama, Mobile, AL, USA

Abstract
This brief review assesses the role of Ca^{2+} signaling in lung endothelium in regulation of endothelial permeability. The disconnect between experimental and clinical outcomes to date may be due, in part, to the use of tools which yield information about aggregate permeability or Ca^{2+} responses in lung or in endothelial monolayers. The teaching point of this review is to "unpack the box," i.e. consider the many potential issues which could impact interpretation of outcomes. These include phenotypic heterogeneity and resultant segment-specific permeability responses, methodologic issues related to permeability measures, contributions from Ca^{2+} channels in cells other than endothelium—such as alveolar macrophages or blood leukocytes), Ca^{2+} dynamic patterns, rather than averaged Ca^{2+} responses to channel activation, and the background context, such as changes in endothelial bioenergetics with sepsis. Any or all of these issues might color interpretation of permeability and Ca^{2+} signaling in lung.

Keywords
ARDS, acute respiratory distress syndromes and acute lung injury, calcium, endothelium

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Introduction

While acute lung injury has been extensively investigated, clinical outcomes remain poor. Aside from low volume ventilation, strategies directed at single targets or downstream signaling pathways identified in preclinical studies on acute lung injury have been disappointing when translated to the clinical setting. The disconnect between experimental and clinical outcomes may be due, in part, to the persistent notion when interpreting experimental data that signaling molecules evoke consistent outcomes across the lung. Further clouding our lens for interpretation is the fact that we use tools which yield information about aggregate responses in lung or in endothelial monolayers when assessing the role of Ca^{2+} signaling in lung endothelium in regulation of endothelial permeability. These tools include measures of total lung wet–dry weight ratio, lung weight gain or the more specific filtration coefficient K_f to assess endothelial permeability in vivo,[1] endothelial monolayer resistance to assess permeability in vitro,[2–4] or measures of averaged Ca^{2+} transients to assess mechanisms regulating Ca^{2+} entry (Fig. 1).[5,6]

Averaged permeability measures may mask phenotypic heterogeneity in endothelium within specific lung vascular compartments.[7–10] Similarly, averaged measures of Ca^{2+} responses will mask variability due to heterogeneity in Ca^{2+} channel expression or responsiveness even within one vascular compartment or one field of cells in vitro.[5,11] While not explored in any detail here, we further need to be cognizant of our assumption that in vitro measures of Ca^{2+}-dependent permeability responses in endothelial cell monolayers replicate or predict responses in the intact lung. Potential for plasticity in Ca^{2+} channel expression with cell passage,[11] localization of Ca^{2+} channels in endothelium, and related localization of Ca^{2+}-dependent intracellular mechanical forces and substrate stiffness[12,13] could color outcomes in a heterogeneous way. To date, these latter

Corresponding author:
Mary I. Townsley, Department of Physiology & Cell Biology, University of South Alabama, Mobile, AL, USA.
Email: mtownsley@southalabama.edu

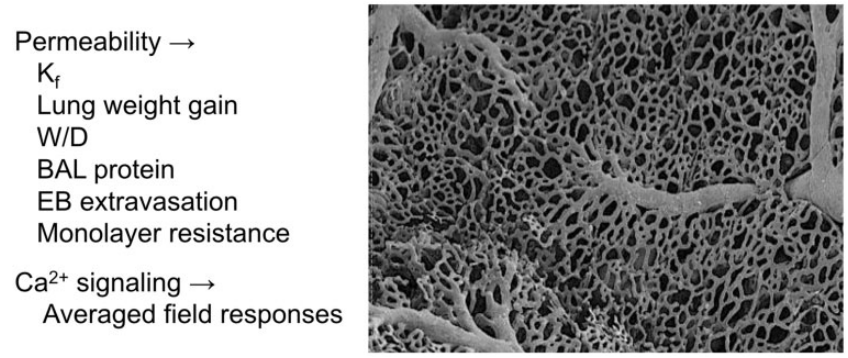

Fig. 1. Measures designed to assess permeability and Ca^{2+} signaling in lung endothelium either in vivo or in vitro typically identify averaged outcomes. Permeability measures include the filtration coefficient (K_f) in isolated lung, lung weight gain at constant perfusion pressure, lung wet–dry weight ratio (W/D), protein content in bronchoalveolar lavage fluid (BAL), extravasation of Evan's blue dye (EB) into lung tissue, and endothelial monolayer resistance.[1] In each case, measures are averaged outcomes in the lung or endothelial monolayer as a whole. Similarly, Ca^{2+} imaging typically only assesses field averaged changes in amplitude over time.[5,62] The scanning electron micrograph of a lung vascular corrosion cast (at the right) elucidates compartments in the lung vascular network, including arteries, capillaries, and veins; the network shown here is that at the pleural surface of the lung. We now know that endothelium in these compartments is phenotypically distinct,[8,10] which may impact both basal permeability responses and those elicited by Ca^{2+} channel activation.

issues have not been explored in any detail in lung endothelium.

This review discusses the potential impact of key issues on interpretation of experimental outcomes related to endothelial permeability and Ca^{2+} signaling: endothelial phenotypic heterogeneity; contributions not related to endothelial cell function; calcium microdomains; and altered context in sepsis. The key teaching point here is to "unpack the box." In other words, do not necessarily interpret outcomes based on the aggregate response. We should question what's inside that aggregate measure—in other words, what's inside the box… and question whether the setting or context may modulate outcomes.

Lung endothelial Ca^{2+} signaling in heart failure

Historical perspective from our own work on lung endothelial barrier function in heart failure highlights the complexities inherent in interpreting permeability measures and Ca^{2+} signaling at the whole organ level. We started this work to try to understand adaptations that might allow individuals with heart failure to compensate for the chronic pulmonary venous hypertension, with increased propensity for pulmonary edema. As research will often do, the trail led us to adaptations in endothelial permeability related to Ca^{2+} signaling, with somewhat surprising outcomes.

In lung endothelium, store-operated channels participate in regulation of endothelial permeability. In cultured rat lung endothelial cells, thapsigargin (TG) or thrombin evoke store depletion, resulting in activation of store-operated channels, endothelial Ca^{2+} influx, loss of adherence junction integrity, and formation of inter-endothelial cell gaps.[14–17] In the intact lung, TG or thrombin increase the filtration coefficient K_f, a measure of water permeability or hydraulic conductivity for the endothelial barrier.[7,16,18] This process requires Ca^{2+} influx into endothelium through store-operated channels. We initially identified a loss of the angiotensin II-dependent permeability responses in lung after pacing-induced heart failure, a model of chronic pulmonary venous hypertension.[19] As angiotensin II elicits store-dependent responses in normal endothelium, we used TG to bypass angiotensin II receptors and directly deplete stores in subsequent studies. We found that the increase in K_f associated with TG-induced store depletion is lost after development of chronic heart failure in both the pacing and AV fistula models.[20,21] The consensus of more current work suggests that store-operated Ca^{2+} channels comprise TRPC1 and TRPC4 proteins, members of the canonical subfamily of transient receptor potential (TRP) proteins.[22]

In parallel, we were investigating the permeability response to high vascular pressure (HiPv) in lung and identified another TRP channel from the vanilloid family—TRPV4—as the target. TRPV4 is gated by mechanical stress, and in many in vitro models, epoxyeicosatrienoic acids or EETs (P450 epoxygenase-derived arachidonic acid metabolites) provide the link between mechanical stress and TRPV4 activation. We subsequently found that the increase in lung K_f with HiPv can be abrogated by pretreatment with a TRPV4 inhibitor or by inhibition of EET synthesis, and is lost in lungs from animals with genetic deletion of TRPV4.[23,24] However, in contrast to the abrogation of the store-dependent permeability response in chronic heart failure, we found that the increased K_f response to 14,15-EET is retained.[20] Note that Ca^{2+} influx via TRPV4 is key to the EET-induced increase in permeability.[7]

Initially, we assumed that the explanation for these disparate outcomes was simple, i.e. that heart failure led to differential downregulation of store-operated TRP channels but retention of TRPV4 expression. Indeed, we did confirm

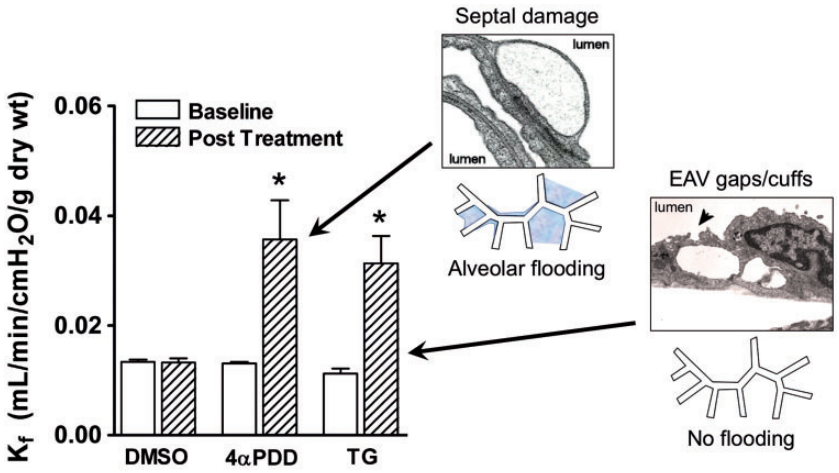

Fig. 2. Segmental permeability responses in lung. Although activation of TRPV4 with 4α-phorbol-12,13-didecanoate (4αPDD) and that of store-operated channels with TG elicits similar increases in the filtration coefficient (K_f) in isolated perfused lungs, these two channels have segment-specific injury patterns. TRPV4 selectively impacts alveolar septal capillaries, leading to derangement of septal endothelium and alveolar flooding. In contrast, TG has no impact in the alveolar septal compartment, but elicits development of inter-endothelial gaps in EAVs, see arrowhead) and perivascular cuffing (not shown). These disparate outcomes provide clear evidence for phenotypic heterogeneity in lung endothelium. Modified from Alvarez et al.[7] and Villalta and Townsley.[36]

that the loss of the permeability response to store-depletion in heart failure was associated with downregulation of endothelial TRPC1 and TRPC4.[20] In contrast, TRPV4 expression is retained in animals and humans with heart failure.[20,24] However, this simple perspective was based in the notion that K_f assesses endothelial barrier function and that all endothelial cells are created equal... Not true. When we "unpacked" that K_f box, we found that the increase in permeability resulting from activation of these two Ca^{2+} channels was focused in distinct vascular compartments in the lung (Fig. 2). Activation of the store-operated TRPC1/4 channel with TG targeted the extra-alveolar compartment, resulting in the formation of inter-endothelial gaps and perivascular cuffs in extra-alveolar vessels (EAV).[7,9] We saw no structural evidence of injury in septal capillaries, nor any evidence of alveolar flooding after activation of store-operated channels. In contrast, activation of TRPV4 with 4α-phorbol-12,13-didecanoate (4αPDD) selectively targeted alveolar septal capillaries. The increase in K_f elicited by TRPV4 activation was associated with injury and derangement of septal capillary endothelium and alveolar flooding.[7] This segregation of injury unmasked clear evidence of phenotypic heterogeneity in lung endothelium, a concept that is now well documented.[8–10,14,25] The corollary of this story is that increases in K_f or lung water may not be due to homogenous lung injury. Equally plausible explanations include segregated or targeted injury to one vascular compartment or merely increased hydrostatic pressures driving filtration.

Are increases in K_f or lung water (or lack thereof) all about the endothelium?

Mechanistically, we relate K_f to the Starling equation, where this coefficient simply predicts the effectiveness of the net Starling force balance in promoting transvascular fluid filtration. However, we need to consider issues related to the method which might impact outcomes in the lung: vascular compliance; stress relaxation; and the extent of the perfused surface area. We and others have documented significant stress relaxation in the very compliant mammalian lung vasculature following a step increase in vascular pressure (reviewed in Parker and Townsley[1]). This means that unless the resultant increase in vascular volume is recognized, the measured K_f can be an overestimate of actual endothelial water permeability. Further, measured K_f is a reflection of filtration through the actual perfused surface area in the lung. When the K_f method is applied consistently, we have found that total K_f increases with lung mass across species in normal lungs from mouse to sheep.[26] On the other hand, if perfused area decreases, as in the case with ligation of lung lobes, the measured K_f will follow even though the intrinsic permeability of the remaining vascular surface area is normal.[27] Thus, measured K_f may underestimate the impact of a challenge on permeability in isolated lung if there is simultaneous derecruitment and loss of perfused surface area.[28] Since other averaged measures used as indices of endothelial permeability (e.g. the lung wet–dry weight ratio, lung weight gain, and Evan's blue dye extravasation) are influenced by transvascular fluid filtration, changes of perfused surface area would impact these measures as well.

Back to Ca^{2+} channels... We have documented involvement of TRPV4 in two clinically relevant models of acute lung injury—HiPv and ventilator-induced lung injury (VILI). In both scenarios, TRPV4 is activated by mechanical stress.[23,24,29] TRPV4 can also be variously activated by heat, acid, EETs and hypotonic-cell swelling.[30,31] The widespread expression of TRPV4, along with the broad array of

compounds and scenarios in which this channel can be activated, means that any role of TRPV4 can be stimulus- and context-dependent. When we considered the potential for synergistic polymodal gating of TRPV4, we found that the pressure threshold for both HiPv and VILI decreases when tissue temperature is increased.[23,29] Since we considered K_f to be a measure of endothelial permeability, we concluded at the time that HiPv and VILI specifically targeted endothelial TRPV4 leading to injury. Perhaps not so simple... The endothelial Ca^{2+} influx associated with HiPv leads to P-selectin surface expression in lung capillaries,[32] raising the possibility of a pro-inflammatory role for endothelial TRPV4. Further, VILI appears to require TRPV4 expression in alveolar macrophages: reconstituting the alveolar macrophage population in TRPV4 null mice with wild-type macrophages completely restores the permeability response to VILI.[33] Similarly, while the increase in lung weight (edema) elicited by administration of platelet activating factor (PAF) was abrogated in TRPV4$^{-/-}$ mice, TRPV4 in blood cells was the real culprit behind the PAF-induced edema rather than TRPV4 in lung parenchyma.[34] An understanding of signaling complexities in any scenario, and thinking outside of the endothelial K_f box to consider all possible options, are critical to correct interpretation of outcomes in acute lung injury.

What really controls Ca^{2+}-dependent outcomes in lung endothelium?

We have shown that direct activation of TRPV4 selectively disrupts the alveolar septal endothelial barrier leading to alveolar flooding.[7,23,24,29] Other Ca^{2+} channels are expressed in lung endothelium, though not all appear to play a role in regulation of endothelial permeability.[35,36] As a case in point, activation of TRPV4 with 4αPDD and depolarization-dependent activation of the α_{1G} T-type voltage-gated Ca^{2+} channel in alveolar septal endothelium lead to apparently equivalent whole-cell Ca^{2+} transients. While TRPV4-mediated Ca^{2+} influx increases permeability, that elicited by T-channel activation selectively recruits endothelial surface expression of P-selectin, without increasing permeability.[37,38] At present, we do not understand the fundamental basis by which Ca^{2+} signals target specific functional outcomes in lung endothelium. In lung capillary endothelium, specificity in Ca^{2+} signaling is particularly challenging due to the attenuated nature of the endothelial barrier.[39] Several possibilities should be considered when attempting to investigate this box.

Proximity of Ca^{2+} channels to their targets? We have little information regarding spatial proximity for T-type Ca^{2+} channels to sites where P-selectin is sequestered in lung endothelium. Further, P-selectin does not appear to be localized to Weibel-Palade bodies in lung microvascular endothelium,[40] so the mechanism underlying P-selectin recruitment to the cell surface with T-channel activation is unclear. We have a little more information regarding TRPV4, which appears to be localized at the base of lung microvascular endothelium.[3] Cell–cell tethering at adherence junctions does not appear to be altered on TRPV4 activation, but rather TRPV4-mediated Ca^{2+} influx elicits activation of MMP2 and MMP9, which contribute to the permeability response.[41] TRPV4-mediated endothelial cell detachment from the basement membrane has been observed in vitro and in vivo.[36,42] Thus, TRPV4-mediated Ca^{2+} influx at the cell base would be poised to effect MMP release and untether cell-matrix integrin bonds. However, two factors potentially limit specificity of TRPV4-mediated Ca^{2+} signals from this perspective: the typical diffusion distance for Ca^{2+} in cytosol (100–500 nm)[43] and the extreme thinness of the septal microvascular endothelial barrier in vivo (100–300 nm).[39] Given these factors, one might predict that TRPV4-mediated Ca^{2+} entry at the endothelial base could elicit P-selectin expression at the apical face of the endothelium, assuming both the channel and the P-selectin source are sited within a finite area across the x-y footprint of the endothelial cell. Since our data do not support that outcome, we conclude that discrete spatial distribution of TRPV4 and the T-type channel is unlikely to solely account for signaling specificity. Other mechanisms must contribute.

Spatial constraints on diffusion due to organellar Ca^{2+} uptake? Given the predicted diffusion distance for Ca^{2+} in cytosol mentioned above, localized uptake into intracellular organelles might constrain the local spatial microdomain for a Ca^{2+} signal elicited by activation of a plasma membrane Ca^{2+} channel, and thus direct specificity. While not a lot of information exists on this potential mechanism in lung endothelium, there are some hints available in the literature. For example, the effectiveness of plasmalemmal Ca^{2+} transients in endothelium in gating nearby Ca^{2+}-activated potassium channels is attenuated by mitochondria in close proximity.[44] Further, interplay between mitochondria and endoplasmic reticulum shapes cytosolic Ca^{2+} transients on activation of store-operated channels.[31,45,46] Others have argued that the major role of mitochondria in endothelium is to modulate Ca^{2+}-dependent signaling, by provision of ATP for ATPase-dependent Ca^{2+} sequestration or extrusion from the cytosol or by serving as a Ca^{2+} sink.[47–49] As an example, in fibroblasts from patients with mitochondrial complex I deficiency, resultant mitochondrial depolarization and blunted ATP synthesis delay recovery of agonist-stimulated cytosolic Ca^{2+} transients.[50] A decreased decay rate could lead to increased time for diffusion and thus increased spatial spread of Ca^{2+} signals. Collectively, these mechanisms should limit Ca^{2+} dispersion and shape the dynamic patterns of Ca^{2+} transients[51] in lung microvascular endothelium. Our own work has documented that inhibition of mitochondrial complex I to impair overall bioenergetic capacity increased endothelial permeability in lung and in lung microvascular endothelial cells, as assessed by K_f and diffusive permeability, respectively.[52,53] Whether this is due to modulation of Ca^{2+} microdomains remains unclear.

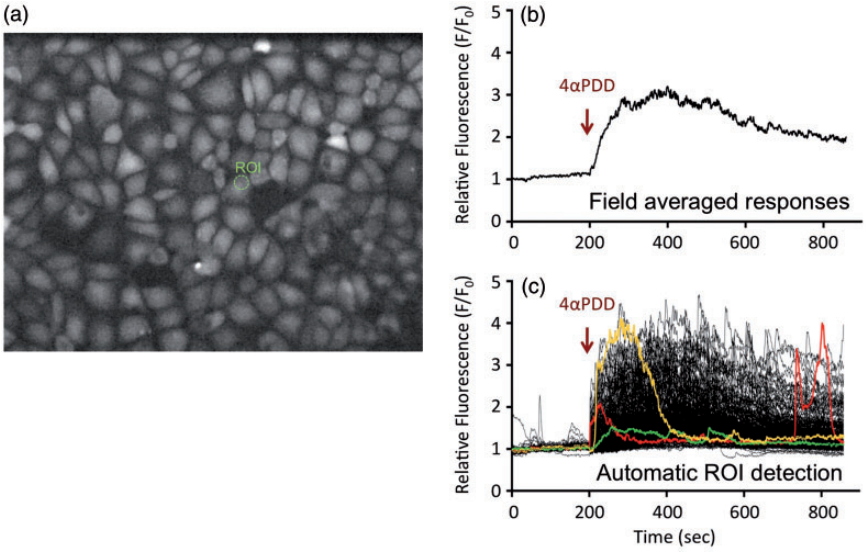

Fig. 3. Ca^{2+} dynamics in lung endothelium. Lung microvascular endothelium were loaded with Fluo4AM for assessment of Ca^{2+} responses to activation of TRPV4 with 4αPDD (a). Dynamic patterning in Ca^{2+} signals elicited by TRPV4 activation is masked when analyzing field-averaged Ca^{2+} responses (b). The dynamics (c) recorded using automated ROI analysis show substantial diversity in the temporal responses elicited by activation of TRPV4 with 4αPDD. In this analysis, each line shows the Ca^{2+} signal over time in an individual ROI. Several ROI responses are color-coded to highlight the diversity in local Ca^{2+} responses after TRPV4 activation. Such attention to individual response coding shows that while signal amplitude after channel activation in individual ROIs does vary, there is much more variability in the temporal pattering, i.e. when a transient appears, its duration and its rate of decay.

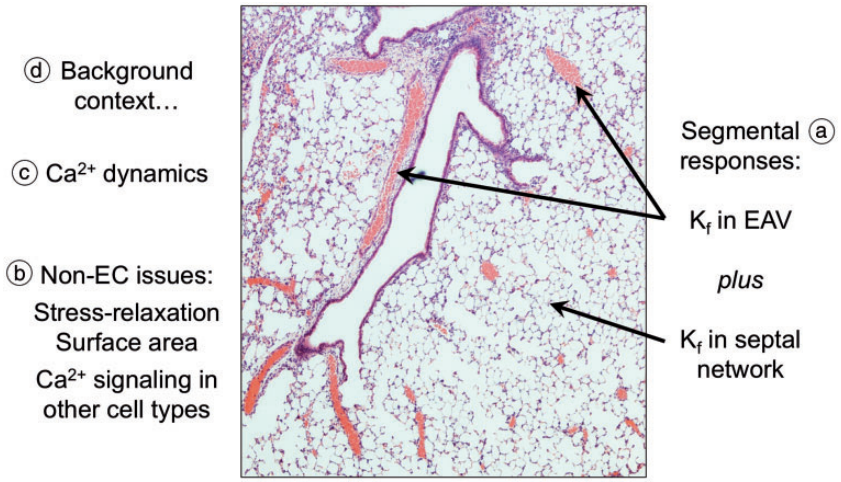

Fig. 4. Summary of issues in unpacking the Ca^{2+} dependent permeability box. These issues are many, as noted on this image of a hematoxylin and eosin-stained lung section. (a) The filtration coefficient K_f in an isolated lung is in reality the sum of those in all perfused vascular compartments. So, coefficients in EAV and in septal capillaries are separately dictated by barrier integrity in those compartments. This issue similarly impacts interpretation of other averaged measures of lung edema and permeability. (b) Issues not related to the endothelium can color interpretation. These include stress relaxation and changes in surface area which can impact the measure of permeability per se. In addition, Ca^{2+} channels in cells other than endothelium (e.g. alveolar macrophages, blood leukocytes, and/or airway epithelium) might, in reality, contribute to the apparent permeability response in lung. (c) Ca^{2+} dynamic patterns, rather than averaged Ca^{2+} responses to channel activation, might yield more specific information regarding the contribution of any Ca^{2+} channel to regulation of lung permeability. Nonetheless, not all Ca^{2+} signals target pathways which regulate permeability. (d) The background context, such as changes in endothelial bioenergetics with sepsis, might color interpretation and integration of Ca^{2+} signals in lung. Responses to challenge with Ca^{2+} channel agonists might be lost or amplified, or perhaps the specificity of those signals might be altered. A small portion of this image appeared in black and white in Townsley and Stevens.[63]

Dynamic patterning of Ca^{2+} transients? We have observed dynamic patterning in Ca^{2+} signals elicited by TRPV4 activation in monolayers of lung microvascular endothelium which is masked when analyzing field-averaged Ca^{2+} responses (Fig. 3). The dynamics recorded using automated region of interest (ROI) analysis show substantial diversity in the temporal responses elicited by activation of TRPV4 with 4αPDD. While signal amplitude after channel activation in individual ROIs does vary, there is much more variability in the temporal patterning, i.e. when a transient appears, its duration, and its rate of decay. In coronary arteries, substance P-mediated vasodilation was found to correlate precisely with dynamic Ca^{2+} patterns in the endothelium, even though there was poor correlation of vasodilation with Ca^{2+} responses averaged across the field of cells.[6] While critical to unpack, understanding this box will require development of tools to assess the integrity of the endothelial barrier and Ca^{2+} signaling with a high degree of spatial and temporal specificity. We will need to explore whether specific cellular targets which regulate endothelial permeability in EAVs and in septal capillaries are regulated solely by changes in Ca^{2+} signal amplitude or whether regulation is dependent upon critical frequency coding in Ca^{2+} transients.

Complexities in sepsis and acute lung injury

Attributing outcomes in sepsis and acute lung injury to activation of one Ca^{2+} channel can be problematic, even when the direct impact of channel activation seems clear cut. For example, while we have a good understanding of TRPV4's role in simple models with direct channel activation, studies in more complex models of acute lung injury, such as that elicited by chemical inhalation exposure or in models of sepsis have not yielded a consistent picture of TRPV4's involvement.[54–57] A further complication in the setting of sepsis may be the resultant alteration in endothelial "context" for interpreting Ca^{2+} channel activation. For example, sepsis and trauma are commonly associated with mitochondrial bioenergetic dysfunction. Serum from patients with trauma and/or sepsis has deleterious impact on bioenergetics of vascular or pulmonary endothelial cells in culture.[58–61] If mitochondria are indeed critical to shaping of Ca^{2+} microdomains in normal lung endothelium, then endothelial bioenergetic impairment in sepsis could increase the spread of Ca^{2+} signals and impair Ca^{2+} signaling specificity. Yet another box to unpack…

Conclusion

In summary, there are many issues to consider when "unpacking" the Ca^{2+}-dependent permeability box (Fig. 4), including potential contributions from differential segmental responses in the lung vasculature, methodologic issues, contributions from other cells in lung beyond endothelial cells, dynamic patterning in Ca^{2+} transients, and the background state of the lung and lung endothelium in disease.

Conflict of interest

The author(s) declare that there is no conflict of interest.

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