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CRITICAL SIGNALING EVENTS IN THE MECHANOACTIVATION OF HUMAN MAST CELLS VIA P.C492Y-ADGRE2

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Abstract

A role for the adhesion G-protein coupled receptor ADGRE2 (EMR2) in mechanosensing was revealed by the finding of a missense substitution (p.C492Y) associated with familial vibratory urticaria (VU). In these patients, friction of the skin induces mast cell hyper-degranulation through p.C492Y-ADGRE2, causing localized hives, flushing and hypotension. We have now characterized the responses and intracellular signals elicited by mechanical activation in human mast cells expressing p.C492Y-ADGRE2 and attached to dermatan sulfate, a ligand for ADGRE2. The presence of p.C492Y-ADGRE2 reduced the threshold to activation and increased the extent of degranulation along with the percentage of mast cells responding. Vibration caused PLC activation, transient increases in cytosolic calcium, and downstream activation of PI3K and ERK1/2 by $G\beta\gamma$, $G\alpha_{q/11}$ and $G\alpha_{i/o}$ -independent mechanisms. Degranulation induced by vibration was dependent on PLC pathways, including calcium, PKC and PI3K but not ERK1/2 pathways, along with pertussis toxin (PTX)-sensitive signals. In addition, mechanoactivation of mast cells stimulated the synthesis and release of PGD₂, a previously unreported mediator in VU, and ERK1/2 activation was required for this response together with calcium, PKC and, to some extent,

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AUTHOR CONTRIBUTIONS

Conceptualization: ANN, AO, DDM; Data Curation: ANN; Formal Analysis: ANN, GB, YB, AO; Funding Acquisition: DLK, DDM; Investigation and Methodology: ANN, GB, YB, MGS, AT, SEB; Resources: HDK, SEB; Supervision: AO; Visualization: ANN, AO; Writing - Original Draft Preparation: ANN, AO; Writing - Review and Editing: ANN, GB, YB, MGS, AT, SEB, HK, DLK, DDM, AO

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PI3K. Our studies thus identify critical molecular events initiated by mechanical forces and potential therapeutic targets for patients with VU.

Keywords

vibratory urticaria; ADGRE2; mast cells; degranulation; calcium; PKC; PGD₂

INTRODUCTION

Adhesion G protein-coupled receptors (aGPCRs) represent the second largest class of receptors within the GPCR superfamily. They are involved in cell-to-cell and cell-to-matrix interactions and are thought to play important roles in immunity, tumorigenesis, reproduction and development (Langenhan et al., 2013). ADGRE2, or EMR2 (epidermal growth factor-like module-containing mucin-like hormone receptor-like 2), is an aGPCR that binds dermatan sulfate (DS) (Stacey et al., 2003) and is predominantly expressed in human myeloid cells.

ADGRE2 regulates neutrophil function and survival (Chen et al., 2011, Yona et al., 2008); and induces macrophage differentiation and expression of pro-inflammatory mediators (I et al., 2017, Kwakkenbos et al., 2002). Recently, we found that ADGRE2 is expressed in human mast cells and that a missense variant (p.C492Y) of ADGRE2 identified in patients with autosomal dominant vibratory urticaria (VU) is associated with disease presentation. These patients exhibit mast cell hyperreactivity upon mechanical stimuli resulting in localized hives, increased histamine levels in serum and increased extracellular tryptase staining in the dermis. Vibratory stimulation *in vitro* causes degranulation of mast cells derived from patients' progenitors and enforced expression of the p.C492Y-ADGRE2 variant in mast cells reproduces this phenotype. These results provided a link between p.C492Y-ADGRE2 and vibration-induced mast cell degranulation and supported a function for this receptor in sensing physical forces (Boyden et al., 2016), as has been reported for other aGPCR family members (Purcell and Hall, 2018, Scholz et al., 2016). However, the intracellular signals activated by mechanical stimulation of ADGRE2 and other aGPCRs remain elusive.

In the present study, we investigate the mechanisms of p.C492Y-ADGRE2 activation in mast cells with the dual purpose of identifying the signals causing the pathological presentation of VU and gaining insights into vibration-induced signal transduction that may be common to other mechanosensing aGPCRs. We show that ADGRE2 activation by physical stimulation, particularly the p.C492Y variant, assembles functionally distinct signaling events that enhance sensitivity to vibration. Vibration-induced signals include activation of phospholipase C (PLC) and calcium mobilization, which were essential for downstream activation of phosphoinositide 3-kinase (PI3K) and extracellular signal-regulated kinase (ERK1/2) pathways. While calcium and protein kinase C (PKC) were required for degranulation, ERK1/2 pathways were essential for the release of prostaglandin D₂ (PGD₂) which increased in serum of patients with VU after vibratory challenge. Our data contribute

to the understanding of pathways activated by aGPCR-mediated mechanosensing, and identify potential therapeutic targets for treatment of patients with VU.

RESULTS AND DISCUSSION

Characterization of vibration-induced degranulation

We sought to further characterize the responses to physical stimulation using the human mast cell line LAD2 transfected with normal or mutated-ADGRE2 (Figure 1a), which resulted in similar efficiencies of transfection (>70%; Figure S1a and in (Boyden et al., 2016)). We first examined the effect of vibratory stimuli of various strengths in cells attached to dermatan sulfate (DS) on the release of β -hexosaminidase as a read-out for degranulation. Speeds lower than 1,500 rpm caused minor degranulation in cells expressing nonmutated (NM)-ADGRE2, while cells expressing p.C492Y-ADGRE2 readily degranulated at 750 rpm or higher (Figure 1b and c, and Figure S1b, d and e d). Optimal differences in degranulation between the two variants were observed between 750 to 1,500 rpm, while higher stimulus strengths caused substantial degranulation in all cells, diminishing the differences between variants (Figure 1c), a result that was not due to cellular damage as viability was >90% after vibration at 2,000 rpm (Figure S1c). In agreement with clinical observations in VU, the results demonstrate that the p.C492Y-ADGRE2 variant requires lower threshold for activation of mast cells in response to vibration.

To gain further insights into the heterogeneity of individual cell degranulation to vibration, we monitored the exposure of the granule marker CD63 onto the cell surface using confocal microscopy. This method efficiently demonstrated increased cell surface expression of CD63 during degranulation in cells stimulated with thapsigargin or with IgE/antigen (Figure S2a and b). Vibration also increased anti-CD63 surface staining, particularly on cells expressing p.C492Y-ADGRE2 (Figure 1d and e), although an increase was also seen in cells with NM-ADGRE2 at higher speeds (Figure S2c). Similar to β -hexosaminidase release (Figure 1b and c), stronger mechanical stimulation reduced the differences in CD63 expression between NM and p.C492Y-ADGRE2 variants (Figure S2c). Analysis of responding cells (i.e. cells showing CD63 surface staining above background), indicated that both the magnitude of degranulation on a per cell basis (Figure 1f) and the percentage of responding cells (Figure 1g) were markedly increased when expressing p.C492Y-ADGRE2.

Degranulation was observed when p.C492Y-ADGRE2 cells were attached to immobilized DS, a reported ligand for ADGRE2 (Stacey et al., 2003) or 2A1, an antibody that recognizes a sequence in the NTF (Figure 1a) and ligates the receptor in macrophages (Huang et al., 2012), but not to chondroitin sulfate A, an analog of DS, polylysine, hyaluronic acid or heparan sulfate (Figure S1d). These and our reported findings (Boyden et al., 2016) suggest that binding of ADGRE2 to immobilized DS or to 2A1 is required, along with mechanical forces, to elicit degranulation in mast cells, as in static conditions no significant degranulation occurred (Figure 1b). Vibration-induced responses in p.C492Y-ADGRE2 cells were similar at concentrations of immobilized DS ranging from 0.1 to 100 μ g/mL (Figure S1e and S3c), consistent with the observed maximal adherence of cells to DS within this range (Boyden et al., 2016). In other mechanosensing aGPCRs, activation is also thought to require both ligation and a mechanical load, as ligand binding by itself causes no detectable

signaling (Karpus et al., 2013, Langenhan et al., 2013, Scholz et al., 2015) or inhibits signaling (Petersen et al., 2015). A proposed model of activation of aGPCRs suggests that the N-terminal fragment (NTF) normally prevents spontaneous activation and that dissociation of the NTF from the C-terminal fragment (CTF) (Figure 1a) is required for signaling to occur (Purcell and Hall, 2018, Scholz et al., 2016). Consistent with this model, the presence of p.C492Y destabilized the non-covalent interaction between the NTF and CTF in mast cells, rendering the receptor more prone to dissociating the NTF after a vibratory stimulus (Boyden et al., 2016), a hypothesis also consistent with the lower threshold of activation in cells with p.C492Y (Figure 1b and Figure S1b), and recent results showing that proteolytic clipping of the NTF of ADGRE2 by alpha/beta-tryptase heterotetramers makes mast cells susceptible to vibration-triggered degranulation (Le et al., 2019).

Vibration causes calcium mobilization in mast cells

Although other aGPCR members have also been viewed as mechanosensors (Scholz et al., 2016), there is limited information on how such receptors signal. Calcium is viewed as one potential messenger in aGPCR-mediated mechanosensing (Clapham, 2003, Scholz et al., 2015, Scholz et al., 2016) and also a critical signal for mast cell degranulation (Gilfillan and Tkaczyk, 2006). Following this lead, we determined that in mast cells loaded with Fura-2 AM, vibration induced a rise in cytosolic calcium, particularly in p.C492Y-ADGRE2 cells, that was maximal immediately after vibration (~0.85 μM in nonmutated- and ~1.5 μM in p.C492Y-ADGRE2 cells) and declined thereafter (Figure 2a). These transient calcium spikes resembled the typical transient calcium mobilization reported to occur after GPCR activation in mast cells. GPCR-induced calcium responses are associated with a distinct degranulation pattern that causes rapid and brief vascular reactions *in vivo* (Gaudenzio et al., 2016) which are consistent with the reactions to a vortex challenge in patients with VU (Boyden et al., 2016, Metzger et al., 1976), and contrast with the more sustained calcium responses mediated by IgE receptor stimulation (Figure S2d) that result in compound exocytosis and slower but stronger and more prolonged reactions in mice than those produced by GPCRs (Gaudenzio et al., 2016).

Similar to the populational analysis, confocal single-cell measurements using Fluo-8, which readily detected calcium elevations with the expected kinetics after stimulation of the IgE receptor or thapsigargin treatment in LAD2 cells (Figure S2d and e) (Suzuki et al., 2014), showed enhanced calcium responses to vibration in cells with p.C492Y-ADGRE2 (Figure 2b–d), and this increase was abolished in calcium free media (Figure 2c). Since ligation of ADGRE2 by 2A1 activates PLC- β in monocytic cells (I et al., 2017) and PLC activity leads to calcium release via the production of inositol 1,4,5-trisphosphate (IP₃), we explored the involvement of this pathway in the calcium responses. Unlike an inactive structural analog (U73343), inhibition of PLC by U73122 or inhibition of IP₃ channels by 2-Aminoethoxydiphenyl borate (2APB) blunted the calcium responses in p.C492Y-ADGRE2 cells (Figure 2c and d). Our data thus implicate PLC and mobilization of intracellular calcium in the transients induced by vibration. A contribution of extracellular calcium influx cannot be excluded, since 2APB is also an inhibitor of SOCE and, to a degree, of transient receptor potential (TRP) isoforms (Bootman et al., 2002).

As in degranulation, the expression of p.C492Y-ADGRE2 increased both the magnitude of the calcium responses per cell basis (i.e. in cells with intensities above baseline after vibration) (Figure 2d), and the number of cells with positive responses to vibration (Figure 2e). Of note, only in cells expressing p.C492Y-ADGRE2, did maximal Fluo-8 intensity correlate with the extent of degranulation after vibration at 750 rpm (Figure 2f and g), consistent with the conclusion that vibration normally causes calcium fluxes via ADGRE2 that translate into limited or no degranulation, but both processes are enhanced and linked when the p.C492Y variant is present.

Even though calcium is essential for mast cell degranulation, the strength and/or duration of calcium changes can determine the overall functional consequences. For instance, activation of the IgE receptor by low-affinity antigens induce lower calcium responses and do not render important degranulation as high-affinity antigens do. They, however, transmit different sets of signals with distinct functional outcomes (Suzuki et al., 2014). Overall, the observations support the concept that the structure of ADGRE2 allows mast cells to discriminate the strength of mechanical stimulation and tune the responses accordingly as vibration triggers calcium responses even in cells with the normal variant. Such discrimination of the mechanical strength may be of importance for the immunomodulatory functions of mast cells, much like has been reported for the concentration (Gonzalez-Espinosa et al., 2003) or affinity of antigens (Suzuki et al., 2014) in IgE-receptor mediated activation. However, the molecular and physiological basis for this tuning of the responses, as well as the relevant type of physiological physical stimulus, need further investigation. For instance, polymerization of extracellular matrix components, which occurs during wound healing, may constitute a mechanical stimulus for aGPCRs *in vivo*, as reported (Petersen et al., 2015); or the crawling of skin parasites that can bind skin glycosaminoglycans (Merida-de-Barros et al., 2018) could mimic a vibration stimulus (stretching or treading). It is tempting to speculate that various physical forces may cause limited but advantageous mast cell responses for the host (i.e. recruitment of immune cells, regulation of wound healing, induction of itch/pain sensing, etc.) without causing a full mast cell response that could be damaging.

Vibration of mast cells with p.C492Y-ADGRE2 induces activation of ERK1/2 and PI3K

We next investigated whether other signaling pathways are activated by vibration. Similar to GPCRs and unlike IgE receptor-mediated activation (Kuehn and Gilfillan, 2007, Mocsai et al., 2003), vibration did not elicit detectable phosphorylation of SRC kinases or spleen tyrosine kinase (SYK) (Figure S3a) in p.C492Y-ADGRE2 mast cells. However, vibration prominently increased pathways commonly associated with agonist-stimulated GPCRs (Gilfillan and Tkaczyk, 2006), such as phosphorylation of protein kinase B (AKT) (Figure 3a and Figure S3b), known to be activated in a PI3K-dependent manner (Manning and Toker, 2017); and phosphorylation of ERK1/2 (Figure 3b and Figure S3c). However, other MAPK pathways, including JNK and p38 (Figure 3b) were not activated. The extent of AKT and ERK1/2 phosphorylation induced by vibration was robust and comparable to that induced by activation of mast cells via the IgE receptor (Figure S3d).

PKC stimulation occurs downstream PLC activation. Since PKC can mediate PI3K (Kawakami et al., 2004, Ziemba and Falke, 2018) or MEK/ERK1/2 pathway activation (Mendoza et al., 2011), we examined its involvement in the regulation of these pathways. Pretreatment with a pan-PKC inhibitor, Gö6983, somewhat reduced AKT phosphorylation induced by vibration, albeit the differences did not reach statistical significance (Figure 3c); and did not affect ERK1/2 (Figure 3d) phosphorylation, suggesting that PI3K and ERK1/2 pathways are activated by mechanisms largely independent of PKC.

We also examined the potential cross-interactions between PI3K and ERK1/2 pathways. Inhibition of MEK/ERK1/2 did not prevent vibration-induced phosphorylation of AKT (Figure 3c) but blunted ERK1/2 phosphorylation (Figure 3d). In contrast, the pan-inhibitor of PI3K, wortmannin, markedly diminished AKT activation (Figure 3c) and ERK1/2 phosphorylation (Figure 3d). LY294002, a less potent PI3K inhibitor only partially reduced ERK1/2 phosphorylation (Figure 3d). Although we cannot exclude potential off-target effects of wortmannin, the results suggest a directional crosstalk between these pathways where activation of PI3K contributes to ERK1/2 activation, but not vice versa.

PLC activation and calcium mobilization are critical for both PI3K and ERK1/2 activation

We then examined the involvement of PLC stimulation on vibration-induced PI3K and ERK1/2 phosphorylation. An inhibitor of PLC blocked both AKT (Figure 4a) and ERK1/2 (Figure 4b) phosphorylation, while a structural analog of this inhibitor did not. Blockade of calcium fluxes, but not inhibition of PKC, obliterated ERK1/2 and AKT phosphorylation (Figure 4a and b), suggesting that activation of PLC by vibration and consequent calcium mobilization are required for both PI3K and ERK1/2 activation. Given that PI3K may also regulate ERK1/2 phosphorylation (Figure 3d), calcium could activate ERK1/2 partly through PI3K-dependent pathways. Other points of interaction upstream the molecules affected by those inhibitors are possible since MEK/ERK1/2 and PI3K/AKT signaling cascades crosstalk in multiple points (Mendoza et al., 2011) (see summary in Figure 6d).

A similar chain of events for ADGRE2 activation was reported in human monocytic cell lines by crosslinking ADGRE2 with immobilized 2A1 antibody (I et al., 2017) in the absence of mechanical forces. Although it is unclear why crosslinking of ADGRE2 would activate monocytic cells in static conditions while in mast cells vibration is required, both studies reinforce the notion of a consensus chain of signaling events elicited by ADGRE2 activation that are initiated by PLC activation and calcium fluxes, followed by PI3K and MAPK cascades, which in the case of mechanically stimulated mast cells, were restricted to ERK1/2.

Initiation of GPCR signaling involves heterotrimeric G-proteins, although in aGPCRs the specific subtypes have not been well characterized (Hamann et al., 2015). Activation of PLC usually depends on $\beta\gamma$, α_q or α_{11} G-subunits (Boyer et al., 1994). Inhibition of $\beta\gamma$ by gallein, α_q and α_{11} subunits by YM254890, or $\alpha_{i/o}$ subunits by pertussis toxin (PTX) did not significantly prevent the activation of AKT or ERK1/2 (Figure 4c and d). The same concentrations of PTX and YM254890 were however effective in reducing Mas-Related G-protein receptor member X2 (MRGPRX2)-mediated degranulation (Figure S4) (Subramanian et al., 2013). Thus, PLC-dependent activation of PI3K and ERK pathways

induced by vibration is independent of these subunits, although involvement of other α subtypes such as α_{16} is possible, as reported (I et al., 2017).

Signaling requirements for vibration-induced mast cell responses

Because of the potential implications for patients with VU, we next investigated which signaling pathways are required for vibration-induced degranulation. Using confocal imaging of CD63 externalization (Figure 5a and b) and/or β -hexosaminidase release (Figure 5c) in the presence of various inhibitors, we determined that PLC activation and consequent calcium mobilization and PKC activation are all necessary for degranulation. Furthermore, downstream of PLC and calcium, PI3K pathways only partly contributed to degranulation (Figure 5b and c) while ERK1/2 was dispensable (Figure 5a–c). Degranulation was also blocked by PTX (Figure 5c), although AKT and ERK1/2 signaling was PTX-independent (Figure 4c and d), suggesting that degranulation depends on both $\alpha_{i/o}$ -sensitive and insensitive pathways (Figure 6d). The specific nature of the $\alpha_{i/o}$ (PTX)-mediated events, as for other GPCRs in mast cells (Subramanian et al., 2013), remains to be elucidated.

Since vibration prominently activated ERK1/2 but had no role in degranulation, we interrogated its involvement in other mast cell responses, particularly PGD₂ release. PGD₂ is one of the most abundant eicosanoid products formed by mast cells as an early *de novo* mediator (Gilfillan and Tkaczyk, 2006) and ERK1/2 is required for its production via activation of phospholipase A2 (Kuehn et al., 2008). Indeed, vibration induced PGD₂ release, particularly in cells with p.C492Y-ADGRE2, was blunted by MEK/ERK1/2 inhibition (Figure 6a). In addition, calcium mobilization and PKC were key to this response, with a partial contribution of PI3K (Figure 6a and b). Blockade of $\alpha_{i/o}$, but not α_q , slightly reduced PGD₂ release, suggesting a contributory role for $\alpha_{i/o}$ -dependent signals. However, PGD₂ release induced by vibration was especially dependent on PLC-dependent pathways, highlighting the key role for PLC and downstream signals in mechanoactivation of mast cells.

PGD₂, an unrecognized mediator in VU

Mirroring the increase in PGD₂ production by mast cells after vibration *in vitro*, we found that in patients with VU, unlike normal subjects, PGD₂ was elevated in the venous return of the arm early after vibration. PGD₂ remained elevated over baseline 30 min after the vortex challenge (Figure 6c). Although confirmation in larger cohorts is needed, the findings suggest a potential role for PGD₂ in the pathology of VU. PGD₂ can mediate immediate hypersensitivity processes causing hypotension and flushing but it can also play anti-inflammatory roles (Boyce, 2007, Kulinski et al., 2016), and reduce vascular permeability by tightening the endothelial barrier (Nakamura et al., 2017). Since the increase in PGD₂ levels appeared to be sustained longer (Figure 6c) than those of histamine (Boyden et al., 2016, Epstein and Kidd, 1981, Ting et al., 1983), one hypothesis would be that PGD₂ downregulates the responses to vibration, contributing to the transient nature of VU pathology. It would be of interest to determine whether PGD₂ is not generated in patients with vibratory angioedema (Keahey et al., 1987), who unlike those with in VU, have long-lasting responses.

Concluding Remarks

Herein, we have identified the signaling pathways activated by physical forces in mast cells expressing p.C492Y-ADGRE2, a variant associated with familial VU that renders skin mast cells more susceptible to friction (Boyden et al., 2016). Mechanical signaling encompasses PLC activation as a key early step for mast cell degranulation and PGD₂ release; and a parallel PTX-sensitive pathway, also essential for degranulation. The requirement for PLC resonates with the reported gain of PLC γ 2 function in a form of inherited cold urticaria where mast cells are also key (Ombrello et al., 2012). Thus, exploration of the role of distinct PLC isoforms and PLC-derived signals in other physical urticarias may be of interest. Downstream of PLC, PKC and calcium were critical for vibration-induced degranulation and eicosanoid production, whereas ERK1/2 was only required for PGD₂ release. The distinct requirement for these signals on degranulation and PGD₂ release are generally consistent with their roles in IgE receptor- or GPCR-activation (Gilfillan and Tkaczyk, 2006, Kimata et al., 2000, Xing et al., 1997), although their crosstalk appears to be distinct for ADGRE2 (Figure 6d). Our findings not only add to the knowledge of the enigmatic signaling events initiated by mechanical stimulation, their hierarchy and cross-interaction, but also may help design novel therapeutic approaches for VU and give clues into mechanisms for other physical urticarias.

MATERIALS AND METHODS

Further details on the experimental procedures can be found in Supplementary Methods.

Patients

Patients with VU were enrolled and evaluated at the NIH Clinical Center under a protocol approved by the Institutional Review Board of the NIAID (09-I-0126). All subjects provided written informed consent. VU symptoms in a clinical setting were elicited as described (Boyden et al., 2016).

Cell activation

LAD2 cells (Kirshenbaum et al., 2003) transfected with the *ADGRE2* constructs by nucleofection (Cruse et al., 2013) were plated overnight on wells coated with 100 μ g/mL DS (chondroitin sulfate B, Sigma-Aldrich). After gentle washing, cells were resuspended in 100 μ L of pre-warmed HEPES buffer and vibrated at 750 rpm (or as indicated) for 5–20 min on an orbital shaker (Eppendorf ThermoMixer C) at 37 °C. In some experiments, cells were treated with the indicated concentrations of inhibitors or vehicle (0.1% DMSO) 20 min before and during vibration.

Mast cell mediators and signaling

Degranulation was determined as the percentage of β -hexosaminidase released into the media (Kuehn et al., 2010) 20 min after vibration. Alternatively, cells plated in DS-coated imaging chambers (Ibidi #80826), were vibrated for 5 min and anti-CD63-APC was added (1:20). Confocal images were acquired over time using a Leica TCS SP8 microscope and processed using ImageJ, selecting Region of Interest (ROI) Manager to determine anti-CD63-APC mean fluorescent intensity per cell.

PGD₂ in cell-free supernatants or serum samples was determined by competitive ELISA (Cayman Chemicals). Phosphorylation of signaling proteins were determined by Western blotting after vibration for 5 min as described (Boyden et al., 2016, Tkaczyk et al., 2002).

Calcium flux measurements

Cells attached to DS-coated chambers were loaded or not with 5 μ M Fura-2 AM (Life Technologies) or Fluo-8 AM (Abcam), washed and vibrated at 750 rpm for 5 min. Measurements of Fura-2 fluorescence were done using a Perkin Elmer Wallac 1420 Victor2 microplate reader as described (Cruse et al., 2013, Gryniewicz et al., 1985) and changes in fluorescence of calcium-bound Fluo-8 by confocal microscopy. Prior to imaging, anti-CD63-APC at 1:20 dilution was added to simultaneously track mast cell degranulation. Mean fluorescent intensity per cell was measured using ROI Manager, and baseline fluorescence was subtracted from each individual cell.

Statistical analysis

Statistical analysis was conducted using GraphPad Prism (version 7.01). Unpaired Student's t-test was used for most comparisons unless otherwise indicated. A p value <0.05 was considered significant. All experiments were done 2 independent times and performed at least in triplicate.

SUPPLEMENTARY MATERIAL

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

2APB	2-Aminoethoxydiphenyl borate
ADGRE2	Adhesion G protein-coupled receptor E2
aGPCR	Adhesion G protein-coupled receptors
AKT	protein kinase B
A.U.	arbitrary units
CTF	C-terminal fragment
ECM	extracellular matrix

EMR2	epidermal growth factor (EGF)-like module-containing mucin-like hormone receptor-like 2
DAG	diacylglycerol
DS	dermatan sulfate
ERK1/2	extracellular signal-regulated kinase
GAIN	GPCR autoproteolysis-inducing domain
IP3	inositol triphosphate
NM	nonmutated
NTF	N-terminal fragment
PGD₂	prostaglandin D ₂
PKC	Protein Kinase C
PLC	phospholipase C
SA	streptavidin
SOCE	store operating calcium channels
SYK	spleen tyrosine kinase
ROI	region of interest
Vb	vibration
VU	vibratory urticaria

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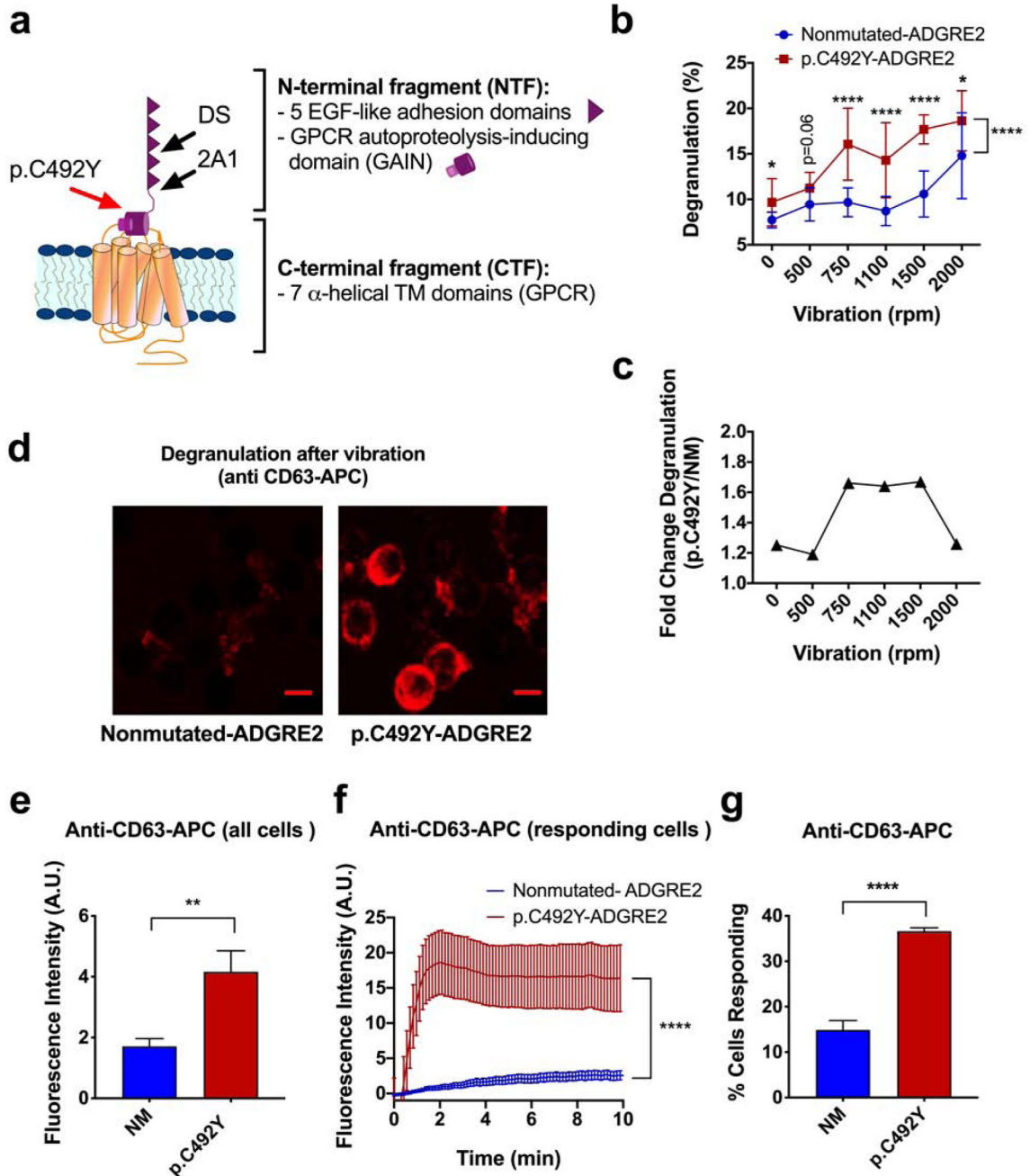


Figure 1. Characterization of vibration-induced degranulation.

(a) Depiction of ADGRE2. The NTF and CTF, translated as a single polypeptide and self-cleaved in the GAIN domain in the ER, remain non-covalently linked (Kwakkenbos et al., 2002). Arrows indicate the location of p.C492Y and binding sites for DS and the 2A1 antibody. (b) β -hexosaminidase release induced by vibration (20 min) in nonmutated (NM)- or p.C492Y-ADGRE2 cells. Data are mean \pm SD. Student t-tests were used for the point by point comparisons and a two-way ANOVA for comparison between the curves (side bracket). (c) Fold changes in degranulation (from data in b). (d) Images of CD63 surface expression 5 min after vibration (750 rpm). Scale bar=10 μ m. (e) Anti-CD63-APC

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fluorescent intensity 10 min after vibration; (n 77). **(f)** Anti-CD63-APC intensity after vibration in responsive cells; (n=10). **(g)** Percentage of responsive cells. Data are mean \pm SEM in **e-g**. *p<0.05; ** p<0.005; **** p<0.0001.

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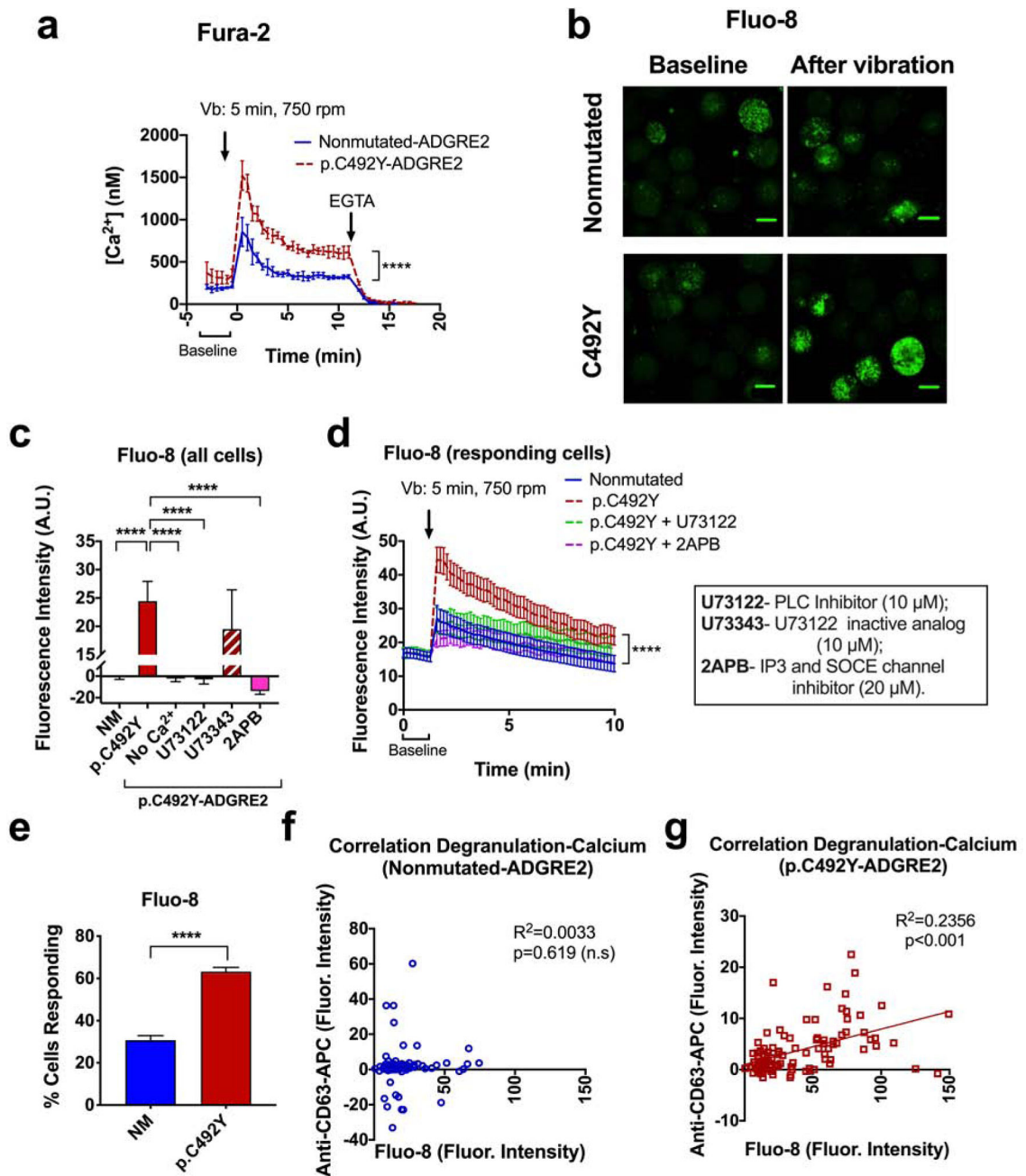


Figure 2. Vibration causes transient calcium mobilization.

(a) Changes in intracellular calcium measured by Fura-2 in NM- or p.C492Y-ADGRE2 cells. Data are mean±SD of 2 experiments performed in triplicate. (b) Images of changes in intracellular calcium after vibration (Vb) using Fluo-8. Scale bar=10 μm (c, d) Average Fluo-8 fluorescence intensity of all cells 5 min after vibration (c) or in responding cells (with signals above baseline) overtime (d), with or without extracellular calcium, and in cells pretreated with inhibitors, as indicated. Data are mean±SEM (n = 19). (e) Percentage of responsive cells. Data are mean±SEM (n = 77 cells). (f–g) Pearson correlation between calcium changes (Fluo-8 intensity) and degranulation (anti-CD63-APC intensity) from n=4

experiments normalized to average intensities. A.U., arbitrary units. ****p<0.0001. Two-way ANOVA was used in **a** and **d**.

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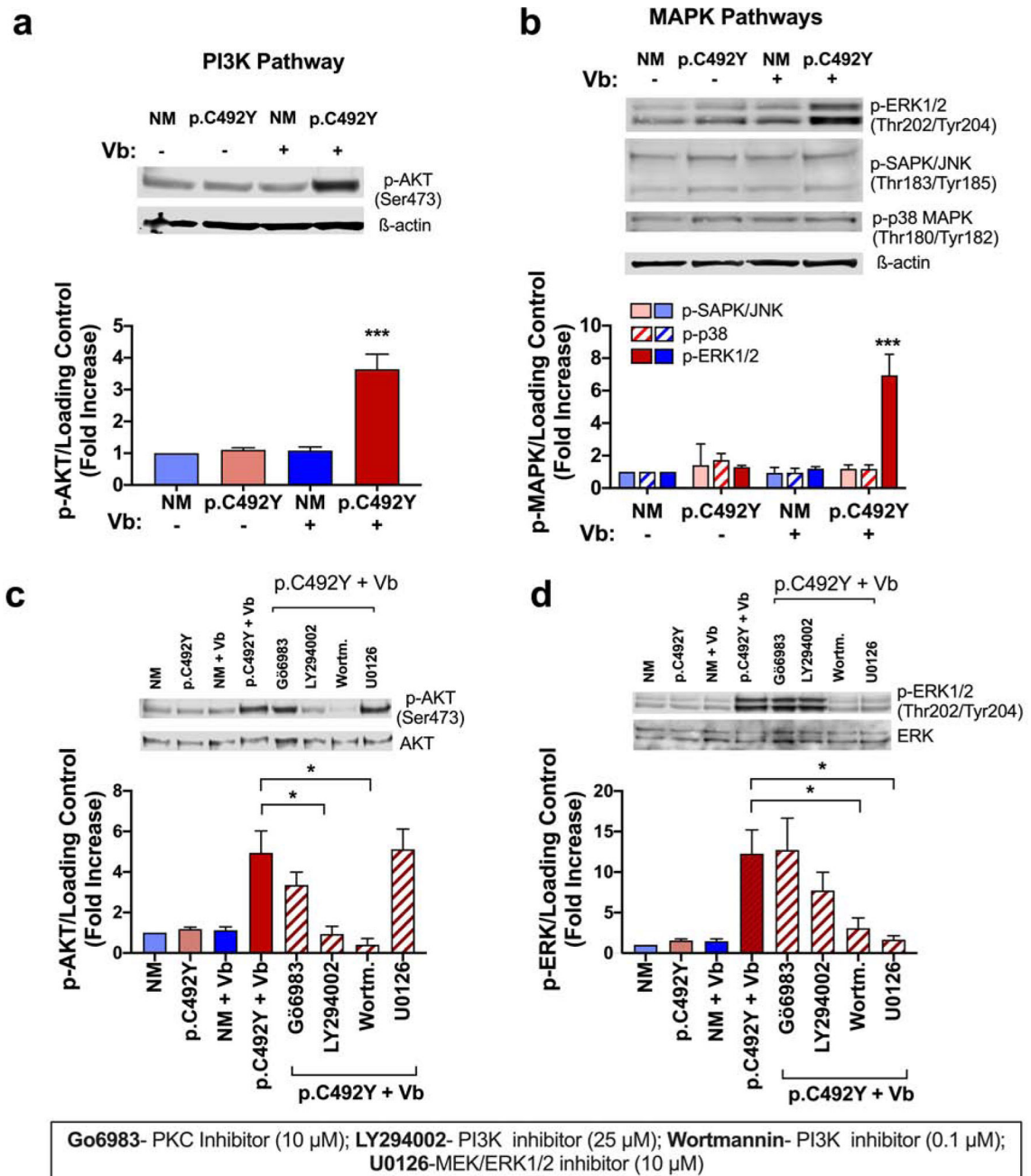


Figure 3. Activation of PI3K and ERK1/2 signaling pathways and their potential crosstalk by mechanoactivation in mast cells with p.C492Y-ADGRE2.

DS-bound cells expressing nonmutated (NM)- or p.C492Y-ADGRE2 were lysed after vibration (+Vb) for 5 min at 750 rpm. **(a, b)** Phosphorylation of AKT **(a)**, ERK1/2, SAPK/JNK and p38 **(b)**. **(c, d)** Effects of inhibition of PKC, PI3K, and MEK1/2 on AKT **(c)** and ERK1/2 **(d)** phosphorylation. Inhibitors were added 20 min before vibration. The histograms in **a-d** show the quantification of band intensities (normalized by total AKT/ ERK1/2 protein or β -actin) from n = 3 experiments and expressed as fold change compared to

non-vibrated, NM-ADGRE2- expressing cells. Data are mean±SEM. * p<0.05; ***
p 0.0001.

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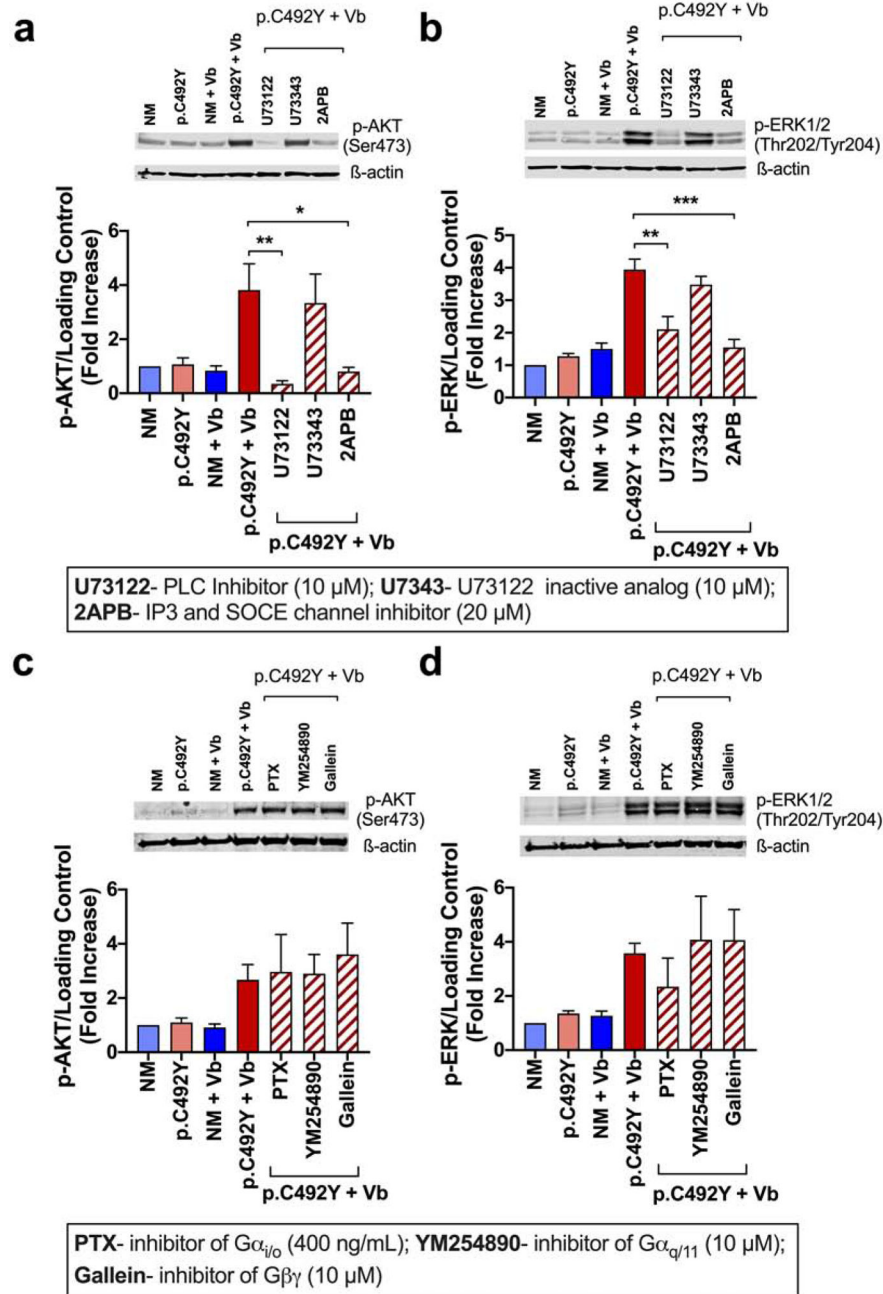


Figure 4. PLC activation and calcium mobilization are critical for PI3K and ERK1/2 activation independently of $\beta\gamma$, $\alpha_{q/11/14}$ and $\alpha_{i/o}$ G-protein subunits.

Effects of inhibitors for PLC, IP3 and SOCE channels (**a and b**), or inhibitors of G-protein subunits $\beta\gamma$, $\alpha_{i/o}$ and $\alpha_{q/11}$ (**c and d**), as indicated, on vibration-induced AKT (**a, c**) and ERK1/2 phosphorylation (**b, d**). DS-attached LAD2 cells expressing nonmutated (NM)-ADGRE2 or p.C492Y-ADGRE2 were treated with inhibitors for 20 min and vibrated 5 min at 750 rpm. Histograms represent band intensities (normalized by total AKT/ERK1/2 protein or β -actin) from n = 3 experiments and calculated as fold change compared to non-vibrated, NM-ADGRE2 expressing cells. Data are mean \pm SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

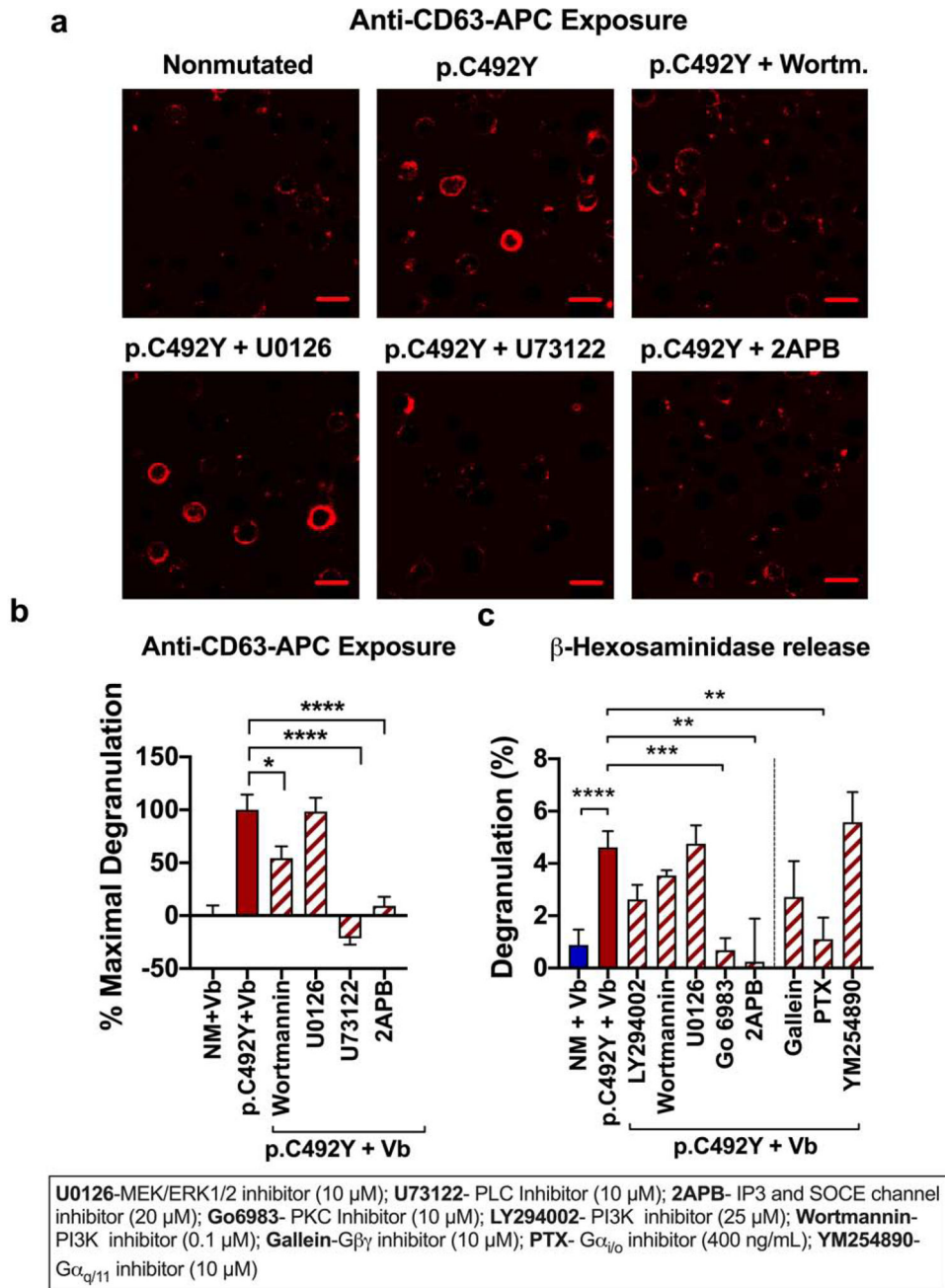


Figure 5. PLC/calcium and PKC axis and PTX-sensitive pathways are critical for vibration-induced degranulation, while MEK/ERK1/2 is dispensable.

LAD2 cells expressing NM-ADGRE2 or p.C492Y-ADGRE2 plated on DS-coated dishes were treated with the indicated inhibitors for 20 min before vibration. (a) Representative confocal images of anti-CD63-APC on the membrane after vibration for 5 min. Scale bar=20 μ m. (b) Quantification of degranulation from data shown in a. Fluorescent intensity of anti-CD63-APC in individual cells (n> 264 cells) was determined, and expressed as % decrement () of the maximal response (i.e. fluorescent intensity of cells expressing p.C492Y-ADGRE2), being the minimal response fluorescent intensity of anti-CD63-APC in

cells with NM-ADGRE2. (c) β -hexosaminidase release after 20 min vibration. Data are mean \pm SEM, n 12 *p<0.05; **p<0.005; ***p<0.001; ****p<0.0001.

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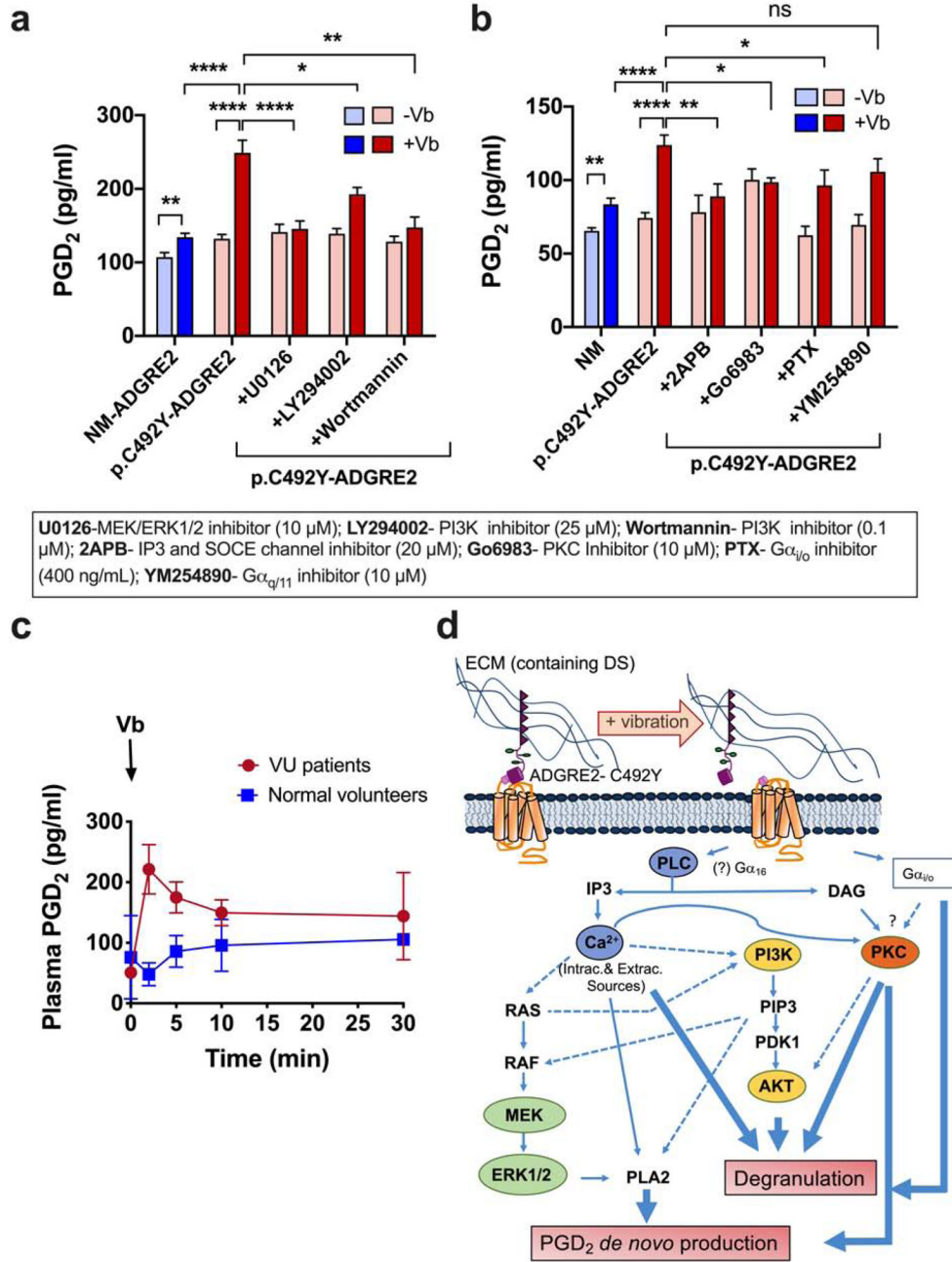


Figure 6. Vibration-induced PGD₂ release is dependent on PLC/calcium, PKC and MEK/ERK1/2; and vortex challenge in patients with VU increases serum PGD₂. (a, b) PGD₂ release induced by vibration in mast cells expressing NM or p.C492Y-ADGRE2 in the presence or absence of the indicated inhibitors. Supernatants of vibrated (750 rpm) and non-vibrated samples after 10 min. Data are mean±SEM; n = 9. *p<0.05; **p<0.005; ****p<0.0001. (c) PGD₂ in the serum of 2 patients with VU and 2 control subjects after a vibratory challenge. (d) Representation of the proposed signaling pathways involved in mechanical activation of mast cells. Dotted lines are potential ways of crosstalk

based on our data or the literature (Cullen and Lockyer, 2002, Danciu et al., 2003, Mendoza et al., 2011, Yano et al., 1998).

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