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Amit Jairaman,* Megumi Yamashita,* Robert P. Schleimer,† and Murali Prakriya*

The G-protein–coupled protease-activated receptor 2 (PAR2) plays an important role in the pathogenesis of various inflammatory and auto-immune disorders. In airway epithelial cells (AECs), stimulation of PAR2 by allergens and proteases triggers the release of a host of inflammatory mediators to regulate bronchomotor tone and immune cell recruitment. Activation of PAR2 turns on several cell signaling pathways of which the mobilization of cytosolic Ca\textsuperscript{2+} is likely a critical but poorly understood event. In this study, we show that Ca\textsuperscript{2+} release-activated Ca\textsuperscript{2+} (CRAC) channels encoded by stromal interaction molecule 1 and Orai1 are a major route of Ca\textsuperscript{2+} entry in primary human AECs and drive the Ca\textsuperscript{2+} elevations seen in response to PAR2 activation. Activation of CRAC channels induces the production of several key inflammatory mediators from AECs including thymic stromal lymphopoietin, IL-6, and PGE\textsubscript{2}, in part through stimulation of gene expression via nuclear factor of activated T cells (NFAT). Furthermore, PAR2 stimulation induces the production of many key inflammatory mediators including PGE\textsubscript{2}, IL-6, IL-8, and GM-CSF in a CRAC channel–dependent manner. These findings indicate that CRAC channels are the primary mechanism for Ca\textsuperscript{2+} influx in AECs and a vital checkpoint for the induction of PAR2-induced proinflammatory cytokines. The *Journal of Immunology*, 2015, 195: 000-000.

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Abbreviations used in this article: AEC, airway epithelial cell; 2-APB, 2-aminoethoxydiphenylborane; BTP2, 3,5-bis(trifluoromethyl)pyrazole; CRAC, Ca\textsuperscript{2+} release-activated Ca\textsuperscript{2+} (CRAC) channels; CSa, cyclosporin A; ER, endoplasmic reticulum; GPCR, G-protein-coupled receptor; IP\textsubscript{3}, inositol 1,4,5-triphosphate; I-V, current voltage; NFAT, nuclear factor of activated T cells; NHBE, normal human bronchial epithelial; PAR2, protease-activated receptor 2; PDBu, phorbol 12,13-dibutyrate; PKC, protein kinase C; PLC, phospholipase C; RNAi, RNA interference; siRNA, small interfering RNA; SOCE, store-operated Ca\textsuperscript{2+} entry; STIM, stromal interaction molecule; TG, thapsigargin; TSLP, thymic stromal lymphopoietin.

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response to ER Ca\(^{2+}\) store depletion, translocate to the junctional ER to interact with Orai channels (22, 23). In immune cells, previous studies have established that CRAC channels encoded by STIM1/Orai1 proteins play a central role in driving Ca\(^{2+}\) signaling that controls the function of T cell, mast cells, B cells, and neutrophils (15–18).

However, the role of CRAC channels in regulating immune functions of the airway epithelium and, in particular, their contributions to the production and release of inflammatory mediators are unknown.

In this study, we show that primary human AECs show robust store-operated Ca\(^{2+}\) entry (SOCE) mediated by the CRAC channel proteins STIM1 and Orai1. Activation of CRAC channels stimulates several critical effector functions in AECs including gene transcription and production of a range of inflammatory modulators. We further find that stimulation of PAR2 receptors produces intracellular Ca\(^{2+}\) elevations that are critically dependent on CRAC channel activity. In turn, CRAC channel-mediated Ca\(^{2+}\) signals induce the production of several proinflammatory mediators IL-8, IL-6, GM-CSF, and PGE\(_2\) in part through stimulation of gene transcription via the nuclear factor of activated T cells (NFAT)–calcineurin signaling axis. These results demonstrate that CRAC channels are a major route of Ca\(^{2+}\) entry in AECs and serve as a key checkpoint for PAR2-mediated generation of inflammatory mediators.

**Materials and Methods**

**Cells and media**

Normal human bronchial epithelial (NHBE) cells were purchased from Lonza (Walkersville, MD) and grown in bronchial epithelial cell growth medium containing various growth factors. BEAS-2B, a bronchial epithelial cell line was a kind gift from Curtis Harris (National Cancer Institute) and were cultured in DMEM/F12 medium containing 5% FBS. A549, an alveolar epithelial cell line, was a kind gift from Dr. Jacob Sznajder (Northwestern University). 1Haeo\(_2\) cells, a bronchial epithelial cell line, was a kind gift from Dr. Alice Prince (Columbia University). Both A549 and 1Haeo\(_2\) cells were cultured in DMEM containing 10% FBS. All the cells were maintained at 37˚C and in 5% CO\(_2\).

**Plasmids and transfections**

The plasmids and RNA interference (RNAi) constructs used in this study were transfected into primary cells and cell lines using Lipofectamine 2000 (Invitrogen) in the dark for 40 min at room temperature in the appropriate culture medium containing 5–10% FBS. After washing away excess dye, cells were incubated in media for an additional 10 min before initiating Ca\(^{2+}\) imaging. Single-cell [Ca\(^{2+}\)]\(_{\text{cyt}}\) measurements were done according to the protocol described previously (25). Image acquisition and analysis was performed using IPlab (Scanalytics, Rockville, MD) and Slidebook (Denver, CO). For data analysis, regions of interest were drawn around single cells, background subtracted, and the \(F_{s,i}/F_{380}\) intensity ratios were determined for each time point. The \(F_{s,i}/F_{380}\) intensity ratios were converted to [Ca\(^{2+}\)]\(_{\text{cyt}}\) using the formula:

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where \(R\) is the \(F_{s,i}/F_{380}\) fluorescence intensity ratio and \(R_{\text{max}} = 9.645\) and \(R_{\text{min}} = 0.268\) were determined by experiment in vitro calibration of Fura-2.

**Table 1. Effect of GPCR and TLR agonists on SOCE in AECs**

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**Reagents and chemicals**

The standard extracellular Ringer’s solution had the following composition (in mmol): 150 NaCl, 4.5 KCl, 10 glucose, 1 MgCl\(_2\), 2 Ca\(_{\text{Cl}}\), and 5 Na-HEPES. pH was adjusted to 7.4 using NaOH. For the Ca\(^{2+}\)-free Ringers solution, Ca\(_{\text{Cl}}\) was excluded from the above composition and Mg\(_{\text{Cl}}\) was increased to 3 mmol. The 20 mmol Ca\(^{2+}\) solution used for electrophysiological recordings contained 20 mmol Ca\(_{\text{Cl}}\) and 130 mmol Na\(_{\text{Cl}}\); other components of this solution were identical to the standard Ringer’s solution. The divalent-free Ringer’s solution contained (in mmol): 150 NaCl, 10 HEDTA, 1 EDTA, and 10 HEPES (pH 7.4). Stock solutions of 2-aminoethoxydiphenylborane (2-APB), 3,5-bis(trifluoromethyl)pyrazole (BTP2), thapsigargin (TG), RO2095, cyclosporin A (CsA), and FK-506 were dissolved in DMSO. PAR2 agonists including type IX trypsin (Sigma-Aldrich) as well as the agonistic peptides SLIGRL and SLIGKV, control peptide LRGILS, and the PAR1 peptide TFLR (all from Tocris Bioscience) were constituted in water. 2-APB, CsA, TG, BTP2, and U73122 were from Sigma-Aldrich. RO2095 [difluoro-N-(5-(4-methyl-1-(5-methyl-thiazol-2-yl)-1,2,5,6-tetrahydro-pyridin-3-yl)-pyrazin-2-yl)-benzamide] was from Synta Pharmaceuticals.

**Intracellular Ca\(^{2+}\) measurements**

NHBE cells and the indicated cell lines were grown on poly-l-lysine–coated glass-bottom dishes (MatTek). Cells were loaded with 2.5 \(\mu\)mol Fura-2 AM (Invitrogen) in the dark for 40 min at room temperature in the appropriate culture medium containing 5–10% FBS. After washing away excess dye, cells were incubated in media for an additional 10 min before initiating Ca\(^{2+}\) imaging. Single-cell [Ca\(^{2+}\)]\(_{\text{cyt}}\) measurements were done according to the protocol described previously (25). Image acquisition and analysis was performed using IPlab (Scanalytics, Rockville, MD) and Slidebook (Denver, CO). For data analysis, regions of interest were drawn around single cells, background subtracted, and the \(F_{s,i}/F_{380}\) intensity ratios were determined for each time point. The \(F_{s,i}/F_{380}\) intensity ratios were converted to [Ca\(^{2+}\)]\(_{\text{cyt}}\) using the formula:

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where \(R\) is the \(F_{s,i}/F_{380}\) fluorescence intensity ratio and \(R_{\text{max}} = 9.645\) and \(R_{\text{min}} = 0.268\) were determined by experiment in vitro calibration of Fura-2.

**NHBE cells were treated with the indicated ligands in a Ca\(^{2+}\)-free Ringer’s solution. Extracellular Ca\(^{2+}\) (2 mmol) was then restored and the amplitude of the [Ca\(^{2+}\)]\(_{\text{cyt}}\) rise was examined. Cells were considered responders if the [Ca\(^{2+}\)]\(_{\text{cyt}}\) elevation was >2× SEM above the resting [Ca\(^{2+}\)]\(_{\text{cyt}}\).**

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pentapotassium salt, $\beta = 0.236$) was determined from the $F_{\text{min}}F_{\text{max}}$ ratio at 380 nm, and $K_d$ is the apparent dissociation constant of Fura-2 binding to Ca$^{2+}$ (135 nmol). Where applicable, rate of SOCE, $-\Delta[Ca^{2+}]_i/\Delta t$ was calculated as the slope of a line fitted to the first three points following addition of 2 mmol Ca$^{2+}$ in the Ca$^{2+}$ imaging trace. Slope was averaged over several cells in the imaging field to generate the relevant bar graphs.

**Western blots**

NHBE and BEAS-2B cells were cultured in six-well plates. At $\sim 70$–80% confluency, cells were washed with cold PBS, following which cell lysis and Western blotting was done using protocols described previously (25). Oral1 and STIM1 proteins were detected using affinity-purified polyclonal Abs and peroxidase-labeled secondary Abs (26, 27).

**Patch clamp measurements**

Patch clamp recordings and analysis of CRAC currents were performed using an Axopatch 200B amplifier (Molecular Devices, Foster City, CA) using standard methods as previously described (28). The holding potential was +30 mV. The standard voltage stimulus consisted of a 100-ms step to $-100$ mV followed by a 100-ms ramp from $-100$ to $+100$ mV applied at 1-s intervals. Cells were pretreated with 1 mmol TG prior to establishment of the seal to deplete stores and activate CRAC channels. Data were leak-subtracted using currents collected in 50 $\mu$M La$^{3+}$ to block CRAC channels.

**NFAT-luciferase assay**

Endogenous NFAT activity in cells was measured using the PGL3–NFAT luciferase reporter construct (29). NFAT activity was normalized to the activity of Renilla luciferase (pRL-Tk-luc). Plasmids were transfected into AECs in a ratio of 20:1 (NFAT/Renilla). At 24 h after transfection, cells were pretreated with the CRAC channel inhibitors BTP-2 and RO2959 for 1 h followed by treatment with 0.5 mmol TG and 50 mmol phorbol 12,13-dibutyrate (PDBu) for 8–10 h. Cells were lysed, and the luciferase activity was determined using the Dual-luciferase Reporter Assay Kit (Promega) and a single-tube luminometer (Berthold Instruments, Germany).

**NFAT translocation assay**

BEAS-2B cells were transfected with NFAT-Luc7-GFP construct and imaged for GFP 24–36 h later. Images were taken in resting 2 mmol Ca$^{2+}$ solution and after depleting ER Ca$^{2+}$ stores with TG (1 mmol, 20 min). The percentage of cells showing NFAT localization to the nucleus was calculated, averaged between several fields, and reported as a bar graph.

**Analysis of cytokine secretion**

AECs (NHBE, BEAS, A549, and IHaeo$^-$ cells) were cultured in 24-well plates. Twelve hours before stimulation, the cell culture medium was changed to one containing 2 mmol Ca$^{2+}$. Cells were treated with specific CRAC channel–activating stimuli (TG and PDBu or PAR2 agonists SLIGRL and SLIGKV), and the levels of various inflammatory mediators were measured using custom ELISA kits (RayBiotech for TNF-\(\alpha\), IL-6, IL-1\(\alpha\), and GM-CSF; Cayman Chemical for PGE\(_2\); R&D Systems for TSLP; and Life Technologies for IL-8 and IL-1\(\beta\)).

**Data analysis**

All bar graphs summarizing the data are reported as mean ± SEM. For data sets involving more than two groups, initial statistical analysis was performed using ANOVA with a confidence interval of 5%. This was followed by two-tailed paired Student t test for comparing different treatment conditions within the set. For data sets with only two groups,
two-tailed paired Student t test was used to compare between control and test conditions.

Results

A screen for GPCR agonists identifies PAR2 receptors as activators of SOCE in the airway epithelium

In what context would CRAC channels be relevant for Ca^{2+} signaling in AECs? CRAC channels are voltage independent and require depletion of ER Ca^{2+} stores for activation (17). There are numerous extracellular stimuli (e.g., growth factors, pathogens, GPCR ligands, etc.) that act in this manner and could be regarded as potential candidates that stimulate CRAC channels in AECs. To examine this, we screened several ligands that are coupled to the generation of IP_3 in NHBE cells. Although several receptors coupled to PLC–IP_3 signaling are thought to be expressed in AECs (30), this screen revealed that only a subset of ligands, including agonists for PAR2 and purinergic P2Y receptors (and to a smaller extent, thrombin and bradykinin), evoked Ca^{2+} signals consistent with SOCE (Table I). PAR2 receptors are important for the ability of AECs to sense allergens and endogenous proteases and for mediating the subsequent inflammatory responses in the lung (31). We therefore sought to define the mechanisms and functional consequences of CRAC channel signaling in AECs following PAR2 activation.

PAR2 activation stimulates Ca^{2+} signaling in AECs by activating CRAC channels

PAR2 activation initiates many cell signaling cascades including IP_3-mediated mobilization of [Ca^{2+}]_cyt (13). However, the pathways involved in generating PAR2 mediated [Ca^{2+}]_cyt elevations, and the functional significance of these Ca^{2+} elevations in AECs are unclear. To address the potential role of store-operated CRAC channels in generating PAR2-mediated Ca^{2+} signals in AECs, we examined the ability of PAR2 ligands to elicit SOCE by imaging cytosolic Ca^{2+} transients using the Ca^{2+} indicator, Fluo-2. Activation of PAR2 by the PAR2-activating peptides SLIGRL and SLIGKV caused a biphasic [Ca^{2+}]_cyt elevation that included a rapid initial rise in [Ca^{2+}]_cyt followed by sustained Ca^{2+} oscillations in NHBE cells (Fig. 1A, 1E and data not shown). The rise in [Ca^{2+}]_cyt following add-back of extracellular Ca^{2+} indicates that Ca^{2+} entry across the plasma membrane is necessary for sustaining PAR2-mediated Ca^{2+} elevations (Fig. 1A). The Ca^{2+} response was specific to PAR2 activation, because administration of control peptide LGIILS or PAR1-specific peptide TFLLR failed to elicit Ca^{2+} signaling (Fig. 1E). Response to SLIGKV was also abolished when cells were pretreated with the PLC inhibitor U73122, consistent with PAR2 activating a G_q-mediated PLC signaling pathway (Fig. 1E). Importantly, the CRAC channel inhibitors BTP2 and RO2959 (32, 33) attenuated both the number of oscillations and the amplitude of the Ca^{2+} response to PAR2 peptides (Fig. 1B–E). These results indicate that PAR2 stimulation evokes a biphasic Ca^{2+} signal the sustained component of which arises from CRAC channel activation.

Previous evidence indicates that the CRAC channel proteins Orai1 and STIM1 are expressed in the lung (21, 34), raising the possibility that these proteins may contribute to Ca^{2+} signaling in AECs. We therefore sought to investigate the contribution of STIM1 and Orai1 to PAR2 evoked Ca^{2+} signals. siRNA-mediated knockdown of STIM1 and Orai1 proteins significantly reduced their expression and inhibited the sustained Ca^{2+} signals in response to the PAR2-activating peptide SLIGKV in the bronchial cell line BEAS-2B (Fig. 1F, 1G, Supplemental Fig. 1A). Similarly, overexpression of a dominant-negative pore mutant of CRAC channels, E106A Orai1 (35), significantly reduced SLIGKV-activated Ca^{2+} response (data not shown). These results indicate that CRAC channels encoded by STIM1 and Orai1 are essential for mobilizing PAR2-evoked Ca^{2+} signals.

PAR2 activation by the serine protease trypsin stimulates CRAC channel-mediated Ca^{2+} signals in AECs

In the airway epithelium, PAR2 is activated by endogenous proteases including trypsin, mast cell tryptase, and human airway trypsin-like proteases that are released during inflammation and injury (31). We therefore tested the effects of trypsin on Ca^{2+} signaling in AECs. Administration of type IX trypsin produced a sustained Ca^{2+} signal only when Ca^{2+} was present in the external bath solution. The sustained component of the [Ca^{2+}]_cyt elevation was abrogated by the CRAC channel inhibitor BTP2 (Fig. 2A, 2B) without affecting store release. Likewise, overexpression of E106A Orai1, which functions as a dominant-negative inhibitor of CRAC channels (35) as well as the knockdown of CRAC channel proteins STIM1 and Orai1, attenuated trypsin-induced Ca^{2+} signal (Fig. 2C, 2D). Taken together, these results show that PAR2 activation in response to both synthetic peptides and enzymatic cleavage by trypsin mobilizes Ca^{2+} signaling in primary human AECs through activation of CRAC channels.

**STIM1 and Orai1 contribute to SOCE in human AECs**

The ability of CRAC channel antagonists to suppress PAR2-evoked Ca^{2+} signals implies that human AECs express the machinery for CRAC channels. To directly address this issue, we next examined

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**FIGURE 2.** The PAR2 agonist, type IX trypsin, evokes Ca^{2+} elevations by activating CRAC channels. (A) Type IX trypsin (100 nmol) activates SOCE in NHBE cells. SOCE was activated using the same protocol as in Fig. 1A. The CRAC channel inhibitor BTP2 abrogates trypsin-induced Ca^{2+} elevations in NHBE cells (mean ± SEM of n = 17–25 cells, three independent experiments). Slope of the line (used to calculate rate of SOCE) following addition of 2 nmol Ca^{2+} is indicated for the control condition. (B) Summary of the rate of Ca^{2+} influx for SOCE shown in (A). (C) siRNA knockdown of STIM1 or Orai1 inhibits trypsin-mediated SOCE in BEAS-2B cells. (D) Summary of the rates of trypsin-mediated Ca^{2+} influx shown in (C) and in cells transfected with dominant-negative E106A Orai1 (mean ± SEM of n = 16–26 cells; three independent experiments). **p < 0.01, ***p < 0.001. Con, control.
We next sought to resolve the molecular machinery of CRAC channels in AECs. CRAC channels are activated by STIM proteins (STIM1 and -2) and encoded by the Orai proteins (Orai1, -2, and -3) (36). Western blots showed the expression of both STIM1 and Orai1 proteins in NHBE and BEAS-2B cells (Fig. 3H, Supplemental Fig. 1A). Overexpression of dominant-negative E106A Orai1 significantly abrogated SOCE in various AECs (Fig. 3F, 3I). Moreover, knockdown of STIM1 and Orai1 using siRNA significantly reduced the protein expression and inhibited both the rate and the amplitude of SOCE in NHBE cells, indicating that these isoforms do not appreciably contribute to SOCE in human AECs (Fig. 3G). We were unable to directly study the expression of Orai2 and Orai3 isoforms due to nonspecificity of the commercially available Abs we tested (data not shown). Nevertheless, together with the functional evidence presented in the previous

![Figure 3](http://www.jimmunol.org/)

**FIGURE 3.** Characterization of store-operated CRAC channels in human AECs. (A–C) ER Ca\(^{2+}\) stores were depleted with 1 μmol TG in a nominally Ca\(^{2+}\)-free solution. Readdition of 2 mmol Ca\(^{2+}\) to the external bath solution evoked SOCE. (A) SOCE is acutely blocked by 2 μmol La\(^{3+}\). (B) A low dose of 2-APB (5 μmol) facilitated SOCE. (C) A high dose of 2-APB (50 μmol) inhibited SOCE. (D) Pretreatment with the CRAC channel inhibitor BTP2 (500 nmol) abolished SOCE in NHBE cells (n = 21–26 cells; three independent experiments). (E) Knockdown of STIM1 and Orai1 expression in NHBE cells significantly inhibited SOCE (n = 21–26 cells; three independent experiments). Summary of the rates of SOCE in NHBE cells following treatment with the CRAC channel inhibitors BTP2 (500 nmol) and RO2959 (500 nmol) and expression of dominant-negative E106A Orai1 (n = 21–42 cells; three experiments) (F) and following siRNA-mediated knockdown of STIM1-2, Orai1–3 (mean ± SEM of n = 22–34; three experiments) (G). (H) Western blots showing expression of Orai1 and STIM1 in NHBE cells. (I) Summary of rates of SOCE in BEAS-2B cells (n = 31–48 cells; three independent experiments). (J) Whole-cell patch-clamp recordings of \(I_{\text{CRAC}}\) in BEAS-2B cells. Cells were pretreated with TG to deplete ER Ca\(^{2+}\) stores. The external solution was periodically switched between 20 mmol Ca\(^{2+}\) and a divalent-free (DVF) solution. In 20 mmol Ca\(^{2+}\), the current exhibits a current-voltage (I-V) relationship with strong inward rectification and positive reversal potential (>600 mV), consistent with the known biophysical hallmarks of CRAC channels (37). \(^*p < 0.05, \^{**}p < 0.01, \^{***}p < 0.001.\) Con, control.
section, these results indicate that the CRAC channel proteins STIM1 and Orai1 are essential for conferring SOCE in human AECs. We therefore focused our studies on the functional roles of STIM1 and Orai1 in the airway epithelium.

CRAC channels have a distinct electrophysiological profile characterized by an inwardly rectifying current-voltage (I-V) relationship, low permeability to large monovalent cations such as Cs⁺, and depotentiation in divalent-free solutions (37). Patch-clamp recordings of store-operated currents in BEAS-2B cells following treatment with TG to deplete ER Ca²⁺ stores showed the presence of a cation current consistent with these well-known properties of CRAC channels (17, 25) (Fig. 3J). In 20 nmol Ca²⁺, the I-V relationship showed strong inward rectification and an extremely positive reversal potential. In divalent-free solution, the I-V relationship revealed a reversal potential of \( \sim +50 \) mV, indicating low permeability to internal Cs⁺ (Fig. 3J). Similar results were seen in the airway epithelial cell line A549 (data not shown). These results indicate that SOCE in airway cells exhibits the canonical biophysical properties of CRAC channels encoded by STIM1 and Orai1.

CRAC channel activation stimulates cytokine and chemokine production in AECs

AECs play a vital role in host defense by producing inflammatory mediators such as TSLP, IL-6, PGE₂, IL-33, IL-8, and RANTES in response to a variety of stimuli including infectious agents, allergens, and other bioactive molecules (38–41). Although Ca²⁺ signals have been implicated in the generation of inflammatory mediators from AECs, the Ca²⁺ signaling pathways that mediate this process remain poorly understood (7, 20, 42).

To address a potential role for CRAC channels in the production of inflammatory mediators from AECs, we initially performed a multiplex cytokine screen in NHBE cells following activation of CRAC channels by the SERCA inhibitor TG (Supplemental Fig. 2). Previous studies have shown that Ca²⁺ signaling pathways in immune cells including T cells often work in synergy with protein kinase C (PKC)–dependent signaling to induce cytokines (43). Therefore, in these experiments, we also costimulated PKC by administering the phorbol ester, PDBu. Key hits from this screen were further examined by individual ELISA kits. These tests revealed that several key inflammatory mediators are regulated by CRAC channel activation in NHBE cells, including TSLP, IL-6, TNF-α, IL-1β, and GM-CSF (Fig. 4A, 4C–F). CRAC channel activation also induced the production of the arachidonic acid metabolite PGE₂ from AECs (Fig. 4B). We also confirmed IL-6, TNF-α, and PGE₂ production in various AEC lines (Supplemental Fig. 3A–C). The production of these inflammatory mediators was prevented in Ca²⁺-free medium and by the CRAC channel inhibitor BTP2 (Fig. 4A–F). Furthermore,
knockdown of STIM1 and Orai1 inhibited CRAC channel–induced production of TNF-α and PGE2 (Fig. 4B, 4C). Thus, these results implicate a causal role for Ca2+ influx through CRAC channels in the production of inflammatory cytokines from AECs. Interestingly, whereas maximal induction of TNF-α and IL-6 required costimulation of NHBE cells with both TG and PDBu, TSLP and PGE2 were induced maximally by TG alone, suggesting that Ca2+ signals differentially regulate cytokine production depending on the presence of specific costimulatory signals.

In addition to the cytokines, CRAC channel activation also caused significant increase in the production of chemokines IL-8 and RANTES, which was blocked by the CRAC channel inhibitors BTP2 and RO2959 (Fig. 4G, 4H). The multiplex cytokine screen further revealed that although AECs also produce IFN-γ and CXCL1, these factors are not induced by CRAC channels, indicating that only a subset of inflammatory mediators produced by AECs are regulated by CRAC channels. Collectively, these results establish a key role for CRAC channels as an important route of Ca2+ entry for the production of cytokines and chemokines in the airway epithelium.

PAR2 agonists activate CRAC channels to induce cytokine production in AECs

Previous studies have established that PAR2 activation in AECs induces the production of a wide range of cytokines including IL-6, GM-CSF, TSLP, and chemokines IL-8 and eotaxin, the growth factor PDGF, arachidonic metabolites PGE2 and PGD2, as well as the enzyme MMP-9 (8–10, 40, 44). The dual findings that PAR2 activation mobilizes Ca2+ signals through CRAC channels (Figs. 1, 2) and that CRAC channel activation with TG stimulates cytokine production (Figs. 4, 5) therefore led us to consider a role for CRAC channels in the production of inflammatory mediators by PAR2 agonists. These tests revealed that administration of the PAR2 agonist peptide SLIGRL resulted in a significant increase in the production of PGE2, IL-6, GM-CSF, and IL-8 (Fig. 5A, 5C, 5E, 5F). PGE2 was induced in 2 h, whereas induction of IL-6, GM-CSF, and IL-8 occurred after 8–24 h of PAR2 activation. The PAR2-mediated production of these factors was significantly inhibited by the CRAC channel antagonist BTP2 (Fig. 5A, 5C, 5E, 5F). Interestingly, PAR2 activation by SLIGRL did not evoke TNF-α induction (not shown). This is in contrast to cell stimulation with TG/PDBu, which was very effective in evoking TNF-α production (Fig. 4D). The reasons for this difference need to be further investigated, but could be because the amplitude and duration of Ca2+ elevations in response to PAR2 activation are insufficient to induce TNF-α. PGE2 and IL-6 induction in response to SLIGRL were significantly inhibited by combined knockdown of STIM1 and Orai1 (Fig. 5B, 5D). Knockdown of STIM1 or Orai1 alone, however, did not significantly diminish the production of these cytokines (data not shown). We suspect that this is
due to the low knockdown efficiency of protein expression in NHBE cells (~50% for both proteins as seen by Western blot, Fig. 3H), which would be expected to only partially inhibit SOCE. Similarly, trypsin produced a significant increase in PGE2 production that was attenuated by the CRAC channel blocker BTP2 (Fig. 5A). Thus, these findings strongly suggest that CRAC channels contribute to the production and release of inflammatory modulators in response to PAR2 activation.

**PAR2 receptor stimulation results in IL-6 and IL-8 production through the activation of NFAT**

By what mechanism does Ca2+ influx through CRAC channels regulate PAR2-mediated production of cytokines? In many immune cells, cytokine induction is regulated transcriptionally by the transcription factor NFAT (45, 46). Ca2+ elevations activate NFAT through the protein phosphatase calcineurin, which then causes NFAT to move into the nucleus and bind to target DNA sequences (43). Although AECs express NFAT proteins (47), the specific targets of NFAT and the physiological relevance of this signaling remains unknown. To explore whether induction of cytokines by PAR2 activation occurs through the calcineurin–NFAT pathway, we measured PAR2-induced cytokine levels in the presence of the calcineurin inhibitor CsA, a widely used small-molecule inhibitor of calcineurin/NFAT signaling (48). Induction of both IL-6 and IL-8 was significantly inhibited by CsA (Fig. 6A). By contrast, the NF-κB inhibitor caffeic acid had no effect on induction of IL-6 or IL-8, indicating that under these experimental conditions, the induction of the cytokines primarily occurs through NFAT-dependent gene transcription (Supplemental Fig. 3D). Moreover, induction of PGE2 and GM-CSF was not affected by CsA, suggesting that the induction of these cytokines in response to PAR2 activation is not significantly regulated by calcineurin/NFAT signaling. These findings highlight a novel role for NFAT-dependent gene expression in the induction of specific cytokines and chemokines in response to PAR2 signaling.

**NFAT regulates cytokine and chemokine production in response to CRAC channel activation**

To explore the role of NFAT in CRAC channel-mediated cytokine production more directly, we stimulated NHBE cells with TG and PDBu for 12 h in the presence or absence of CsA and evaluated cytokine induction through the calcineurin–NFAT pathway (Fig. 6B). CsA caused an almost complete inhibition of TNF-α and IL-6 production, implicating a key role for NFAT in induction of these cytokines (Fig. 6B). CsA also partially inhibited production of PGE2 and IL-8, suggesting that NFAT contributes to the production of these mediators (Fig. 6B). The partial inhibition of PGE2 induction in this case stands in contrast to the lack of CsA effect seen following 2 h of PAR2 stimulation (Fig. 6A), suggesting that at longer time points, PGE2 production is influenced by NFAT-mediated gene expression, possibly through transcriptional regulation of the enzymes mediating PGE2 generation (49). Interestingly, although the promoter elements of GM-CSF are believed to exhibit NFAT binding, the induction of this cytokine in response to both PAR2 and TG/PDBu was unaffected by CsA, suggesting that not all cytokines induced by CRAC channels are regulated through the calcineurin–NFAT pathway (Fig. 6B).

NFAT is normally phosphorylated at rest and resides in the cytoplasm in the inactive state. Ca2+-free solution and by BTP2, indicating that Ca2+ entry through CRAC channels was required to trigger nuclear translocation of NFAT (Fig. 7A, 7B). Moreover, examination of NFAT-dependent gene transcription using a luciferase reporter assay indicated that CRAC channel activation stimulated NFAT-dependent luciferase expression in NHBE cells (Fig. 7C). Luciferase activity was abrogated in the absence of external Ca2+ and by the CRAC channel antagonists BTP2 and RO2959 (Fig. 7C) without affecting cell viability (Supplemental Fig. 4). Importantly, siRNA-mediated suppression of STIM1 and Orai1 expression significantly inhibited NFAT-dependent gene transcription (Fig. 7D). The calcineurin inhibitors CsA and FK506 also strongly inhibited induction of NFAT-luciferase activity consistent with the well-known role of calcineurin in controlling NFAT activation (Fig. 7C). Thus, these results highlight a broader role for NFAT in the generation of inflammatory mediators from the airway epithelium (Fig. 8).

**Discussion**

Ca2+ signals regulate many key physiological processes in AECs, including activation of Ca2+ activated Cl− conductances, ciliary beat frequency, and mucous and surfactant production (19, 50,
There is now growing evidence that Ca\(^{2+}\) signals also play an important role in the induction of inflammatory mediators from the airway epithelium in response to pathogens and allergens and may be involved in orchestrating inflammatory responses in several airway diseases including cystic fibrosis, asthma, and acute lung injury (7, 20, 52). Despite these observations, our understanding of how Ca\(^{2+}\) signals are generated in human AECs and how they are linked to effector functions remains incomplete.

Knowledge of molecules that comprise the functional architecture of the Ca\(^{2+}\) signaling network and the mechanisms by which these proteins shape Ca\(^{2+}\) signals is important to gain a full understanding of how Ca\(^{2+}\) signals modulate downstream inflammatory responses in human AECs. In this study, we show that CRAC channels encoded by Orai1 and STIM1 mediate a critical role in generating Ca\(^{2+}\) signals in response to PAR2 activation and regulate the production of several key inflammatory mediators in the airway epithelium, in part by activating NFAT-dependent gene expression. These results highlight CRAC channels as a key checkpoint for transducing responses from proteases and for the generation of inflammatory mediators from the airway epithelium and identify CRAC channels as a potential target for therapeutic treatment of allergic and inflammatory airway diseases.

**CRAC channels are a major route of Ca\(^{2+}\) entry in AECs**

We show that primary human AECs exhibit SOCE and store-operated currents that share the pharmacological and biophysical hallmarks of CRAC channels (37). These features include block by low concentrations of (1 to 2 \(\mu\)mol) La\(^{3+}\), modulation by 2-APB, inhibition by the CRAC channel inhibitors BTP2 and RO2959, an inwardly rectifying I-V, fast inactivation, and depotentiation of the Na\(^{+}\) CRAC current (Fig. 3). Analysis of the underlying molecular machinery further reveals that the CRAC channel proteins STIM1 and Orai1 make essential contributions to SOCE in primary human AECs (Fig. 3E–G). Both bronchial and alveolar epithelial cells exhibited STIM1-Orai1–dependent SOCE, suggesting that...
whether PAR2 activation evokes CRAC channel-mediated Ca\(^{2+}\) signaling pathway have been studied in AECs, it is unclear important sensor of allergens and endogenous proteases in the specific set of receptors stimulate CRAC channel activation in AEC lines and corroborate the emerging viewpoint that CRAC channels are a well-conserved mechanism for Ca\(^{2+}\) influx in the lower airways (21, 34, 53). Our data, however, do not rule out a role for other CRAC channel isofoms. Indeed, SOCE was not fully abolished by STIM1 and Orai1 knockdown. Moreover, STIM2 and Orai3 knockdown resulted in a modest inhibition of SOCE (Fig. 3G), raising the possibility that these proteins may be expressed and make additional contributions to SOCE in AECs. More studies using isoform-specific Abs and knockout mice would be needed to test this issue. These issues notwithstanding, our findings unequivocally show that STIM1 and Orai1 are essential for conferring SOCE in human AECs.

**CRAC channels mediate the downstream effects of PAR2 signaling**

A screen for ligands that activate SOCE revealed that only a very specific set of receptors stimulate CRAC channel activation in AECs (Table I). A prominent hit in this screen was PAR2, an important sensor of allergens and endogenous proteases in the lower airways (5, 6, 9). Although many elements of the PAR2 signaling pathway have been studied in AECs, it is unclear whether PAR2 activation evokes CRAC channel-mediated Ca\(^{2+}\) signals. In this study, we find that Ca\(^{2+}\) influx through CRAC channels encoded by STIM1 and Orai1 is needed to maintain the sustained Ca\(^{2+}\) signal that arises from PAR2 activation (Figs. 1, 2). Furthermore, we find that PAR2 activation induces production of PGE\(_2\), IL-6, IL-8, and GM-CSF in AECs in a CRAC channel-dependent manner (Fig. 5). These findings highlight a novel role for CRAC channels as a key control element in the PAR2 signaling response. It is worth noting that although PGE\(_2\) production is seen following brief activation of PAR2 receptors (2 h), IL-6, IL-8, and GM-CSF production required prolonged stimulation of PAR2 (8–24 h). Because PGE\(_2\) evokes bronchodilation through effects on the airway smooth muscle, these results are consistent with the emerging idea that acute responses to PAR2 tend to be protective largely, whereas more sustained responses to PAR2 activation result in induction of cytokines that are proinflammatory, thereby contributing to the pathogenesis of diseases like asthma (12, 54).

**CRAC channels induce the production of inflammatory mediators from AECs**

In the repertoire of inflammatory mediators secreted by AECs, several, including PGE\(_2\), TSLP, IL-6, IL-8, and GM-CSF, are thought to be induced by cytosolic Ca\(^{2+}\) elevations (7, 55, 56). However, the source of Ca\(^{2+}\) signals driving the induction of these proinflammatory mediators remains unclear. We find that CRAC channel activation induces the production of a host of inflammatory mediators from primary bronchial cells including the cytokines IL-6, TSLP, TNF-\(\alpha\), and IL-1\(\beta\), the arachidonic acid metabolite PGE\(_2\), the chemokines IL-8 and RANTES, and the growth factor GM-CSF (Fig. 4). Further examination shows that TNF-\(\alpha\), IL-6, and GM-CSF are specifically secreted only by co-stimulation of CRAC channels with PKC activation, whereas TSLP, PGE\(_2\), and IL-1\(\beta\) are maximally induced by CRAC channel stimulation alone (Fig. 4). These results indicate that cross-talk between signaling pathways can change the specific repertoire of inflammatory mediators released from AECs.

Among the inflammatory mediators that regulate airway inflammation, TSLP and PGE\(_2\) are produced primarily by the airway epithelium. TSLP plays a vital role in effecting a Th2-type airway inflammatory response that is characteristic of allergic diseases like asthma and is known to be induced by various bacterial, viral, and fungal products (1). In contrast, the arachidonic acid metabolite PGE\(_2\) has immunoprotective effects in the airway that include attenuation of bronchoconstriction in exercise or allergen-induced asthma and inhibition of lymphocytic proliferation (57, 58). In this study, we find that CRAC channel activation induces both TSLP and PGE\(_2\) in AECs but at very different time scales: PGE\(_2\) is induced in <2 h, whereas TSLP induction requires at least 48 h of AEC stimulation. These temporal differences indicate that CRAC channels regulate multiple stages of the inflammatory response in AECs, and the net consequences for airway function will depend on balance between the ensuing pro- and anti-inflammatory effector responses. Curiously, whereas CRAC channel activation produced PGE\(_2\), we saw no induction of the leukotrienes (LT\(\beta_4\), LTC\(_4\)-E\(_4\)), which are proinflammatory products of the arachidonic acid pathway (Figs. 4, 5, and data not shown). This is in contrast to findings in mast cells, where CRAC channel activation is a potent and specific trigger for the induction of LTC\(_4\) production (46). CRAC channels may thus activate different arachidonic acid metabolites and produce distinct biological responses depending on the cell type.

**CRAC channels activate NFAT-dependent gene expression in AECs**

Induction of IL-6, TNF-\(\alpha\), PGE\(_2\), and IL-8 in response to CRAC channel activation was suppressed by the calcineurin antagonist, CsA (Fig. 6), indicating that these cytokines are transcriptionally regulated by the calcineurin–NFAT pathway. This is consistent with observations that the promoter regions of a number of cytokine genes including IL-6, IL-8, and TNF-\(\alpha\) harbor NFAT

**FIGURE 8.** Model for the Ca\(^{2+}\)-dependent signaling pathway activated by PAR2. Activation of PAR2 receptors by serine proteases (trypsin) and peptide agonists (SLIGRL and SLIGKV) evokes PLC stimulation, leading to IP\(_3\) production and ER store release through the opening of IP\(_3\) receptors. Release of Ca\(^{2+}\) from intracellular stores leads to activation of STIM1, which opens CRAC channels comprised of Orai1 subunits. Ca\(^{2+}\) influx through CRAC channels activates calcineurin–NFAT signaling to transcriptionally induce IL-6 and IL-8 production, likely in conjunction with other pathways. BTP2 and RO2959, CRAC channel inhibitors; U73122, PLC\(\beta\) inhibitor.
binding sites (43) and reveals an important role for NFAT as a CRAC channel–dependent regulator of cytokine production in AECs. In line with this interpretation, PAR2-mediated induction of IL-6 and IL-8 was also significantly inhibited by blocking the NFAT signaling pathway. Interestingly, whereas CsA inhibited IL-8 production in response to both PAR2 agonists and TG/PDBu to the same extent, the induction of IL-6 by PAR2 activation was only partially inhibited by CsA. We speculate that this difference may reflect the possibility that additional NFAT–independent mechanisms activated by PAR2 signaling come into play to drive IL-6 induction. Moreover, not all inflammatory mediators activated by PAR2 signaling require NFAT gene expression (e.g., PGE2 and GM-CSF; Fig. 6A), suggesting that the induction of these mediators occurs through alternate Ca2+-regulated pathways. One possibility is that Ca2+-mediated stimulation of calcium-dependent phospholipase A2 may drive the induction of PGE2 (59), something that remains to be tested. Taken together, our results highlight a hitherto unappreciated role for NFAT as an important mediator of downstream PAR2 signaling in the airway epithelium.

Based on these results, we propose a model for PAR2 receptor signaling in the airway epithelium in which activation of these receptors, either by peptides or endogenous proteases, causes Gq PLCβ-PtdIns3,0-mediated store release, leading to the generation of long-lasting Ca2+ signals through the activation of store-operated CRAC channels. The ensuing Ca2+ influx triggers the activation of transcription factor NFAT, which then translocates to the nucleus and drives expression of IL-6 and IL-8 genes in conjunction with other signaling mechanisms (Fig. 8). CRAC channel activation thus constitutes an important regulatory checkpoint for the induction of inflammatory mediators from the airway epithelium.

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