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Epithelial calcium-sensing receptor activation by eosinophil granule protein analog stimulates collagen matrix contraction

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Abstract

Background—Eosinophils reside in normal gastrointestinal tracts and increase in disease. Receptors for eosinophil derived granule proteins (EDGPs) have not been identified but highly cationic molecules, similar to eosinophil proteins, bind extracellular calcium-sensing receptors (CaSR). We hypothesized that stimulation of CaSR by eosinophil proteins activates epithelial cells.

Methods—Caco2 intestinal epithelial cells, AML14.3D10 eosinophils, wild type human embryonic kidney 293 (HEK293) cells not expressing CaSR (HEK-WT) or CaSR transfected HEK293 cells (HEK-CaSR) were stimulated with an eosinophil protein analog poly-L-arginine (PA) and phosphorylated extracellular signal-regulated kinases 1/2 (pERK) was measured. Functional activation was measured with collagen lattice contraction assays.

Results—Co-culture of Caco2 cells with AML14.3D10 eosinophils augmented lattice contraction compared to lattices containing Caco2 cells alone. PA stimulation of Caco2 lattices augmented contraction. HEK-CaSR stimulation with PA or Ca^{2+} resulted in greater pERK activation than stimulated HEK-WT cells. PA stimulated greater HEK-CaSR lattice contraction than unstimulated lattices. Contraction of PA stimulated and unstimulated HEK-WT lattices did not differ.

Conclusion—Exposure of intestinal epithelia to the EDGP analog, PA, stimulates CaSR dependent ERK phosphorylation and epithelial mediated collagen lattice contraction. We

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speculate that EDGP release within the epithelial layers activates the CaSR receptor leading to matrix contraction and tissue fibrosis.

INTRODUCTION

Tissue remodeling can be characterized by a number of different mucosal responses including changes in barrier function, mucus secretion, muscle contraction and collagen deposition. In these regards, eosinophils may play a key role in the remodeling process as they have been shown to diminish epithelial barrier function, increase mucus secretion and stimulate contraction and collagen deposition (1,2). Eosinophils have been implicated in tissue remodeling in the lung where they are increased in asthma and eosinophil granule proteins have elicited increased expression of pro-fibrotic, transforming growth factor- β (TGF-β), in lung fibroblasts in vitro (3). With respect to the gastrointestinal tract, the relative contribution and mechanistic role of eosinophils and eosinophil derived granule proteins (EDGPs) in tissue remodeling is not certain. Tissue fibrosis, which is one important aspect of tissue remodeling, is characterized by excessive deposition and contraction of the extracellular matrix (ECM) (4). An *in vitro* model, the collagen lattice contraction assay, offers an easily controlled and flexible environment to study factors contributing to matrix contraction (5). While muscle fiber contraction and fibroblast activity has been thought to account for much of this activity *in vivo*, contraction of non-muscle cells, such as epithelia, also occurs in remodeling and epithelial cells also interact with the ECM, but these events are less well defined.

The extracellular calcium-sensing receptor (CaSR) is a G-protein coupled receptor (GPCR), which is a member of the C family of GPCRs. CaSR is expressed on extracellular Ca2+-regulating cells such as parathyroid, renal tubular and bone cells (6). CaSR is also expressed throughout the gastrointestinal system, in basal cells of the esophagus (7), gastric G and parietal cells (8–10), I cells of the duodenum (11) and surface and crypt epithelia of the colon (12). CaSR activation is known to increase intracellular MAP kinase activity including ERK activation (6). Increased ERK activation has been associated with overexpression of profibrotic genes in dermal fibroblasts (13,14), and MAPK inhibitors have been shown to inhibit both fibroblast populated collagen lattice contraction and wound contraction in an *in vivo* rat model (15). It is currently understood that CaSR is a multimodal sensor of multivalent cations such as poly-L-Arginine (PA), polyamines, L-amino acids and pH in addition to ionized Ca^{2+}. Previously we have reported that CaSR activation by an EDGP, major basic protein (MBP), stimulated fibroblast growth factor-9 (FGF9) expression and secretion from an esophageal cell line (16). The aim of the current experiments was to determine if poly-L-Arginine (PA), a previously used

RESULTS

AML14.3D10 eosinophils and eosinophil derived granule protein analog, poly-L-arginine, augment epithelial mediated collagen lattice contraction

Although eosinophils are known to reside adjacent to the gastrointestinal epithelium, their potential role in remodeling of the extracellular matrix via epithelial cells has not been investigated. To address this, we hypothesized that EDGPs stimulate gastrointestinal tissue

contraction and thus measured the impact of eosinophils on epithelial populated collagen lattice contraction. Co-culture of Caco2 cells with AML14.3D10 eosinophils (3D10 eosinophils) led to significant contraction compared to Caco2 cells or 3D10 eosinophils alone as shown in Figure 1 (43.0±1.0% vs. 34.3 ±2.2%, $p<0.02$; Caco2 with eosinophils vs. Caco2 alone). We next used the highly charged MBP analog, PA, in the collagen lattice contraction assay. PA (0.5 μM and 1μM) stimulated a concentration and time dependent contraction of Caco2 populated lattices that was significantly augmented compared to unstimulated controls (Figure 2A and 2B) (46.4±1.6 vs. 36.7±1.3, $p<0.001$; PA vs. media alone). Cell viability of Caco2 and AML14.3D10 cells was assured by trypan blue staining (96%+/− 2% viability in co-cultured, PA stimulated and unstimulated cells).

CaSR expression

Monomeric CaSR is present in the stably CaSR-transfected HEK cells (HEK-CaSR) and the Caco2 cells, but absent in the late passage (p11) HT-29 cells (Supplemental Figure 1 (online)). Under non-reducing conditions higher molecular weight CaSR protein (~250 kDa) was also found in both the HEK-CaSR cells and the Caco2 cells but also absent in late passage HT-29 cells (data not shown).

Eosinophil derived granule protein analog activates epithelial cells

Given these findings we were interested in determining how the EDGPs may be activating intestinal epithelial cells. To date, receptors for EDGPs have not been identified. Knowing that EDGPs and especially the MBP analog, PA, are highly charged cationic proteins, we examined the impact of PA on the CaSR that is known to be expressed on Caco2 cells (17). Phosphorylated extracellular signal-regulated kinase (pERK) was measured to assess for cell activation in response to PA stimulation. Western blot analysis revealed that PA activated pERK at 5 minutes, an effect that was sustained to 1 hour as seen in Figure 3 A and B.

ERK activation by eosinophil granule protein analog is CaSR dependent

We next examined whether CaSR was necessary for PA activation of epithelial cells. Wild type HEK cells, (HEK-WT) which do not express CaSR, were transiently transfected with full length CaSR (HEK-T) (Figure 4A). Stimulation of HEK-T cells with PA, led to pERK activation compared to HEK-WT cells with calcium used as a CaSR positive control and EGF activation as a non-CaSR control (Figure 4B). PA stimulation led to robust ERK signal by 5 minutes as determined by qualitative Western blot analysis (Figure 4C). ERK activation was also evaluated using quantitative chemiluminescent Meso Scale technology (Meso Scale Diagnostics, Gaithersburg, MD). Both PA (1μM) and calcium (20mM) stimulation led to an increase in ERK activation in stably transfected HEK-CaSR cells (Figure 5) compared to HEK-WT cells (fold increase stimulated pERK over unstimulated control: 1.2±0.45 vs. 7.9±1.1, $p<0.01$, HEK-WT vs. HEK-CaSR (PA); 2.0±0.57 vs. 8.9±1.9, $p<0.01$, HEK-WT vs. HEK-CaSR (Ca)) (Figure 5). Both the HEK-WT and HEK-CaSR cells display similar 8–10 fold increases in pERK with EGF stimulation (8.4±0.9 vs. 9.6±2.5, HEK-WT and HEK-CaSR respectively, $p =0.48$), (Figure 5). The same pattern of ERK activation was seen in HEK-T cells and controls (data not shown).

Gain of CaSR function augments cell-mediated collagen lattice contraction in response to granule protein analog stimulation

We next determined if gain of CaSR ligation resulted in a functional impact. PA stimulation (1 μM) significantly increased contraction of collagen lattices populated with HEK-CaSR cells compared to both unstimulated HEK-CaSR lattices and PA stimulated HEK-WT lattices at 24 hours (percent initial surface area; 58.8%±2.9 vs. 71.0%±3.9, PA stimulated HEK-CaSR vs. unstimulated HEK-CaSR lattices respectively, $p<0.01$, and 58.8%±2.9 vs. 71.8%±2.0, PA stimulated HEK-CaSR vs. PA stimulated HEK-WT lattices respectively, $p<0.01$) (Figure 6). PA stimulation did not significantly affect collagen lattice contraction in HEK-WT populated collagen lattices compared to HEK-WT unstimulated control (CTL) lattices (71.8%±2.0 vs. 69.6%±1.0, PA vs. CTL respectively, NS).

DISCUSSION

Eosinophils are present in the gastrointestinal mucosa and can reside directly adjacent to the basolateral surface of epithelial cells (18). During inflammatory states, eosinophils secrete EDGPs into tissue spaces (19). In studies examining pulmonary and gastrointestinal inflammation, eosinophils have been shown to participate in the remodeling process. *In vitro* studies support a role of eosinophils in remodeling as they can stimulate mast cell histamine release, fibroblast cytokine release and muscle contraction. While EDGPs are associated with these roles, no cell surface receptors have been identified and molecular mechanisms have not been fully defined. Here we show that PA, an analog of eosinophil derived MBP, stimulates CaSR dependent activation of the epithelium and epithelial mediated collagen lattice contraction. This work suggests that the EDGP, MBP, may exert biological activity via CaSR.

Mucosal tissues are composed of a variety of molecular and cellular components. In a number of organ systems, extracellular matrices can be remodeled by resident and recruited cells, such as eosinophils, to form scar tissue and to contract. While lung epithelial cells have previously been shown to contract collagen lattices and appear to play a role in tissue remodeling, the role of gastrointestinal epithelial cells in tissue remodeling is less well defined (20). As we have previously described, the collagen lattice contraction assay was used here to provide a functional readout of the remodeling process as it pertains to epithelial-eosinophil interactions (5).

Whereas several studies identified EDGP's cytotoxicity, a process thought to be related to increasing cell membrane permeability (21), little is known about how EDGPs may activate resident and recruited cells in mucosal surfaces in a non-toxic fashion (22). Since CaSR is expressed on the intestinal epithelia along the length of the intestinal tract and is ligated by highly charged molecules, we wondered whether this receptor could provide a link by which epithelial cells sense the extracellular milieu. Results presented here support a dynamic interaction between PA and epithelial cells in which the EDGP analog stimulates robust ERK activation and functional response related to collagen lattice contraction.

Previously we have shown that knockdown of the CaSR in esophageal epithelial cells prevented the stimulation of FGF-9 secretion by an MBP peptide (16). Loss of CaSR

expression by transient transfection with siRNA duplex has also been shown to prevent Ca^{2+} stimulated inhibition of defective Wnt/β-catenin signaling in colon cancer cells (23) and stimulation of bone morphogenetic protein-2 (BMP-2) and Wnt5a from colonic myofibroblasts (24). In the current experiments we have relied on CaSR-transfected HEK cells to examine these responses and compared their response to added calcium in untransfected HEK cells that do not express the CaSR. Usually the HEK-CaSR cells respond maximally with either hormone secretion (25) or intracellular Ca2+ transients at 3–10 mM (26). The two fold increase in Ca2+ required for maximal effect here likely represents reduced expression of the CaSR from lowered transfection efficiency. It is also possible that mutations of the extracellular domain of the CaSR increased the Ca2+ concentration required to activate the receptor, as has been previously reported (27). Our results comparing calcium addition to HEK cells that did not express the CaSR with those with some CaSR expression show very little activation occurred without CaSR expression. Additionally, significant augmentation of lattice contraction with PA stimulation only occurred in cells with CaSR expression. This strongly suggests that the CaSR mediated the activation and contraction responses seen in the HEK-CaSR cells.

While this study provides evidence to support the role of MBP as a ligand for the CaSR, several issues remain to be addressed. First, our previous study exposed an intact epithelial monolayer to PA whereas here epithelial cells are dispersed throughout the collagen lattice (1). Interactions between epithelial cells, collagen and other matrix proteins likely influence their response to PA in a different fashion. Second, direct biochemical evidence of CaSR ligation by EDGPs has not yet been demonstrated. Third, the observed results could be explained by the cationic charge associated with PA and not a specific feature of the EDGP, MBP. Finally, the exact distribution of CaSR protein on epithelial cell lines requires definition. Here we demonstrated the presence of CaSR on Caco2 cells by Western blots using HEK-CaSR cells as a positive control. Consistent with this finding was our previous study showing that HT-29 cells express CaSR protein, which was reduced by RNAi technique (28). As others have reported that the CaSR promoter is epigenetically silenced in several adenocarcinoma cell lines (29) it was important to confirm that the CaSR was present in the Caco2 cells. It is noteworthy that detectable but faint CaSR transcript is present in both Caco2 and HT-29 cells (29), and the current experiments demonstrate CaSR protein in the Caco2 cells. It is likely that with passage the CaSR in the HT29 cells becomes methylated and its expression is lost with increased passage. Indeed, our earlier work showing CaSR protein and its knockdown used cells only from p1-6 (28). Caco2 cells differentiate with confluence, and it has been observed with confluence CaSR expression in Caco2 cells increases (unpublished results), it is likely that selection of clones that express various levels of the CaSR occurs during passage in this cell line. Nevertheless in the current experiments the Caco2 cells expressed the CaSR and it is likely that proteins secreted from the eosinophil cell line were acting as agonist(s) of the CaSR to stimulate wound closure.

In conclusion, our results show that exposure of intestinal epithelia to the EDGP analog, PA, stimulates CaSR dependent ERK phosphorylation and epithelial mediated collagen lattice contraction. We believe that further work is needed to determine whether eosinophil release of MBP activates the CaSR receptor leading to matrix contraction and tissue fibrosis.

METHODS

Cell culture

All cell incubations were performed at 37°C in a humidified incubator with 5% CO2. Caco2 intestinal epithelial cells and human embryonic kidney cells (HEK293) were obtained from American Type Culture Collection (ATCC, Manassas, VA). Caco2 and HEK293 cells lines were maintained in T75 flasks using DMEM medium (Fisher, Pittsburgh, PA) supplemented with 10% fetal bovine serum (FBS) and 100 U/ml penicillin and 100μ/ml streptomycin (Gibco, Grand Island, NY). AML14.3D10 cells (3D10 cells) were generously provided by Cassandra Paul (Wright State University, Dayton, OH) and were maintained in RPMI 1640 medium supplemented with 10% FBS (2 mM L-glutamine, 1 mM sodium pyruvate), 5×10^{-5}M 2-mercaptoethanol (2–ME), 100U/ml penicillin, and 100μ/ml streptomycin (Gibco, Grand Island, NY) as previously reported (1). HEK293 cells stably transfected with CaSR (HEK-CaSR) were grown and maintained under the same conditions as HEK293 wild type cells (HEK-WT). Cells were passaged weekly at subconfluence by trypsinization (0.25%, 1mM EDTA).

CaSR Western Analysis

Early passage, stably transfected HEK-CaSR cells, late passage (p 11) HT-29 adenocarcinoma cells and early passage Caco2 adenocarcinoma cells were processed for protein analysis. In brief, all cells grown on 6 well plates were washed once with ice-cold PBS containing 1 mM sodium orthovanadate and 25 mM NaF, then 100 μL of ice-cold lysis buffer was added (20 mM Tris.HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 25 mM NaF, 1% Triton X-100, 10% glycerol, 1 mM dithiothreitol (DTT), 1 mM sodium vanadate, 50 mM glycerophosphate and 10 μg/ml of aprotinin, leupeptin, calpain inhibitior and Pefabloc (100 μg/ml), the latter being freshly made. After being sonicated for 5 s, lysates were centrifuged at 10,000 g for 10 min at 4°C. Protein concentrations were assessed (HEK-CaSR, HT-29 cells 100 μg; Caco2, 200 μg). Each aliquot was mixed with 2X-SDS-Laemmli gel loading buffer containing 100 mM DTT and denatured at 65°C for 30 min. Proteins were resolved electrophoretically on a 6.5% acrylamide gel in running buffer, and transferred to Immobilon membrane. Membranes were first blocked for 1hr at RT in PBS containing 0.25% TritonX-100 and 5% dry milk, then incubated with anti-CaSR antibody (Abnova) at 1:400 in PBS with 0.25% TritonX-100 and 1% dry milk overnight at 4°C. Blots were washed 5 times for 10 min at RT with PBS with 1% TritonX-100 and 0.15% dry milk, then incubated for 1h with a secondary antibody conjugated with horseradish peroxidase (1:2000) in blocking solution. Blots were washed a second time (5× 10 min) and bands visualized by chemiluminescence according to the manufacturer's instructions (SuperSignal: Pierce Biotechnology, Rockford, IL). Blots were stripped and re-probed with anti-β-actin (Sigma Chemical, St. Louis, MO) at a dilution of 1:500.

Cell stimulation and protein analysis

Adherent Caco2, HEK-WT and wild type HEK cells stably (HEK-CaSR) or transiently transfected (HEK-T) with CaSR were stimulated by adding PA, M_r 5,000–15,000 (Sigma Chemical), calcium or human recombinant epidermal growth factor (EGF) (Gibco) directly to the media overlying cells in a 6 well plate for indicated times. Cells were then lysed and

immunoblotted with phosphorylated extracellular signal-regulated kinase 1/2 (ERK1/2) antibody (Cell Signaling, Beverly, MA) as above. Prior to stimulation, cells were deprived of calcium and serum using high glucose Dulbecco's Modified Eagle Medium without pyridoxine, L-glutamine, or sodium pyruvate (Gibco) and then supplemented with 0.5mM Ca and 1% FBS for 12 hours. Total and phosphorylated ERK 1/2 from cell lysates were quantified using the chemiluminescent Meso Scale Discovery phospho (T/Y: 202/204; 185/187)/total ERK 1/2 Multi-Spot Assay System (Meso Scale Diagnostics, Gaithersburg, MD). Percent ERK 1/2 phosphorylation was calculated according to the manufacturer's instructions.

Collagen lattice contraction assay

Cell populated collagen lattices were prepared using previously reported methods (5). Briefly, collagen solution (3mg/ml) was made by solubilizing type I rat tail collagen (Sigma, St. Louis, MO) in 0.1% acetic acid. AML14.3D10 cells in suspension and trypsinized, suspended HEK and Caco2 cells were mixed as described with 3mg/ml collagen solution and a titrated volume of NaOH to produce a solidified cell populated collagen lattice with neutral pH and cell concentrations of 5×10^5 cells/ml. 0.5ml lattices were poured into wells of a 24 well plate (Costar; Corning Inc., Corning, NY) to solidify. After 30 minutes 0.5ml of culture medium was laid over the lattices, and then lattices were freed from the well walls with a pipet tip. Images were acquired at specific time points following lattice solidification with a digital camera at a fixed distance from the lattice and surface area was calculated using ImageJ software v. 1.32 (http://rsb.info.nih.gov/ij/; National Institutes of Health, Bethesda, MD). Results are reported as a percent of initial lattice surface area or as a percent contraction (100 – percent initial surface area). Lattices were stimulated with PA M_r 5,000–15,000 (Sigma) by using prepared medium with indicated concentration of PA to resuspend cells and in media overlaying lattices.

Transient transfection

HEK293 cells were transiently transfected with full length CaSR cDNA clone in pCMV6-XL4 vector or vector alone (Origene Technologies, Rockville MD) using Polyfect according to the manufacturer's instructions (QIAGEN, Valencia, CA).

Assessment of gene-specific mRNA

Transcriptional analysis of HEK cells for expression of human CaSR was assessed in RNA from monolayers of HEK cells. RT-PCR analysis of mRNA was performed from total RNA as described previously (30) now using intron spanning primers specific for CaSR (forward primer 5'-TAT AGC TGC TGG GTC CT-3' and reverse primer 5'-GCT GGG CTG TTT ATC TC-3', 248 bp fragment) or control β-actin (forward primer 5'-GGA GAA AAT CTG GCA CCA CAC C-3' and reverse primer 5'-CCA CAT CAG CTG GAA GGT GG-3', 133 bp fragment). Each primer was amplified using 35 cycles of PCR at 94°C for 30 s, 58°C for 45s, and 72°C for 1 min with a final extension of 72°C for 7 min. PCRs were then visualized on a 1.5% agarose gel containing 5μg/ml ethidium bromide.

Data analysis

Data are summarized as mean ± SD. Statistical analyses of results were performed by using unpaired Student's t-tests. $p<0.05$ was considered to be significant.\

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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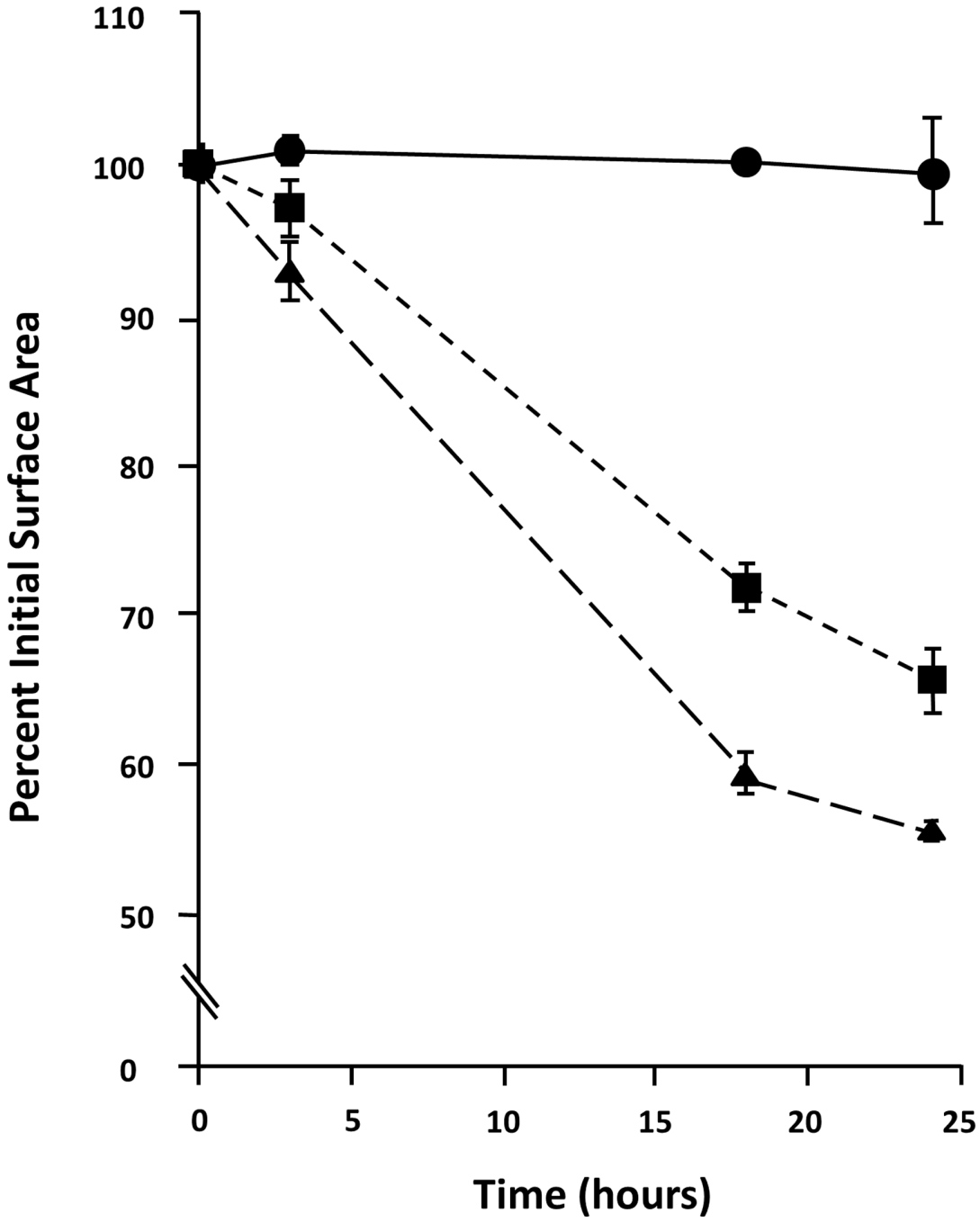

Figure 1.

Eosinophil-epithelial co-culture results in increased collagen lattice contraction

Contraction of collagen lattices populated with 3D10 eosinophils and Caco2 cells over time. 3D10 eosinophils (5×10^5 cells/ml) and Caco2 cells (5×10^5 cells/ml) were suspended in collagen lattices either alone or in co-culture. Lattice surface area was measured at indicated time points. Contraction is reported as mean percent initial surface area ±SD. Lattice contraction was augmented when Caco2 cells were suspended with 3D10 eosinophils (triangle) compared to Caco2 cells alone (square). Lattices with 3D10 eosinophils alone

(circle) and acellular collagen lattices (not shown) did not contract ($p<0.02$ Caco2 with eosinophils vs. Caco2).

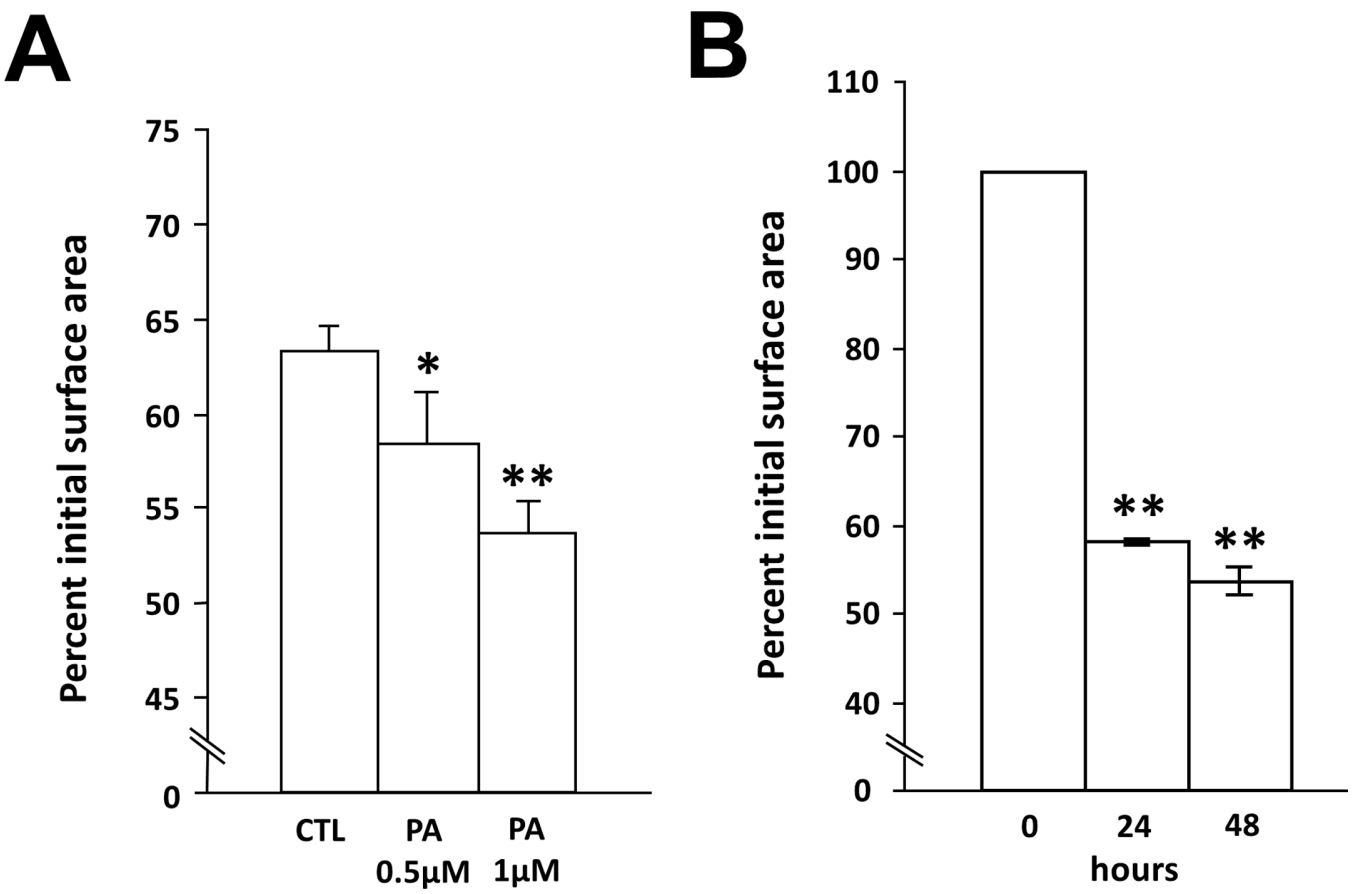

Figure 2.

Eosinophil granule protein analog stimulation of epithelial cells results in increased collagen lattice contraction. (**A**) Contraction of Caco2 populated collagen lattices exposed to 0.5μM and 1μM poly-L-arginine (PA) or media alone (CTL). Lattice surface area was measured at 48 hours. PA treated lattices display concentration dependent augmentation in contraction compared to untreated lattices (*p=0.01, **p<0.01). (**B**) Contraction of Caco2 populated collagen lattices exposed to 1μM PA over time (initial time point vs. 24 and 48 hours **p<0.01). Percent initial surface areas at 0, 24 and 48 hours following lattice solidification are displayed. All values represent mean percent initial surface area ±SD of five lattices from one of two representative experiments.

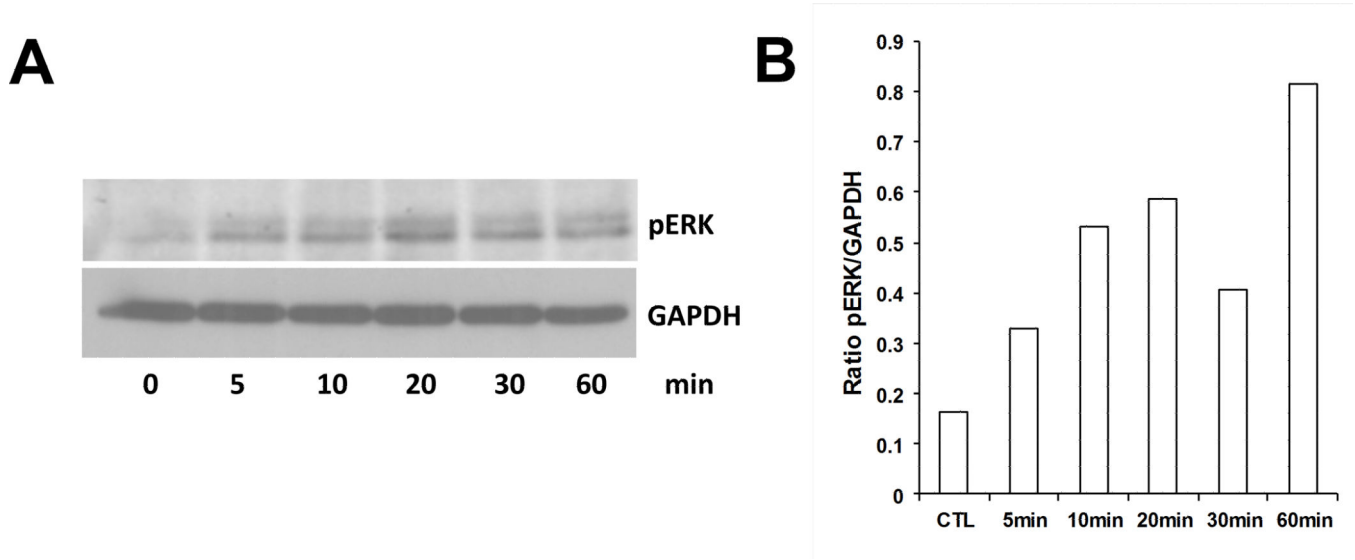

Figure 3.

Eosinophil granule protein analog stimulates epithelial ERK phosphorylation

Influence of poly-L-arginine (PA) on ERK 1/2 phosphorylation in Caco2 epithelial cells. (**A**) Caco2 cells were stimulated with PA (1μM) for indicated times. Cell lysates were harvested and western blot performed for phosphorylated ERK 1/2 (pERK). GAPDH indicates equivalent protein loading. (**B**) Densitometry analysis displaying ratio pERK/GAPDH.

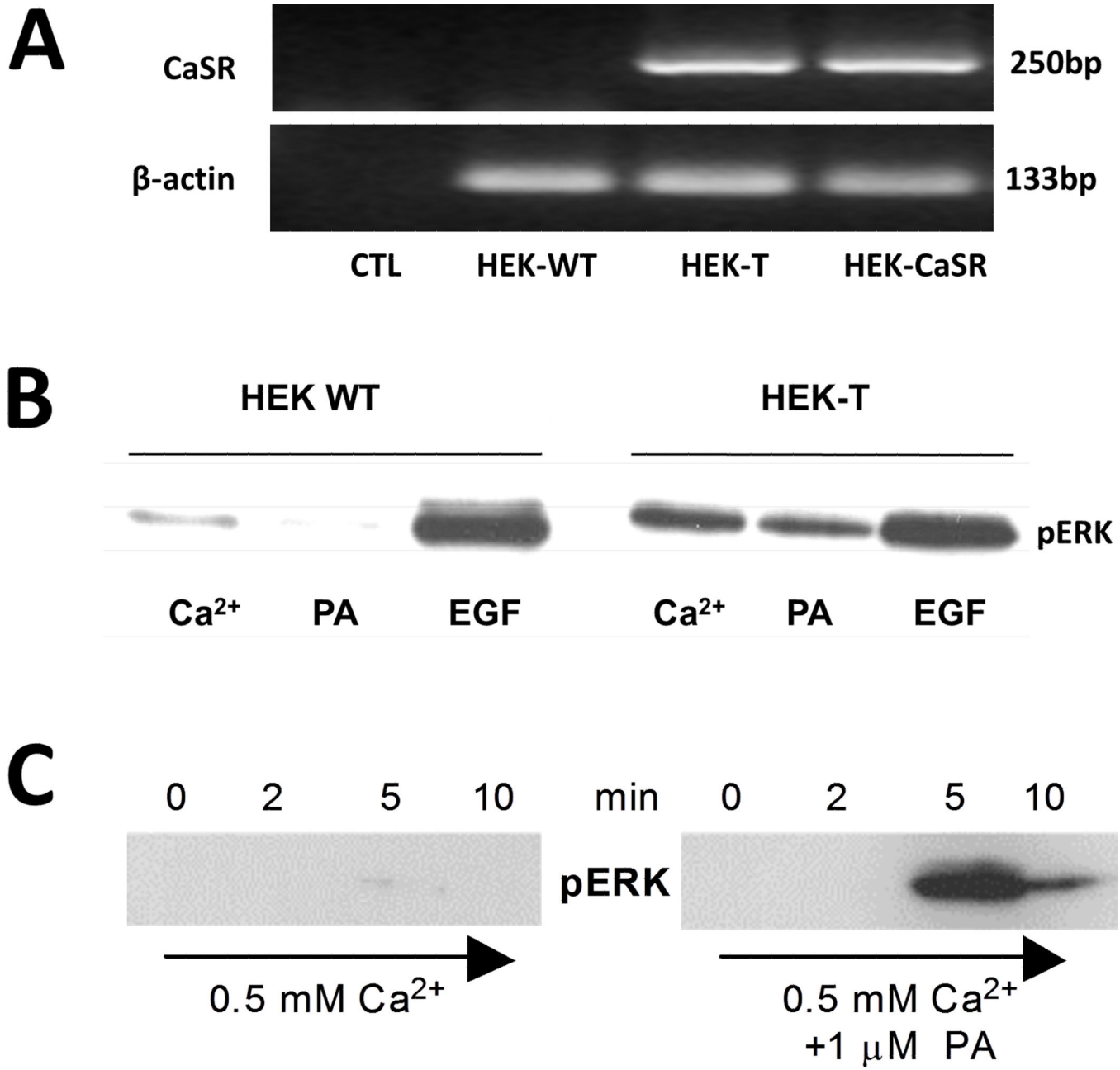

Figure 4.

Epithelial ERK phosphorylation by PA is CaSR dependent

Influence of Ca, PA and EGF on wild type and CaSR transfected HEK cells. (**A**) mRNA was harvested and RT-PCR for CaSR and β-actin performed on wild type HEK cells (HEK-WT), transiently CaSR transfected HEK cells (HEK-T), and HEK cells stably transfected with CaSR (HEK-CaSR). CTL represents negative control without mRNA. (**B**) Wild type HEK cells (HEK-WT) and CaSR transiently transfected HEK cells (HEK-T) (Origene, Rockville, MD) were exposed to Ca (20mM), PA (1μM) or EGF (100ng/ml) for 5 minutes and western blot on cell lysates was performed for pERK. In CaSR transiently transfected HEK cells (HEK-T) both 1μM PA and 20mM calcium induce ERK activation along with

100ng/ml EGF. (**C**) Time course of ERK phosphorylation (pERK) with PA (1μM) stimulation and media control in a HEK-CaSR cell line that stably expresses CaSR.

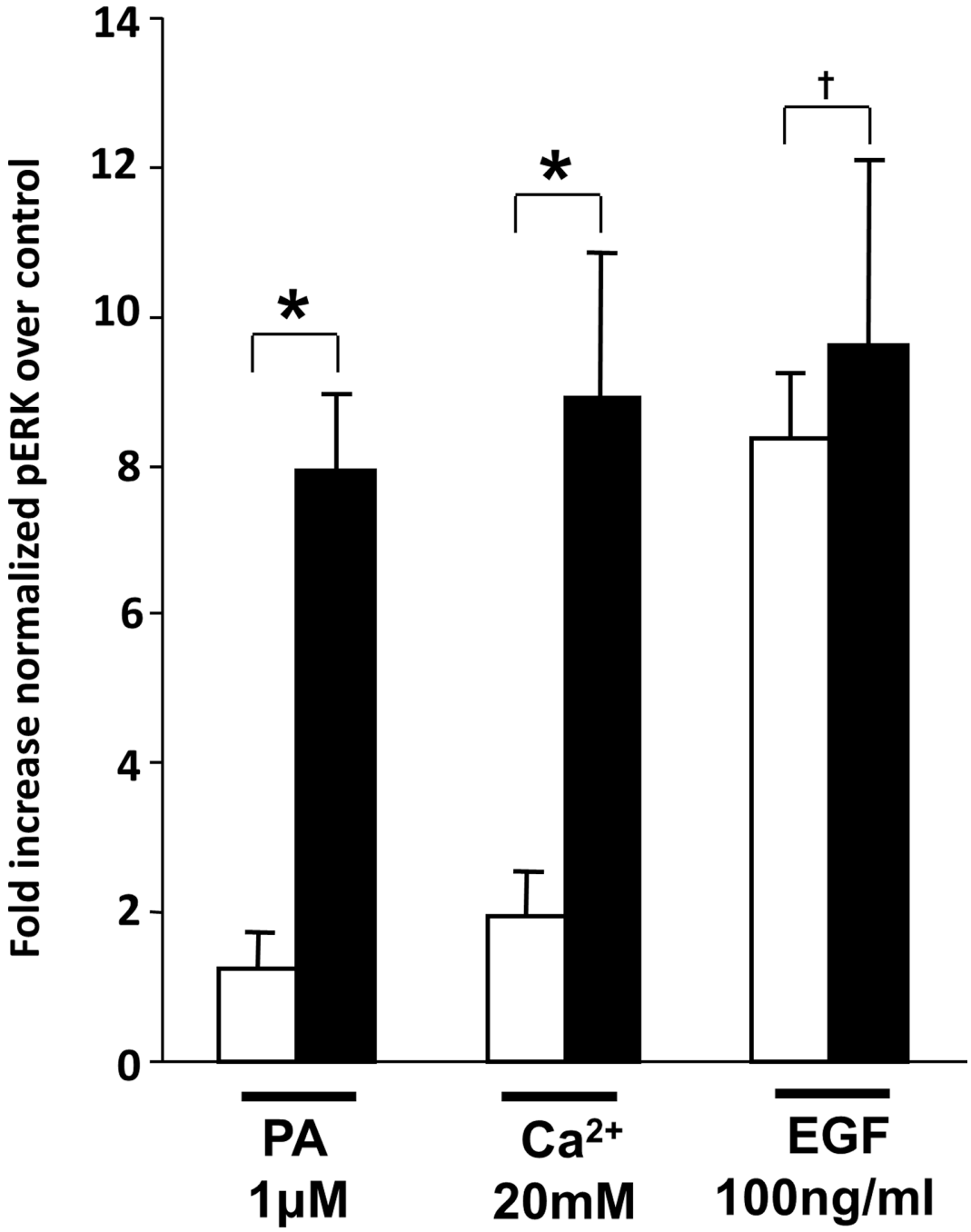

Figure 5.

Epithelial ERK phosphorylation by PA is CaSR dependent. Fold increase in percent ERK phosphorylation (pERK/total ERK) over control following 5 minute stimulation of wild type HEK cells (white bar) and HEK cells stably transfected with CaSR (black bar) with PA, Ca and EGF. Phospho-ERK and total ERK were quantified from cell lysates using chemiluminescence (Meso Scale Discovery). Results reflect the mean ± SD of 5 independent experiments (*p<0.01, †Not significant).

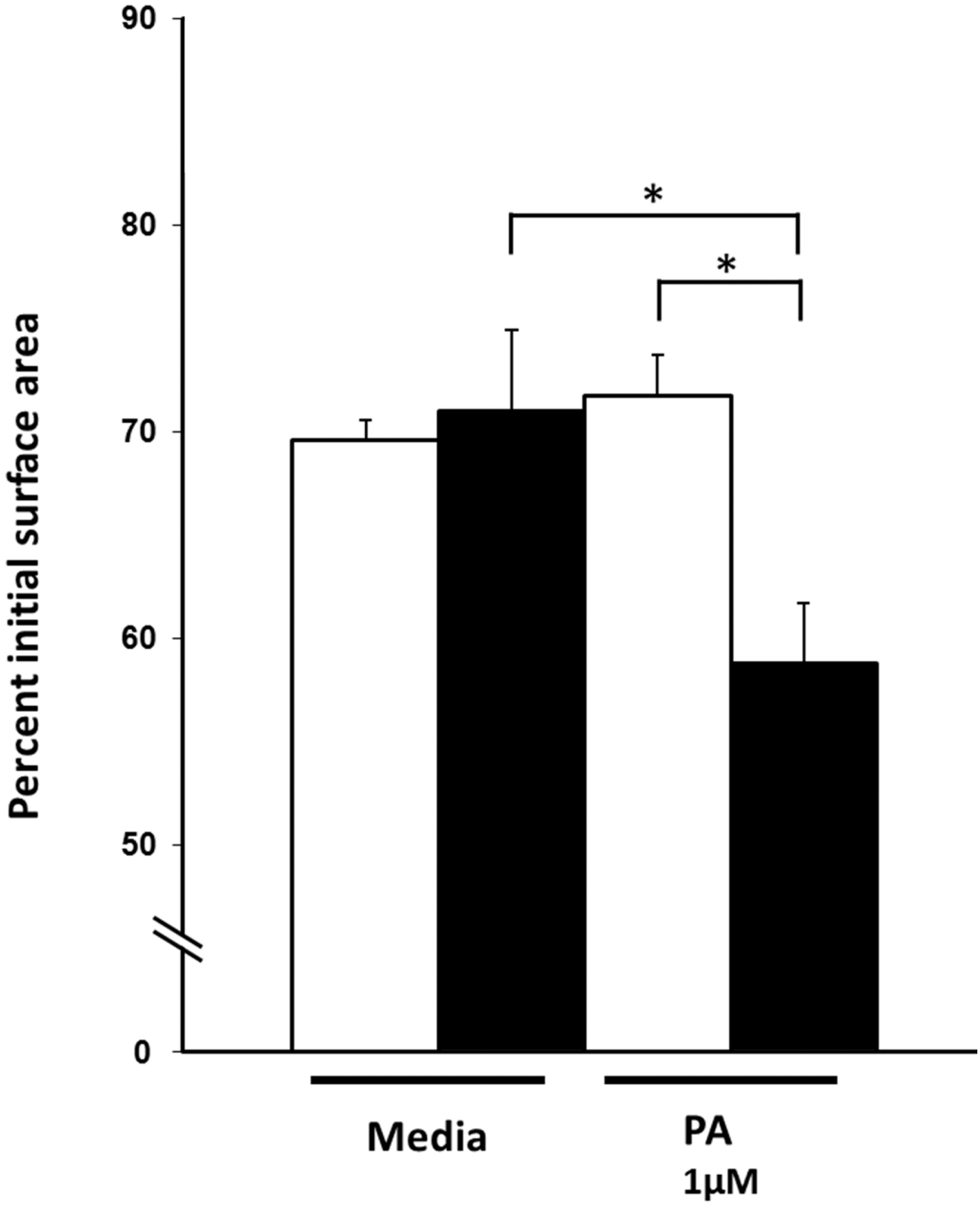

Figure 6.

CaSR contributes to collagen lattice contraction. Contraction of HEK-WT (white bar) and HEK-CaSR (black bar) populated collagen lattices stimulated with PA and media control. HEK-WT and HEK-CaSR cells were suspended in collagen lattices at 5×10^5 cells/ml and exposed to 1μM PA or not. Percent initial lattice surface area at 24 hours is reported. Values represent means ±SD of five lattices (*$p<0.001$).