

German cockroach extract activates protease-activated receptor 2 in human airway epithelial cells

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Background: The German cockroach has been reported to act as an allergen that might be associated with a protease reaction in asthma. However, the molecular identities of the antigens in German cockroach extract (GCE) with protease activity and the protease-activated receptors (PARs) that are activated by GCE in human airway epithelial cells have not been characterized.

Objective: We investigated the direct effect of GCE on Ca^{2+} signaling in human airway epithelial cells and the type of PARs activated by GCE.

Methods: The Ca^{2+} -sensitive dye Fura2 was used to determine intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) by means of spectrofluorometry.

Results: GCE induced a baseline type of $[\text{Ca}^{2+}]_i$ oscillations in a dose-dependent manner. The oscillations persisted for long periods of time in the absence of Ca^{2+} entry across the plasma membrane, suggesting that the observed $[\text{Ca}^{2+}]_i$ increases were due to Ca^{2+} release from intracellular stores. Accordingly, after depleting endoplasmic reticulum Ca^{2+} with thapsigargin, an endoplasmic reticulum Ca^{2+} ATPase inhibitor, the GCE-mediated $[\text{Ca}^{2+}]_i$ signals were abolished. Whereas desensitization of PAR-1, PAR-3, and PAR-4 had no effect on GCE-mediated Ca^{2+} mobilization, no GCE-mediated $[\text{Ca}^{2+}]_i$ increase was observed after desensitization of PAR-2.

Conclusions: These results indicate that GCE has a direct effect on human airway epithelial cells, in particular generating $[\text{Ca}^{2+}]_i$ oscillations through Ca^{2+} release from thapsigargin-sensitive Ca^{2+} stores through activation of PAR-2. (J Allergy Clin Immunol 2004;113:315-9.)

Key words: German cockroach, Ca^{2+} signaling, protease-activated receptor, allergen, human airway epithelial cell

Sensitization and exposure to cockroach allergens are associated with the development of asthma¹ and might be associated with increased asthma-related morbidity in

Abbreviations used

$[\text{Ca}^{2+}]_i$: Intracellular Ca^{2+} concentration
ER: Endoplasmic reticulum
GCE: German cockroach extract
GPCR: G protein-coupled receptors
IP₃: 1,4,5-Tris-inositolphosphate
PAR: Protease-activated receptor
PAR-2AP: PAR-2 agonist peptide
PMCA: Plasma membrane Ca^{2+} pump
PSS: Physiologic salt solution

inner-city areas.² The allergenicity of cockroach extract has been demonstrated in human subjects by means of skin tests, bronchial provocation tests, and RASTs.^{3,4} Immunoblot analysis identified several allergenic components in German cockroach extract (GCE) with molecular weights of 12.5 to 110 kDa,⁵ and it is now generally accepted that the major German cockroach allergens are Bla g 1 (molecular weight, 25-30 kDa) and Bla g 2 (molecular weight, 36 kDa).⁶ Subsequently, several additional allergens, such as Bla g 4 (ligand-binding protein or calycin),⁷ Bla g 5 (glutathione transferase),⁸ and Bla g 6 (tropoin),⁹ have been cloned, and their functions were studied.

Protease-activated receptors (PARs) are 7-transmembrane-spanning, G protein-coupled receptors (GPCRs) that activate Gq to release $\text{G}\alpha_q$ and $\text{G}\beta\gamma$. $\text{G}\alpha_q$ activates phospholipase C β to generate 1,4,5-tris-inositolphosphate (IP₃) in the cytosol to release Ca^{2+} from the endoplasmic reticulum (ER). Ca^{2+} release from the ER leads to activation of store-operated Ca^{2+} channels in the plasma membrane, and Ca^{2+} release and influx increase the intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$). Subsequently, the plasma membrane Ca^{2+} pump (PMCA) removes Ca^{2+} from the cytosol to reduce Ca^{2+} toward resting levels until $[\text{Ca}^{2+}]_i$ stabilizes at a plateau, the level of which depends on the relative ratio between the activity of store-operated Ca^{2+} channels and the PMCA.¹⁰

Activation of PARs involves receptor recognition by a protease and cleavage of the receptor at a specific enzymatic site located at the extracellular NH₂ terminus.¹¹ Recent reports have suggested that serine proteases derived from mite allergens, such as Der p 3 and Der p 9,¹² or cysteine protease from Der p 1¹³ induce cytosine production by airway epithelial cells and that this is par-

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tially mediated by PAR-2, resulting in a nonallergic inflammatory response. Although Bla g 2 was identified as an inactive aspartic protease on the basis of critical amino acid substitutions at the active site, serine protease activity has been suggested in crude extracts of *Blattella germanica*.^{14,15} However, it is unclear whether the antigens in GCE contain protease activity, and the identities of the PARs that interact with GCE in human airway epithelial cells are unknown. In the present work we investigated the direct effect of GCE on human airway epithelial cells in terms of Ca^{2+} signaling and attempted to identify the PAR involved in generating the $[\text{Ca}^{2+}]_i$ signals. Our work demonstrates that GCE generates $[\text{Ca}^{2+}]_i$ oscillations by means of Ca^{2+} release from the thapsigargin-sensitive Ca^{2+} stores through activation of PAR-2.

METHODS

Materials

Fura2-acetoxymethyl ester was purchased from TefLabs (Austin, Tex). Thrombin, trypsin, soybean trypsin inhibitor, and histamine were purchased from Sigma (St Louis, Mo). The PAR-2 agonist peptide SLIGRL-NH₂ was purchased from Tocris (Ellisville, Mo). Dulbecco modified Eagle medium and Ham's F-12 medium in a 1:1 ratio, BSA, penicillin, and streptomycin were from GIBCO BRL (Life Technologies, Grand Island, NY).

Cell culture

Cells from the A549 cell line were purchased from the American Type Culture Collection (Manassas, Va) and were cultured in Dulbecco modified Eagle medium and Ham's F-12 medium (1:1) containing 10% FBS and antibiotics. For dye loading, the cells were seeded on polylysine-coated cover slips at 4×10^6 cells/mL per 60-mm dish and were used at 80% confluency.

Preparation of GCE

Thirty grams of live or frozen German cockroaches was pulverized in liquid nitrogen. The defatted sample in 200 mL of a 1:1 volume of ethyl ether/ethyl acetate was extracted with slow stirring at 4°C overnight in PBS (pH 7.4) containing 6 mmol/L 2-mercaptoethanol and 1 mg/mL 1-phenyl-3-(2-thiazolyl)-2-thiourea to prevent melanization.¹⁶ The extract was then centrifuged at 10,000g for 30 minutes at 4°C, and the supernatant was finally filtered through a 0.22- μm filter. The supernatant had negative E-toxate assay results for endotoxin. Total protein in the extract was 2.78 mg/mL, as evaluated with a Bradford assay reagent (Bio-Rad, Hercules, Calif). The Bla g 1 concentration was measured by means of sandwich ELISA, and the GCE stock contained 0.63 U/mL Bla g 1.

$[\text{Ca}^{2+}]_i$ measurement

The composition of the extracellular physiologic salt solution (PSS) used in this study was as follows: NaCl, 140 mmol/L; KCl, 5 mmol/L; MgCl_2 , 1 mmol/L; CaCl_2 , 1 mmol/L; HEPES, 10 mmol/L; and glucose, 10 mmol/L titrated to pH 7.4 with NaOH. Ca^{2+} -free media was Ca^{2+} -free PSS that contained 1 mmol/L EDTA and 1 mmol/L ethyleneglycol-bis-(β -aminoethylether)-N,N,N',N'-tetraacetic acid. The osmolality of the extracellular solution (measured with a FISKE 110 osmometer), was 310 mOsm. Cells were incubated for 2 hours in PSS containing 5 $\mu\text{mol/L}$ fura2-acetoxymethyl ester in the presence of Pluronic F-127 to enhance dye loading. Changes in $[\text{Ca}^{2+}]_i$ were measured by means of fura2 fluorescence, with excitation wavelengths of 340 nm and 380 nm, respectively, and an emission wavelength of 510 nm at room temperature. Background fluorescence

was subtracted from the raw signals at each excitation wavelength before calculating the fluorescence ratio as follows:

$$\text{Ratio} = F_{340}/F_{380}.$$

The emitted fluorescence was monitored with a CCD camera (Photon Technology International Inc, Lawrenceville, NJ) attached to an inverted microscope. Fluorescence images were obtained at 4-second intervals. All data are presented as means $\pm$ SEM.

RESULTS

GCE induces $[\text{Ca}^{2+}]_i$ oscillations in a dose-dependent manner

We hypothesized that GCE might activate PARs in human epithelial cells, thus increasing $[\text{Ca}^{2+}]_i$ and, consequently, cytokine release. To characterize the Ca^{2+} signals evoked by GCE, we measured $[\text{Ca}^{2+}]_i$ at various GCE concentrations, from 20 ng/mL to 1 $\mu\text{g/mL}$, in human airway epithelial A549 cells. Treatment with GCE alone evoked $[\text{Ca}^{2+}]_i$ oscillations (Fig 1, A), with each spike lasting about 1 minute. As is well documented for other Gq-coupled receptors,¹⁷ the frequency of the oscillations was dependent on the concentration of GCE (Fig 1, B): $1.0 \pm 0.05/10^3$ seconds at 20 ng/mL ($n = 7$), $2.0 \pm 0.24/10^3$ seconds at 50 ng/mL ($n = 7$), $6.0 \pm 0.63/10^3$ seconds at 500 ng/mL ($n = 7$), and $9.2 \pm 1.72/10^3$ seconds at 1 $\mu\text{g/mL}$ ($n = 7$). However, the amplitude of the spikes was unaffected by the GCE concentration in the range of 20 ng/mL to 1 $\mu\text{g/mL}$. At concentrations of greater than 1 $\mu\text{g/mL}$, GCE induced a typical biphasic $[\text{Ca}^{2+}]_i$ increase (Fig 1, A); that is, there was a rapid transient $[\text{Ca}^{2+}]_i$ increase followed by a sustained plateau. Occasionally, a spike plateau type of response was observed at a GCE concentration of 1 $\mu\text{g/mL}$.

$[\text{Ca}^{2+}]_i$ oscillations are independent of Ca^{2+} entry

Gq-coupled agonists induce 2 types of $[\text{Ca}^{2+}]_i$ oscillations, those that are acutely dependent on Ca^{2+} influx and those that largely involve Ca^{2+} release and reuptake into the ER with minimal contribution of Ca^{2+} influx.¹⁰ To identify the type of $[\text{Ca}^{2+}]_i$ oscillations and to determine the source of the $[\text{Ca}^{2+}]_i$ increase induced by GCE, cells were treated with 1 $\mu\text{g/mL}$ GCE for 6 minutes in the presence of external Ca^{2+} and then for 6.5 minutes with nominally Ca^{2+} -free media, and finally, Ca^{2+} was added back to the medium ($n = 6$; Fig 1, C). The $[\text{Ca}^{2+}]_i$ oscillations persisted in Ca^{2+} -free media, suggesting that GCE-induced $[\text{Ca}^{2+}]_i$ oscillations are of the type independent of acute Ca^{2+} entry.

$[\text{Ca}^{2+}]_i$ increase is due to Ca^{2+} release from the thapsigargin-sensitive store

The major internal Ca^{2+} store is in the ER. This store can be selectively depleted by thapsigargin, a specific inhibitor of the ER/SR- Ca^{2+} ATPase (SERCA) pump. The cells were incubated in Ca^{2+} -free medium to prevent Ca^{2+} influx and allow depletion of the stores. Treatment with thapsigargin resulted in an initial slow Ca^{2+} increase because of inhibition of SERCA and leakage of Ca^{2+} from the ER to the cytosol, which is more typical in

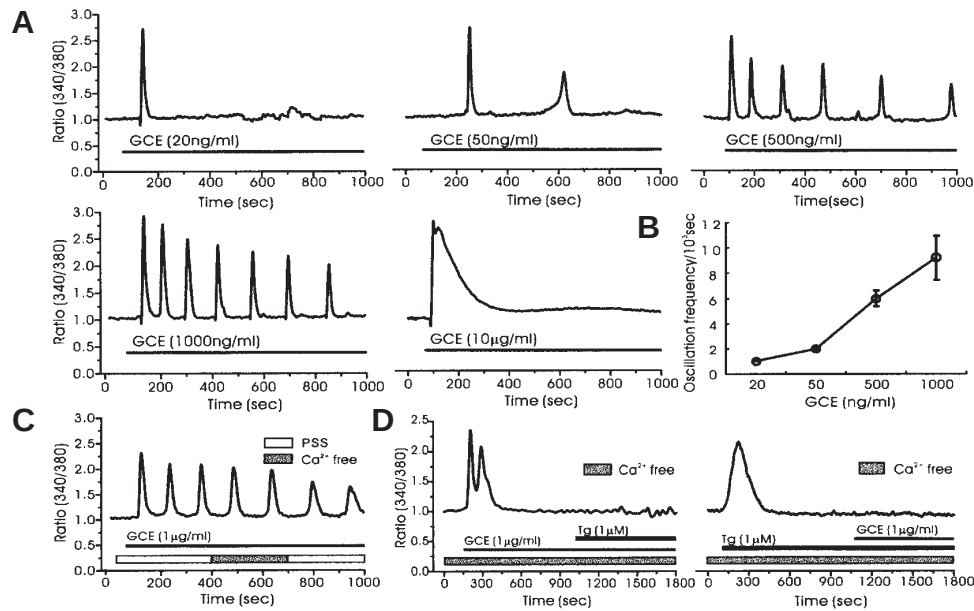


FIG 1. Characterization of GCE-mediated Ca^{2+} signaling in human airway epithelial cells: **A**, $[\text{Ca}^{2+}]_i$ oscillations induced by various concentrations of GCE ranging from 20 to 1000 ng/mL; **B**, dose-response curve of oscillation frequency; **C**, effect of extracellular Ca^{2+} on $[\text{Ca}^{2+}]_i$ oscillations; **D**, effect of 1 $\mu\text{mol/L}$ thapsigargin on GCE (1 $\mu\text{g/mL}$)-induced Ca^{2+} release in the absence of extracellular Ca^{2+} .

epithelial cells. The high $[\text{Ca}^{2+}]_i$ level activated the PMCA, which pumped Ca^{2+} out of the cells to reduce $[\text{Ca}^{2+}]_i$ to basal level. Depletion of the ER completely prevented the Ca^{2+} influx caused by GCE ($n = 6$; Fig 1, D, right trace). In a complementary experiment we found that depleting the ER by means of stimulation with 1 $\mu\text{g/mL}$ GCE in the absence of external Ca^{2+} prevented the Ca^{2+} influx caused by 1 $\mu\text{mol/L}$ thapsigargin ($n = 5$). The combined protocols in Fig 1, D, indicated that GCE increases $[\text{Ca}^{2+}]_i$ by means of Ca^{2+} release from the ER.

Activation of PAR-2 by GCE evokes $[\text{Ca}^{2+}]_i$ increases

In the next stage we set to identify the receptors activated by GCE. Recently, it was reported that GCE has serine protease activity.¹⁵ Therefore we hypothesized that GCE might cleave PARs to activate them and increase $[\text{Ca}^{2+}]_i$. Because specific antagonists for PARs are not available, we used desensitization protocols to identify the PAR mediating the GCE-evoked Ca^{2+} signals. To do so, the cells were repeatedly stimulated with 1 $\mu\text{mol/L}$ thrombin, which activates PAR-1, PAR-3, and PAR-4 but not PAR-2, or 100 nmol/L trypsin, which activates PAR-2. Fig 2, A, shows that the first stimulation with thrombin resulted in a transient increase in $[\text{Ca}^{2+}]_i$, whereas subsequent and repeated stimulation failed to increase the $[\text{Ca}^{2+}]_i$ level, which is indicative of desensitization of the PARs activated by thrombin. After the desensitization, the cells were treated with 2 $\mu\text{mol/L}$ histamine to activate the Gq-coupled H1 histamine receptor or with GCE and PAR-2 agonist peptide (PAR-2AP) to determine whether desensitization of PAR-1, PAR-3, and PAR-4 affected all Gq-coupled

signaling and activation of PAR-2. Fig 2, A, shows that desensitization of PAR-1, PAR-3, and PAR-4 had no effect on the $[\text{Ca}^{2+}]_i$ signal evoked by either histamine ($n = 4$), GCE ($n = 5$), or PAR-2AP ($n = 4$), indicating that the GCE-induced $[\text{Ca}^{2+}]_i$ increase is not mediated by PAR-1, PAR-3, or PAR-4. In contrast, Fig 2, B, shows that after desensitization of all PARs, including PAR-2, by treatment with trypsin, histamine still increased $[\text{Ca}^{2+}]_i$ (left panel, $n = 4$), but GCE did not (right panel, $n = 7$).

In additional protocols we desensitized the GCE-activated receptors by means of repeated stimulation with 2 $\mu\text{g/mL}$ GCE (Fig 2, C). This had no effect on the Ca^{2+} signal evoked by histamine but abolished the response to the supermaximal concentration (300 $\mu\text{mol/L}$) of a specific PAR-2AP ($n = 5$, respectively). Finally, Fig 2, D, shows that the 1 $\mu\text{mol/L}$ serine protease inhibitor, soybean trypsin inhibitor, reversibly inhibited the response to both trypsin and GCE ($n = 4$, respectively). Thus together, the results in Fig 2 identify PAR-2 as the receptor activated by GCE.

DISCUSSION

In the present work we found that GCE had a novel effect on airway epithelial cell lines. Specifically, GCE induced $[\text{Ca}^{2+}]_i$ increase in the form of baseline $[\text{Ca}^{2+}]_i$ oscillations (Fig 1, A). $[\text{Ca}^{2+}]_i$ oscillations are a physiologic phenomenon that mediate many cellular responses, such as fertilization, muscle contraction, synaptic transmission, fluid and electrolyte secretion, learning and memory, and gene transcription.¹⁷ At physiologic concentrations, most agonists that activate GPCRs are able

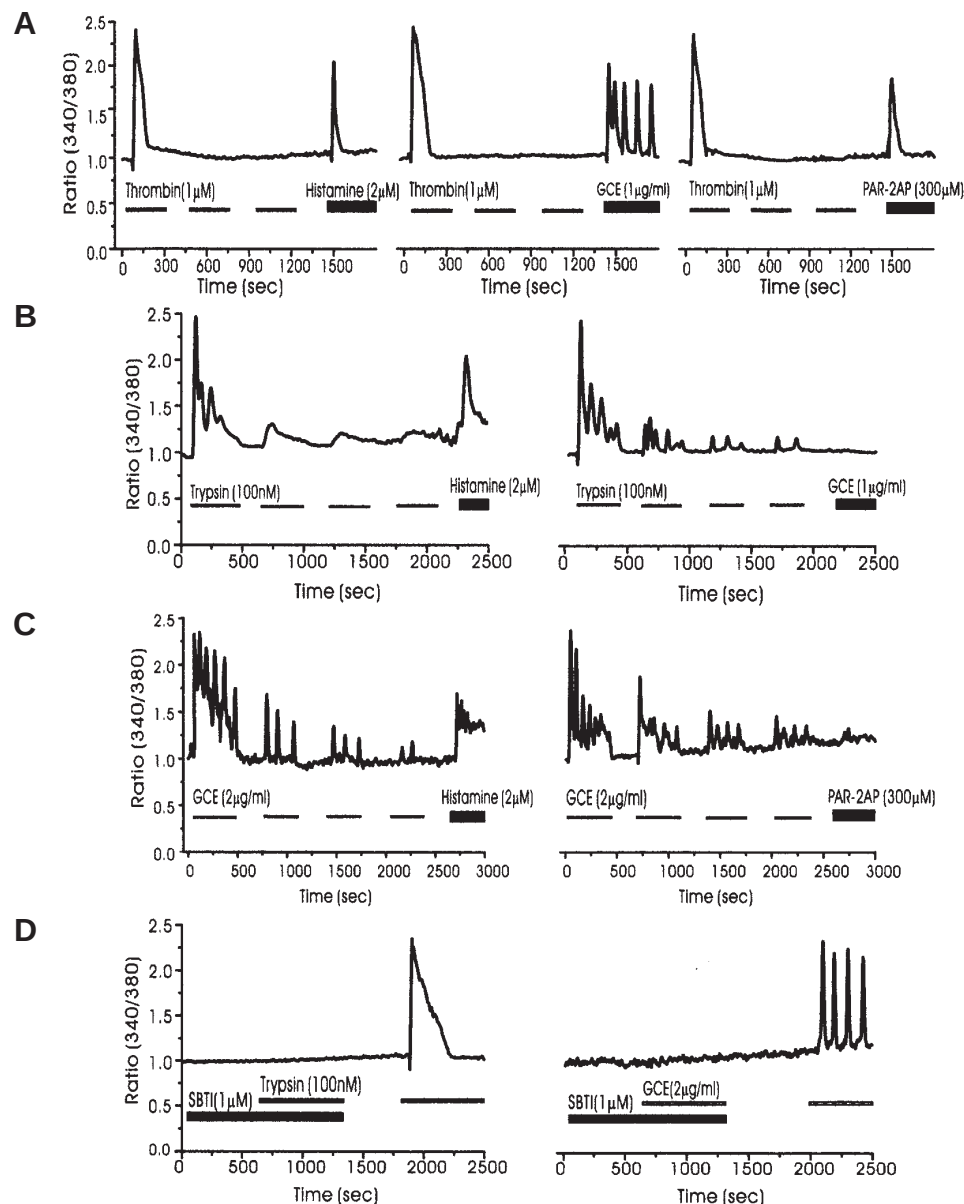


FIG 2. Effect of PAR desensitization on Ca^{2+} mobilization by GCE. **A**, After desensitization by means of repeated stimulation with 1 μ M/L thrombin, cells were treated with 2 μ M/L histamine, 1 μ g/mL GCE, or 300 μ M/L PAR-2AP. **B**, After desensitization with 100 nmol/L trypsin, cells were treated with 2 μ M/L histamine or 1 μ g/mL GCE. **C**, After desensitization with 2 μ g/mL GCE, cells were treated with 2 μ M/L histamine or 300 μ M/L PAR-2AP. **D**, In the presence of 1 μ M/L serine protease inhibitor soybean trypsin inhibitor, neither trypsin nor GCE increased $[Ca^{2+}]_i$ levels.

to induce $[Ca^{2+}]_i$ oscillations, and at greater than physiologic concentrations, they usually induce the typical biphasic $[Ca^{2+}]_i$ increase of a spike and plateau. We found that GCE at the concentration range of 20 ng/mL to 1 μ g/mL triggers $[Ca^{2+}]_i$ oscillations, and at concentrations of greater than 2 μ g/mL, GCE induces a biphasic $[Ca^{2+}]_i$ increase in airway epithelial cells. Recently, the allergens of the house dust mite, Der p 3 and Der p 9, were reported to evoke a $[Ca^{2+}]_i$ increase after an increase in intracellular concentration of IP_3 because of

activation of phospholipase C β .¹² However, these $[Ca^{2+}]_i$ increases were biphasic rather than oscillatory, as we found with the same A549 cells. Thus in the present study GCE induced $[Ca^{2+}]_i$ oscillations, showing repetitive spikes from a constant basal level, which has not been reported previously in human airway epithelial cells. Among other epithelial cells, pancreatic acini showed a similar pattern of $[Ca^{2+}]_i$ oscillations when induced by cholecystokinin, an agonist that stimulates the release of pancreatic enzymes.¹⁸

GCE induced $[Ca^{2+}]_i$ oscillations in a dose-dependent manner (Fig 1, B). Oscillation frequency plays a key role in the transduction of various signals.¹⁹ Our work demonstrates that increasing concentrations of GCE increase the frequency of Ca^{2+} oscillations, with no measurable effect on their amplitude. This behavior can be best explained by the quantal properties of Ca^{2+} release,¹⁸ with increasing concentrations of IP_3 reducing the threshold for initiation of Ca^{2+} release.

The most notable finding of the present study is that GCE specifically activates PAR-2 in human airway epithelial cells. Using desensitization protocols, we demonstrated that GCE activated PAR-2 but not PAR-1, PAR-3, or PAR-4. Hence thrombin completely desensitized all PARs except PAR-2; however, histamine, a ligand for the GPCRs linked to Gq or GCE, still release Ca^{2+} . By contrast, after desensitization with trypsin, stimulation with GCE did not cause a $[Ca^{2+}]_i$ increase, suggesting that GCE activates exclusively PAR-2. Recently, Pomes et al¹⁴ found that the cockroach allergen Bla g 2 has an aspartic protease activity. Moreover, Bhat et al¹⁵ reported that GCE contains serine protease activity and promotes TNF- α -induced IL-8 expression. Taken together, our results indicate that GCE has a novel protease activity, which specifically cleaves the extracellular domain of PAR-2 that is linked to phospholipase C β to eventually mobilize Ca^{2+} . It will be of particular interest to identify the molecular identity of the protease activity in the near future.

The $[Ca^{2+}]_i$ oscillations evoked by GCE depend on Ca^{2+} release from intracellular thapsigargin-sensitive stores (Fig 1), a conclusion similar to that reported before for activation of PAR-2.¹⁷ Little is known about the exact physiologic role of $[Ca^{2+}]_i$ oscillations in human airway epithelial cells. However, changes in $[Ca^{2+}]_i$ are intimately involved in various stress responses and the inflammatory response through activation of IL-8 production and the nuclear factor κB (NF- κB) and the ERK pathways.^{20,21} It is therefore likely that the GCE-induced $[Ca^{2+}]_i$ responses activate similar pathways to regulate the release of pro-inflammatory cytokines and trigger the immune response seen in GCE-related asthma. If this can be shown to be the case, development and use of anti-PAR-2 ligands might provide new modalities for the treatment of GCE-related immune responses, complications, or both.

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