

Ca²⁺ signalling is critical for autoantibody-induced blistering of human epidermis in pemphigus*

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Summary

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Conflicts of interest

The authors declare they have no conflicts of interest.

Data Availability Statement

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

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Background Pemphigus is a severe bullous autoimmune skin disease. Pemphigus foliaceus (PF) is characterized by antidesmoglein (Dsg) 1 IgG causing epidermal blistering; mucosal pemphigus vulgaris (mPV) by anti-Dsg3 IgG inducing erosions in the mucosa; and mucocutaneous pemphigus vulgaris (PV) by affecting both, with autoantibodies targeting Dsg1 and Dsg3.

Objectives To characterize the Ca²⁺ flux pathway and delineate its importance in pemphigus pathogenesis and clinical phenotypes caused by different antibody profiles.

Methods Immunoprecipitation, Ca²⁺ flux analysis, Western blotting, immunofluorescence staining, dissociation assays and a human skin *ex vivo* model were used. **Results** PV IgG and PF IgG, but neither Dsg3-specific monoclonal antibody (AK23) nor mPV IgG, caused Ca²⁺ influx in primary human keratinocytes. Phosphatidylinositol 4-kinase α interacts with Dsg1 but not with Dsg3. Its downstream target – phospholipase-C- γ 1 (PLC) – was activated by PV IgG and PF IgG but not AK23 or mPV IgG. PLC releases inositol 1,4,5-trisphosphate (IP3) causing IP3 receptor (IP3R) activation and Ca²⁺ flux from the endoplasmic reticulum into the cytosol, which stimulates Ca²⁺ release-activated channels (CRAC)-mediated Ca²⁺ influx. Inhibitors against PLC, IP3R and CRAC effectively blocked PV IgG and PF IgG-induced Ca²⁺ influx; ameliorated alterations of Dsg1 and Dsg3 localization, and reorganization of keratin and actin filaments; and inhibited loss of cell adhesion *in vitro*. Finally, inhibiting PLC or IP3R was protective against PV IgG-induced blister formation and redistribution of Dsg1 and Dsg3 in human skin *ex vivo*.

Conclusions Ca²⁺-mediated signalling is important for epidermal blistering and dependent on the autoantibody profile, which indicates different roles for signalling complexes organized by Dsg1 and Dsg3. Interfering with PLC and Ca²⁺ signalling may be a promising approach to treat epidermal manifestations of pemphigus.

What is already known about this topic?

- Autoantibody-induced Ca²⁺ signalling and activation of phospholipase C in keratinocytes has been reported as the first signalling pathway in pemphigus.
- Ca²⁺ influx correlates with the presence of autoantibodies targeting desmoglein (Dsg) 1.

- The role and exact mechanisms of the Ca²⁺ signalling pathway and its role in pemphigus pathology are unclear.

What does this study add?

- There is an important role of the phospholipase C/Ca²⁺ signalling pathway in the pathogenesis of pemphigus.
- Relevant signal components of the Ca²⁺ pathway are characterized in detail.
- Dsg1 and Dsg3 are found to interact with signalling molecules, organizing signalling complexes that differ with respect to the presence of phosphatidylinositol 4-kinase α , the most upstream activator of the Ca²⁺-influx pathway, assigning different roles to Dsg1- and Dsg3-mediated signalling.

What is the translational message?

- Current therapies for pemphigus have a delayed onset of action. Rapidly effective therapeutic approaches that directly stabilize desmosomal adhesion are desirable.
- While targeting the Ca²⁺ flux is problematic, the role of phospholipase C in the influx identifies this molecule as a potential target for specific molecular therapy approaches.
- Elucidating the mechanism of Dsg1-dependent regulation of Ca²⁺ influx in keratinocytes helps us to understand the different blistering patterns and the phenotypic variability seen in pemphigus.

Bullous autoimmune diseases of the pemphigus group cause disruption of the skin and/or mucosal barrier. The main variants of pemphigus are mucosal pemphigus (mPV), which affects the mucosa only; mucocutaneous pemphigus vulgaris (PV), which also causes epidermal blistering; and the less severe variant, pemphigus foliaceus (PF), where blistering is restricted to the epidermis.^{1,2} Pemphigus may lead to severe complications and high mortality if untreated.³ Current first-line treatments are systemic high-dose corticosteroids combined with immunosuppressants or rituximab; however, these have an delayed onset of action and may cause severe adverse reactions.⁴ Other treatment options include intravenous IgG or immunoadsorption, which also have drawbacks, such as the limited availability of donor IgG,⁵ general immunosuppression or short-term effectiveness.^{2,5,6} Therefore, rapid-acting and effective treatment options that promote the stabilization of desmosomal adhesion in keratinocytes until the formation of new autoantibodies is inhibited are highly desirable.⁷ The main goals of current research remain a better understanding of the pathophysiology of pemphigus and the identification of more specific treatment options.

Pemphigus is caused primarily by autoantibodies against the desmosomal cadherins desmoglein (Dsg) 1 for PF, Dsg3 for mPV, and Dsg1 and Dsg3 for PV;^{1,2,8} however, it is also caused by antibodies targeting other proteins.^{9,10} Desmosomes are robust adhesive cell–cell contacts,¹¹ in which desmosomal cadherins are connected to plaque proteins, which, in turn, link the desmosome to the keratin cytoskeleton.^{12,13} Desmosomes provide mechanical stability and also partake in cell signalling.¹⁴ In pemphigus, loss of desmosomal adhesion is

caused by both direct inhibition of cadherin interaction and intracellular signalling.^{7,15–18}

It is well known that the clinical pemphigus phenotypes correlates with autoantibody profiles. Anti-Dsg1 IgG modulates specific signalling pathways and is required for epidermal loss of adhesion and blistering.^{1,19,20} In human keratinocytes, PV IgG has been demonstrated to activate phospholipase C- γ 1 (PLC), generating inositol 1,4,5-trisphosphate (IP3), and induce Ca²⁺ influx.²¹ PF IgG induces Ca²⁺ influx even under conditions when Dsg2 or Dsg3 were depleted, indicating a Dsg1-dependent mechanism.²² In line with this, an interaction between Dsg1 and phosphatidylinositol 4-kinase α (PI4K), an upstream kinase of PLC,²³ had been predicted.²⁴ Further downstream, IP3 is well established to activate the IP3 receptor (IP3R), which releases Ca²⁺ from the endoplasmic reticulum (ER) into the cytosol.²⁵ Finally, cytosolic Ca²⁺ activates protein kinase C (PKC),²⁶ which has been shown to be important in pemphigus IgG-induced loss of keratinocyte adhesion and skin blistering, resulting in depletion of Dsg3, keratin retraction and the loss of cell adhesion *in vitro*.^{13,27–31} However, the exact mechanisms involved in the Ca²⁺ pathway and its pathogenic role in pemphigus remain unknown and were therefore investigated in this study.

Materials and methods

Cell culture

For *in vitro* experiments, primary normal human epidermal keratinocytes (NHEK) in passage 2–6 or HaCaT cells were used and cultured as previously described.³¹ Cells were

incubated with vehicle or mediators at a dilution of 1 : 50 in dimethyl sulfoxide (DMSO) for 1 h before IgG treatment. See Table 1 for details of the PV IgG used in this study.

Immunostaining

Cells were grown on glass coverslips and fixed with 2% paraformaldehyde for 5 min or in ethanol (at -20 °C) shaken on ice for 30 min and acetone (at -20 °C) for 3 min. Paraformaldehyde-fixed cells were permeabilized with 1% Triton X-100 in phosphate-buffered saline (PBS) for 5 min and blocked with 3% bovine serum albumin (BSA) and 1% normal goat serum in PBS for 30 min. Primary antibodies were applied overnight at 4 °C. Cy3-coupled goat antirabbit/mouse/human secondary antibodies (Dianova, Hamburg, Germany) were incubated with Alexa FluorTM 488 phalloidin (Life Technologies, Carlsbad, CA, USA) for 1 h and with 4',6-diamidino-2-phenylindole (DAPI) 1 : 10 000 for 15 min. The cover slips were mounted with n-propyl gallate 2% and evaluated with a SP5.II confocal microscope equipped with a × 63 NA 1.4 PL APO objective (Leica, Wetzlar, Germany). After heating to 60 °C for 30 min, skin slices were processed in a similar way, except that they were permeabilized for 1 h. Samples were washed three times with PBS between each step of the staining protocol except between the transition from ethanol to acetone.

Ratiometric intracellular Ca²⁺ measurements

Fura-2-acetoxymethyl ester (Thermo Fisher Scientific, Waltham, MA, USA) was used to measure intracellular Ca²⁺ in real time, as described previously.²² For each independent experiment, the signals from eight of 15 randomly selected cells were evaluated (occasional nonresponding cells were not included; very rare oscillating cells and weak responders were included, n = 4).

Co-immunoprecipitation

NHEKs were cultured in T75 flasks, washed once with PBS + 10 g L⁻¹ ethylenediaminetetraacetic acid and twice with PBS. For cell lysis, 1 mL Ca²⁺-free PBS with 68.5 mmol L⁻¹ half-normal NaCl + 1% TritonTM X-100 + 1% nonoxinol-

40 + 0.1% sodium dodecyl sulfate (SDS) + CompleteTM (Merck, Kenilworth, NJ, USA) was used, shaking for 15 min on ice. Buffer without SDS, 0.5% Triton X-100 and 0.1% nonoxinol-40 was used for washing. The cells were mechanically detached and sheared with a 5 mL syringe and 10 G needle (B. Braun, Melsungen, Germany) 10 times. The resulting suspension was centrifuged at 4 °C for 15 min at 17 949 g, and the pellet was removed. The amount of protein was determined with a commercial Pierce BCA protein assay kit (Thermo Fisher Scientific). The supernatant was added to buffer-washed agarose G beads (Millipore, Burlington, MA, USA) adding about 600–1000 µg protein. After 1.5 h on a rotator, the beads were removed via centrifugation for 2 min at 4 °C (6797 g) and the supernatant was mixed with 1.5 µL antibody or normal rabbit IgG, 1 mmol L⁻¹ Ca²⁺ and 0.5 mmol L⁻¹ Mg²⁺, and rotated for 3 h. The mixture was added to washed beads and rotated at 4 °C overnight. The beads were washed three times (1 min at 4 °C; 959 g). Proteins were released from the beads by washing three times with 27 µL Lämmli buffer at 95 °C, and beads were then removed by centrifugation (5 min at 4 °C; 6797 g).

Cell lysis, gel electrophoresis and Western blotting

Cells were cultured in 24-well plates. Lysates were fractionated into a soluble and insoluble fraction using Triton extraction buffer. Lysis electrophoresis and Western blotting were performed as described previously.³¹ Antibodies were incubated overnight at 4 °C in 5% BSA in Tris-buffered saline with Tween 20 1 : 1000, except for anti-pPKCα (1 : 20 000).

Dispase-based dissociation assay

Dissociation assays were performed as described previously,³¹ subjecting the cells to IgG for either 2 h or 24 h.

Human skin samples

Skin biopsies from donors who had been deceased for < 24 h were used. Each piece of epidermis (~4 cm²) was excised from the shoulder region and divided into ~1 cm² pieces.

Either 50 µL U-73122 (4 µmol L⁻¹), Xest (2 µmol L⁻¹) in DMSO/PBS 1 : 50 or vehicle were injected intraepidermally, followed by floating incubation as described previously.³² After 1 h incubation, either PV2 or IgG control was injected. Injections were performed as described previously.³²

Haematoxylin and eosin staining

Samples were embedded in tissue freezing medium (Leica Biosystems, Nußloch, Germany) and 7-µm sections were done using a CryoStarTM NX70 Kryostat (Thermo Fisher Scientific). The resulting sections were stained with haematoxylin and eosin according to standard protocols. For morphometric analysis, images were captured at × 200 magnification using a light DM18 microscope (Leica).

Table 1 Pemphigus vulgaris (PV) IgG used in this study

IgG	ELISA score for anti-Dsg1	ELISA score for anti-Dsg3
	IgG (U mL ⁻¹)	IgG (U mL ⁻¹)
PV1	1207.00	3906.00
PV2	57.60	177.00
PV3	981.00	698.00
mPV1	0.00	849.00
mPV2	0.00	2293.00
PF	215.34	6.26

Dsg, desmoglein; ELISA, enzyme-linked immunosorbent assay; mPV, mucosal pemphigus; PF, pemphigus foliaceus.

Statistical analysis

Data were analysed in Excel (Microsoft, Redmond, WA, USA) and compared using a one- or two-way ANOVA followed by a Bonferroni post-hoc test (for Gaussian-distributed samples) using GraphPad Prism (GraphPad Software, La Jolla, CA, USA). Significance was assumed with a *P*-value of ≤ 0.05 . Data are shown as mean (SEM).

Results

Pemphigus IgG induces desmoglein 1-dependent Ca²⁺ flux via inositol 1,4,5-triphosphate receptor and Ca²⁺ release-activated channel *in vitro*

Firstly, we confirmed that the main components of the Ca²⁺ pathway are expressed in human keratinocytes. As outlined

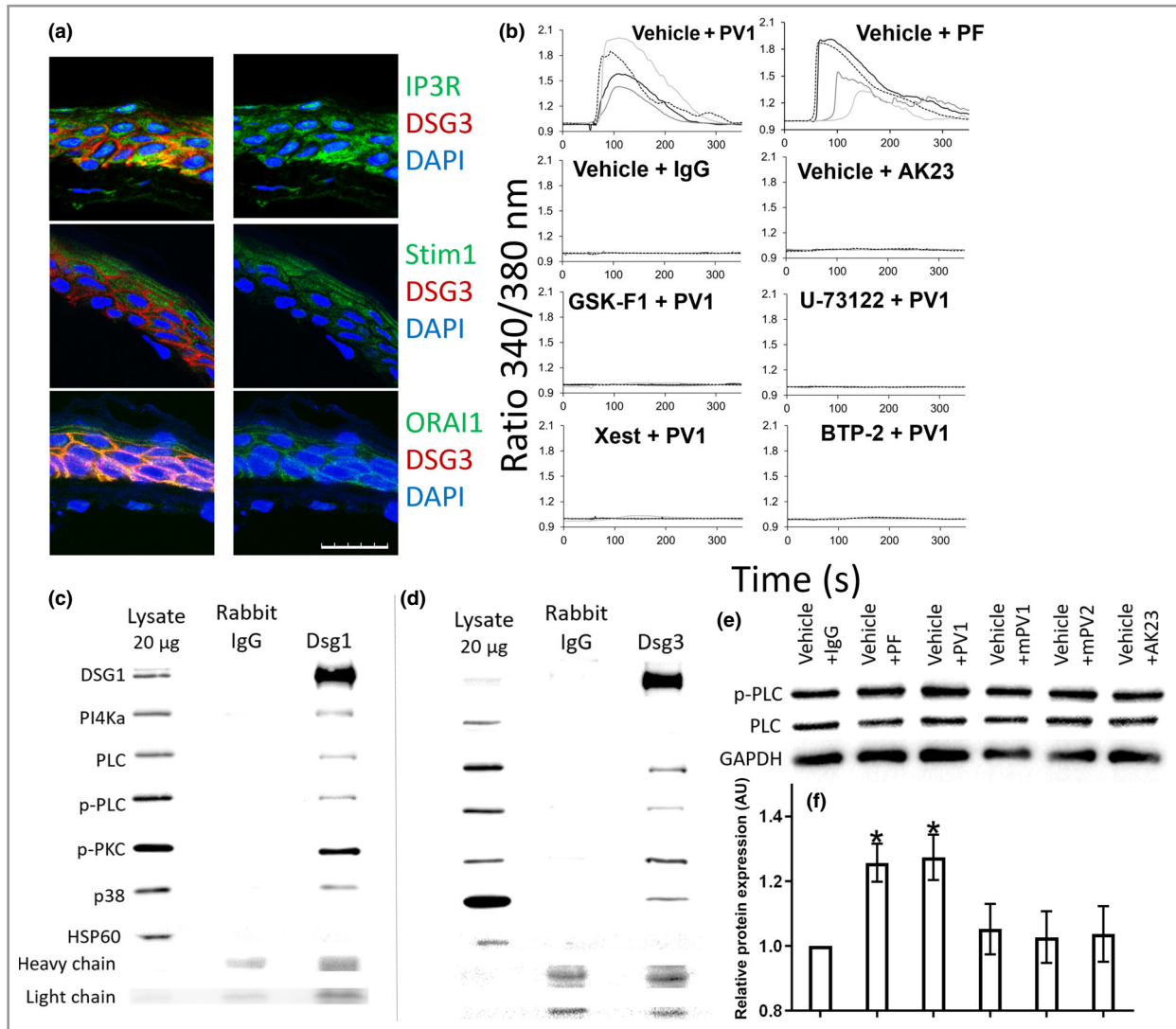


Figure 1 (a) Immunostaining of Ca²⁺ channel proteins in human skin sections (*n* = 3, scale bar 25 μm). (b) Ca²⁺ flux measurements upon IgG addition performed using fura-2-acetoxymethyl ester in normal human epidermal keratinocyte (NHEK) cells. (c) Co-immunoprecipitation experiments using NHEK cells, precipitating with antidesmoglein (Dsg) 1 IgG (*n* ≥ 3). (d) Co-immunoprecipitation experiments using NHEK cells, precipitating with anti-Dsg3 IgG (*n* ≥ 3). (e) Representative Western blot showing the phosphorylation of phospholipase C-γ1 (PLC) after 30 min of IgG treatment. (f) Quantification of PLC phosphorylation in a Western blot after 30 min of IgG treatment (*n* = 6). Error bars represent the SEM. The asterisk indicates a significant difference compared to the control condition. AU, arbitrary units; DAPI, 4',6-diamidino-2-phenylindole; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HSP, heat shock protein; IP3R, inositol 1,4,5-triphosphate receptor; mPV, mucosal pemphigus; ORAI1, calcium release-activated calcium channel protein 1; PF, pemphigus foliaceus; PI4Kα, phosphatidylinositol 4-kinase α; PKC, protein kinase C; PV, pemphigus vulgaris; STIM1, stromal interaction molecule 1.

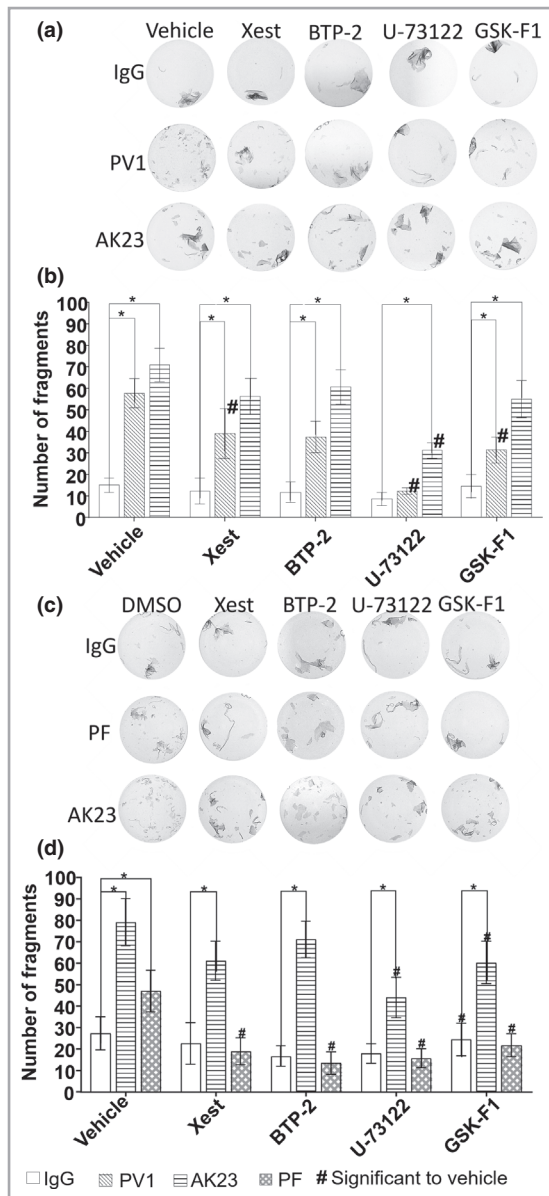


Figure 2 (a) Representative dissociation assay results after treatment with pemphigus vulgaris (PV) 1 IgG for 24 h. (b) Quantification of dissociation assay results after treatment with PV1 IgG for 24 h ($n = 6$). (c) Representative dissociation assay results after treatment with pemphigus foliaceus (PF) IgG for 24 h. (d) Quantification of dissociation assay results after treatment with PF IgG for 24 h ($n = 6$). Error bars in the represent the SEM. The asterisk indicates a significant difference compared to the control condition. DMSO, dimethyl sulfoxide.

above, IP3R redistributes Ca²⁺ from the ER to the cytosol, and low ER Ca²⁺ activates stromal interaction molecule 1 (STIM1) in the ER membrane. Secondly, STIM1 contacts calcium release-activated calcium channel protein 1 (ORAI1) to form the Ca²⁺ release-activated channel (CRAC),³³ to replenish the Ca²⁺ store in the ER.^{34,35} We saw that IP3R and ORAI1 were evenly distributed in all epidermal layers, whereas STIM1 was

more prominent in the granular layer (Figure 1a). Similarly to previous studies,³¹ PV and PF IgG (Table 1), but not IgG from healthy volunteers or AK23 or mPV IgG, increased intracellular Ca²⁺ in NHEK cells (Figure 1b and Figure S1a; see Supporting Information). mPV IgG was used in addition to AK23, to avoid clonal differences being overlooked, which has been reported previously.³⁶ In agreement with previous reports,^{22,31} the Ca²⁺ flux correlated with the presence of anti-Dsg1 IgG. The inhibition of CRAC, IP3R, PLC or PI4K stopped the Ca²⁺ influx caused by pemphigus IgG (Figure 1b and Figure S1a). Co-immunoprecipitation demonstrated that PI4K interacts with Dsg1 but not with Dsg3. In contrast, the downstream targets of PI4K, such as activated phosphorylated PKC and PLC, as well as p38 mitogen-activated protein kinase (MAPK), are well known to be involved in the pathogenesis of pemphigus,^{32,37} and are part of both Dsg1- and Dsg3-orchestrated complexes (Figure 1c, d and Figure S1b). This is important because, so far, only the interaction of p38 MAPK with Dsg3 has been shown.^{38,39} PV IgG and PF IgG, but not AK23 or mPV IgG, induced phosphorylation of PLC (Figure 1e, f). Taken together, these data indicate the existence of a Dsg1-specific signalling complex, which, in response to the binding of pemphigus IgG, can initiate a Ca²⁺ flux-dependent pathway.

Inhibition of Ca²⁺ flux is protective against anti-desmoglein 1 IgG-induced pathogenic effects *in vitro* and human skin *ex vivo*

Next, we evaluated the role of Ca²⁺ signalling in the loss of keratinocyte adhesion *in vitro*. PV IgG caused loss of keratinocyte adhesion in dispase-based dissociation assays in NHEKs after 2 h and 24 h and PF IgG after 24 h (Figure 2 and Figure S2; see Supporting Information). mPV did not affect adhesion in NHEKs but was effective in HaCaT cells (Figure S2). Treatment with inhibitors against PI4K, PLC, IP3R or CRAC added 1 h before pemphigus autoantibodies effectively blocked the cell sheet fragmentation after 2 h and 24 h. After 24 h, inhibition of PLC even reduced the pathogenic effect of AK23 on cell adhesion (Figure 2 and Figure S2).

To evaluate the underlying mechanisms, immunostaining for desmosomal and cytoskeletal components was employed. After being subjected to PV IgG for 24 h, immunostaining for Dsg1 and Dsg3 showed a fragmented pattern at cell borders and partially relocated to the cytosol (Figure 3a and Figure S3a–c; see Supporting Information). For PF IgG and mPV IgG the localization of Dsg1 and Dsg3, respectively, was affected. Treatment with inhibitors for PI4K, PLC, IP3R or CRAC ameliorated the pathogenic effects. In contrast to PV IgG-induced fragmentation of Dsg3 staining, mPV IgG-induced fragmentation of Dsg3 staining was not affected (Figure S3).

In addition to reorganization of desmosomal cadherins, keratin filaments retracted from the cell borders after 24 h of pemphigus IgG treatment (except mPV2). All inhibitors ameliorated the effect (Figure 3b and Figure S3d). Similarly,

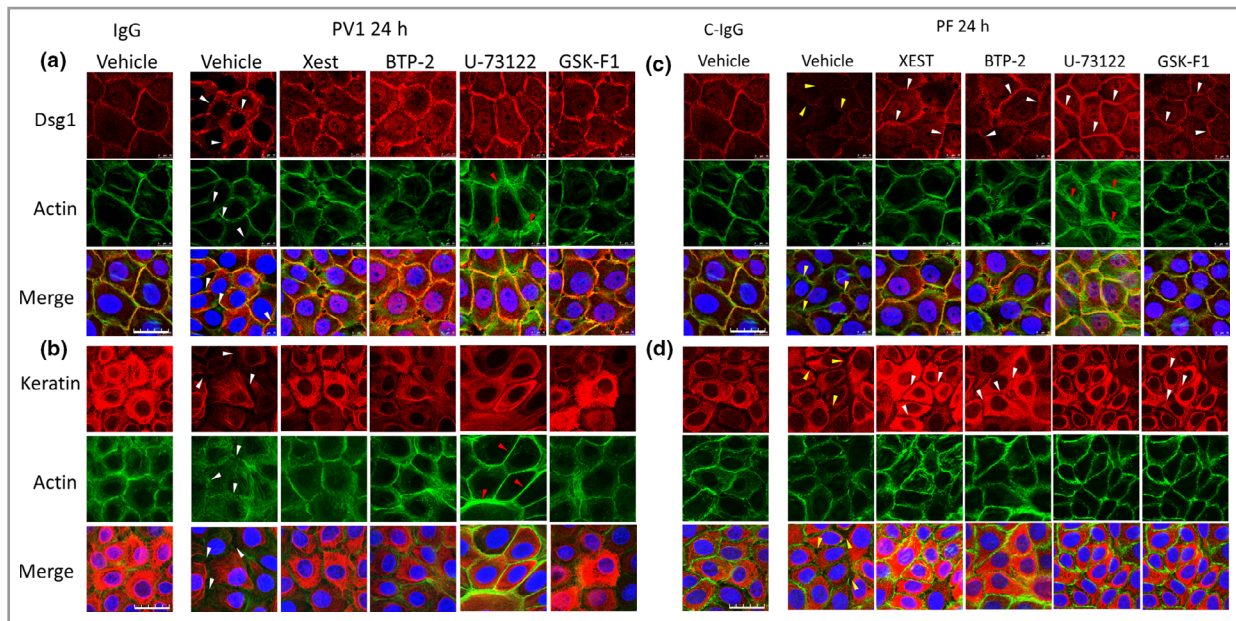


Figure 3 (a) Immunostaining of desmoglein (Dsg) 1 (rabbit phosphorylated antibody; Abclonal, Woburn, MA, USA) in normal human epidermal keratinocyte (NHEK) cells after 24 h pemphigus vulgaris (PV) 1 IgG treatment. (b) Immunostaining of keratin filaments in NHEK cells after 24 h PV1 IgG treatment. (c) Immunostaining of Dsg1 in NHEK cells after 24 h pemphigus foliaceus (PF) IgG treatment. (d) Immunostaining of keratin filaments in NHEK cells after 24 h PF IgG treatment. White arrows indicate pemphigus IgG-induced fragmentation of Dsg1 staining or keratin and actin reorganization; red arrows indicate strengthening of the cortical actin ($n \geq 3$, scale bar 25 μm).

cortical F-actin was disrupted, which was ameliorated by all inhibitors (Figure 3 and Figure S3). Interestingly, F-actin staining often appeared more pronounced after PLC inhibition (Figure 3 and Figure S3).

Finally, we tested whether interference with Ca²⁺ signalling was effective in ameliorating the pathogenic effects of PV IgG in intact human skin *ex vivo*. AK23 and mPV IgG were omitted as, in our model – similar to the situation in patients with pemphigus – both have been shown to cause mucosal blistering only and not epidermal blistering.^{32,40} Human skin samples subjected to PV IgG *ex vivo* showed typical suprabasal blistering with a characteristic tombstone pattern of basal keratinocytes (Figure 4 and Figure S4; see Supporting Information). PV IgG was not only enriched at the blistering sites, but was also detectable in unaffected regions, where it was located at cell borders (Figure S4a). Similarly to cultured keratinocytes Dsg1 and Dsg3 staining was fragmented and redistributed to the cytosol (Figure 4c and Figure S4b), while F-actin staining was almost abolished at blistering sites (Figure 4d). Importantly, blistering was completely blocked by inhibition of PLC or IP3R (Figure 4 and Figure S4).

Discussion

The data presented here demonstrate, for the first time, that Dsg1-dependent Ca²⁺ influx plays a central role in the epidermal pathology of pemphigus. Most importantly, inhibition of Ca²⁺ signalling blocked pemphigus IgG-induced loss of keratinocyte adhesion *in vitro* and blistering in human skin *ex vivo*. PV IgG- and PF IgG-containing autoantibodies against Dsg1

induced PLC activation and Ca²⁺ influx, which were required for the reorganization of Dsg1 and Dsg3, as well as of the keratin and actin cytoskeleton (Figure 5). These observations are in line with early reports demonstrating that PLC-mediated Ca²⁺ influx is the earliest signalling event in pemphigus.²¹ The second novelty of this study is that Dsg1 and Dsg3 form signalling complexes in which they associate with several molecules involved in pemphigus pathogenesis such as PLC, PKC and p38 MAPK. Previously, only the association of p38 MAPK with Dsg3 has been demonstrated.³⁸

PLC appears to be one of the most promising pharmaceutical targets of this pathway. For interfering with PV IgG-induced loss of cell adhesion *in vitro*, inhibition of PLC was most efficient. After 24 h of incubation, loss of adhesion was completely abolished. Moreover, loss of adhesion induced by AK23, which did not cause Ca²⁺ influx or enhance baseline PLC activity, was significantly ameliorated by inhibiting PLC. This may indicate that PLC targets such as PKC might be activated by baseline PLC activity. Alternatively, PLC may regulate other targets involved in pemphigus pathogenesis such as p38 MAPK or actin remodelling via Ras homolog family member A.^{41,42} Finally, it has to be noted that pemphigus IgG against thyroid peroxidase and other targets might also be able to activate PLC isoforms and Ca²⁺ flux,^{9,43,44} which also suggests that inhibition of PLC may be effective to treat pemphigus.

The data presented here suggest that PLC-mediated Ca²⁺ influx is dependent on Dsg1 (Figure 5). In line with this, PV IgG and PF IgG induced PLC phosphorylation and Ca²⁺ influx, whereas AK23 and mPV IgG, both of which target Dsg3 but not Dsg1, did not trigger this pathway. This may be explained

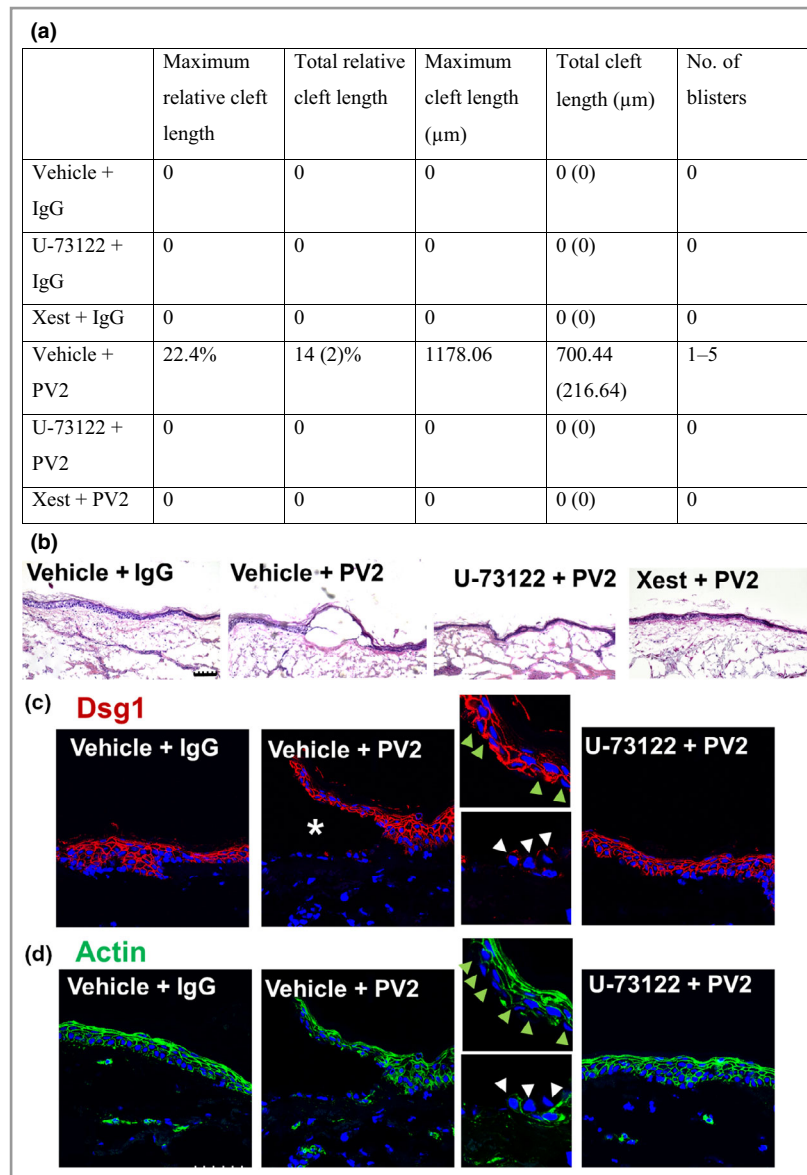


Figure 4 (a) Quantitative evaluation of blister formation in human skin slices *ex vivo*. (b) Representative microscopic images of haematoxylin and eosin staining of human skin slices after 24 h IgG treatment ($n > 3$). (c) Immunostaining of desmoglein (Dsg) 1 (mouse monoclonal antibody; Progen, Heidelberg, Germany) in human skin slices after pemphigus vulgaris (PV) 2 treatment. (d) F-actin staining in human skin slices after PV2 treatment. Each set of images shows a $\times 4$ zoom of the blister roof and bottom. Green arrows indicate missing staining at the cell border; white arrows indicate the remaining basal cells (tombstoning) with reduced staining ($n = 3$, scale bar 50 μm).

by the fact that signalling molecules such as p38 MAPK, PLC and PKC α form signalling complexes with both Dsg1 and Dsg3. In contrast, PI4K α , the most upstream kinase of this pathway, is associated with Dsg1 only. This may also explain why Dsg3 localization is affected by the Ca²⁺ flux pathway, although autoantibodies binding to Dsg3 cannot initiate signalling. We noted that mPV IgG with antibodies against Dsg3 but not Dsg1 were insufficient to cause the loss of adhesion in primary keratinocytes, despite causing severe fragmentation of Dsg3 staining. This reflects the situation in patients where the unaffected skin of mucosal PV also displays severe fragmentation of Dsg3 localization,^{45,46} and the *ex vivo* skin model where

mPV IgG, similar to AK23, does not cause blistering.³² In line with this, fragmentation of Dsg3 staining induced by PV IgG, but not by mPV IgG, was ameliorated by inhibiting the Ca²⁺ flux pathway, indicating that different mechanisms are involved in mucosal PV. Similarly, it was reported that inhibition of p38 MAPK was protective against blistering in skin but not in mucosa.⁴⁷ Nevertheless, the mPV IgG fraction used in our study caused significant cell sheet fragmentation in HaCaT cells, indicating that primary keratinocytes better reflect the role of DSG3 for keratinocyte adhesion.

PKC isoforms are one of the primary downstream targets of the Ca²⁺ pathway. PKC affects cell adhesion in several ways.

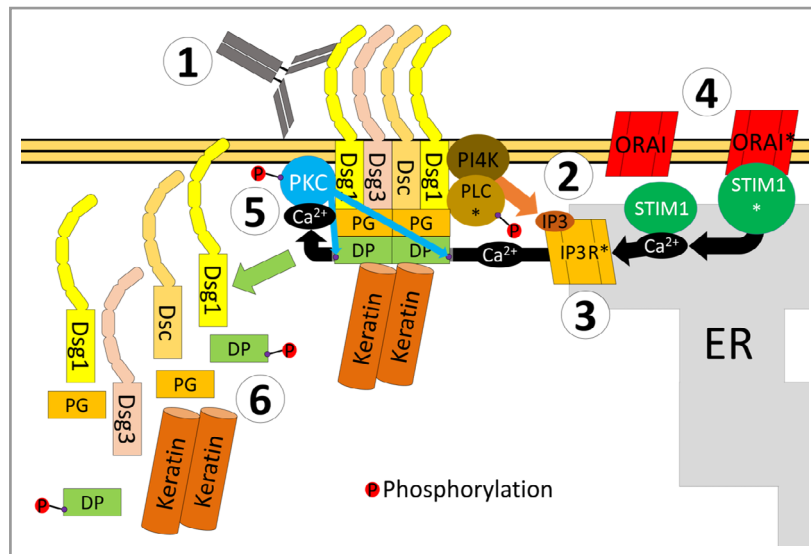


Figure 5 Schematic of the Ca²⁺ flux-dependent signalling pathway in keratinocytes upon pemphigus vulgaris (PV) IgG binding. (1) Binding of pemphigus IgG to desmoglein (Dsg) 1, associated with phosphatidylinositol 4-kinase alpha (PI4K) and phospholipase C-γ1 (PLC), (2) activates PLC and releases inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG). (3) IP3 activates IP3 receptor (IP3R), releasing Ca²⁺ from the endoplasmic reticulum (ER) into the cytosol. (4) Low ER Ca²⁺ activates stromal interaction molecule 1 (STIM1) at the ER membrane. STIM1 contacts calcium release-activated calcium channel protein 1 (ORAI1), forming Ca²⁺ release-activated channel (CRAC), causing Ca²⁺ influx from the extracellular space. With the IP3R still active, this further increases the concentration of Ca²⁺ in the cytosol. (5) Cytosolic Ca²⁺ and DAG activate protein kinase C (PKC), (6) which destabilizes the desmosome by keratin retraction and depletion of Dsg1 and Dsg3. The asterisk indicates a significant difference compared to the control condition. DP, desmoplakin; Dsc, desmocollin; PG, plakoglobin.

The stability of newly formed desmosomes is dependent on extracellular Ca²⁺.⁴⁸ After several days, desmosomes become hyperadhesive and Ca²⁺ independent.^{49,50} In this state, they are also less sensitive to PV IgG.^{27,51} Inhibiting PKC results in a rapid transformation from Ca²⁺-dependent to Ca²⁺-independent desmosomes.^{49,50} Upon PV IgG treatment PKC has been reported to translocate to the desmosomal plaque.²⁶ They phosphorylate desmoplakin, destabilizing the desmosome,^{12,13} alter Dsg3 localization and cause acantholysis in passive immune transfer mouse models of pemphigus.^{29–31,47} Cytoskeletal components such as keratin 8 and 18 are also substrates of PKC,^{52–54} possibly affecting keratin filament retraction. Similarly, PKC affects the actin cytoskeleton, mostly causing disassembly and reduced anchorage,⁵⁵ and possibly modulates desmosome turnover via the actin-binding adducin.⁵⁶

However, results concerning the role of PKC in the regulation of desmosomes are often contradictory. Specifically, inhibiting PKCβ was not sufficient to prevent fragmentation *in vitro*.⁷ In line with this, the targets of PKCβ specifically seem to be of little relevance for cell adhesion.⁵⁴ Inhibiting other PKCs ameliorated PV IgG-induced loss of adhesion *in vitro* but were not sufficient to block blistering in human skin *ex vivo*.^{27,31,40} However, it has to be borne in mind that there are many isoforms of PKC and at least three species are activated by different mechanisms, including diacylglycerol (DAG) and cytoplasmic Ca²⁺ for calcium-dependent PKC (cPKC), DAG for novel (nPKC) and translocation for atypical, respectively.²⁶ At least cPKCs and nPKCs are downstream

targets of PLC and thus may be activated in response to autoantibodies in pemphigus.

Taken together, the data from this study support the hypothesis that PV pathology is dependent on patients' autoantibody profiles, with different anti-Dsg1/Dsg3 ratios and target epitopes playing an important role by eliciting different cellular responses.^{1,19} Similarly, different signalling responses to PV IgG, PF IgG and AK23 have been previously reported to be dependent on the presence of autoantibodies targeting Dsg1, Dsg3 or desmocollin.^{22,31,57} Therefore, the data presented here help to explain why autoantibodies against Dsg1 but not against Dsg3 are required for epidermal involvement and why the phenotypes of PF, PV and mPV are clinically different. These insights may help to develop novel Dsg1-specific treatment strategies for pemphigus.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Figure S1 (a) Ca²⁺ flux measurements upon IgG addition. (b) Co-immunoprecipitation experiments using normal human epidermal keratinocytes.

Figure S2 (a) Representative dissociation assay results after treatment with PV3 for 2 h. (b) Quantification of dissociation assay results after treatment with PV3 for 2 h. (c) Representative dissociation assay results after treatment with PV3 for 24 h. (d) Quantification of dissociation assay results after treatment with PV3 for 24 h.

Figure S3 (a) Immunostaining of desmoglein (Dsg) 3 in normal human epidermal keratinocyte (NHEK) cells after PV1 treatment for 24 h. (b) Immunostaining of Dsg3 in NHEK cells after PV3 treatment for 24 h. (c) Immunostaining of Dsg1 in NHEK cells after PV3 treatment for 24 h. (d) Immunostaining of the cytokeratin filaments in NHEK cells after PV3 treatment for 24 h.

Figure S4 (a) Immunostaining of PV2 with goat antihuman IgG Cy3. (b) Immunostaining of desmoglein 3 in human skin slices after PV2 treatment.