Published in final edited form as:
J Immunol. 2010 December 1; 185(11): 6831–6838. doi:10.4049/jimmunol.1002675.

Protective Endogenous Cyclic Adenosine 5′-Monophosphate Signaling Triggered by Pemphigus Autoantibodies

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Abstract

Pemphigus vulgaris (PV) is an autoimmune skin disease mediated by autoantibodies directed against the cadherin-type cell adhesion molecules desmoglein (Dsg) 3 and Dsg1 and is characterized by loss of keratinocyte cohesion and epidermal blistering. Several intracellular signaling pathways, such as p38MAPK activation and RhoA inhibition, have been demonstrated to be altered following autoantibody binding and to be causally involved in loss of keratinocyte cohesion. In this paper, we demonstrate that cAMP-mediated signaling completely prevented blister formation in a neonatal pemphigus mouse model. Furthermore, elevation of cellular cAMP levels by forskolin/rolipram or β receptor agonist isoproterenol blocked loss of intercellular adhesion, depletion of cellular Dsg3, and morphologic changes induced by Ab fractions of PV patients (PV-IgG) in cultured keratinocytes. Incubation with PV-IgG alone increased cAMP levels, indicating that cAMP elevation may be a cellular response pathway to strengthen intercellular adhesion. Our data furthermore demonstrate that this protective pathway may involve protein kinase A signaling because protein kinase A inhibition attenuated recovery from PV-IgG–induced cell dissociation. Finally, cAMP increase interfered with PV-IgG–induced signaling by preventing p38MAPK activation both in vitro and in vivo. Taken together, our data provide insights into the cellular response mechanisms following pemphigus autoantibody binding and point to a possible novel and more specific therapeutic approach in pemphigus.

Pemphigus with the main variant pemphigus vulgaris (PV) is a blister-forming disease affecting skin and mucous membranes (1). Erosions may develop in the epithelium of mucous membranes only or may be paralleled by blistering within the epidermis. PV patients usually have autoantibodies against the cell adhesion molecules desmoglein (Dsg) 3 corresponding to the involvement of mucous membranes. In those PV patients with additional Dsg1 autoantibodies, skin lesions also develop. Dsgs, like desmocollins, are Ca^{2+}-dependent cadherin-type adhesion molecules that localize to the plasma membrane and are key components of the desmosomal adhesion complex. By interacting with desmosomal cadherins of neighboring cells with the extracellular N-terminal domain and by connecting to the intermediate filament network via adaptor molecules plakoglobin, plakophilin, and desmoplakin, they provide intercellular adhesive strength (2).

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Disclosures

The authors have no financial conflicts of interest.

It is a matter of debate whether loss of intercellular adhesion is the result of direct inhibition of Dsg transinteraction by pemphigus Abs or alterations of intracellular signaling events following Ab binding (3, 4). We have demonstrated that PV autoantibodies are capable to directly inhibit Dsg3 transinteraction, whereas in pemphigus foliaceus, direct inhibition was not detectable (5–7). In addition, a crucial role for multiple signaling events in loss of intercellular adhesion, such as plakoglobin, p38MAPK activation, or RhoA inhibition has been demonstrated (8–10). Up to now, therapy of pemphigus is limited to unspecific suppression of the immune system with according severe adverse events. Taken into account the necessity of an often lifelong treatment, novel and more specific therapy options of pemphigus are highly eligible.

Similar to Dsgs in epithelial cells, the endothelial-specific vascular endothelial (VE) cadherin contributes to adhesion of adjacent endothelial cells lining the lumen of blood vessels, which is a prerequisite to form a selective barrier between the blood and the interstitium (11). Second messenger cAMP is known to be one of the most potent barrier-protective signaling molecules both in vivo and in vitro (12, 13). This is at least in part attributed to increased VE-cadherin–mediated adhesion (14, 15). Therefore, in view of the apparent role of cAMP for enhancing cadherin-mediated intercellular adhesion of endothelial cells, the aim of this study was to investigate whether cAMP may also be involved in the regulation of desmosomal adhesion and may be sufficient to prevent loss of intercellular adhesion induced by pemphigus-IgG. Our data demonstrate that increased cAMP protects against the pathogenic effects of PVAbs both in vivo and in vitro. Furthermore, incubation with PV Abs augmented keratinocyte cAMP levels. This identifies cAMP signaling as part of an intracellular rescue pathway in response to PV Ab-mediated loss of cell adhesion, which may be used as novel treatment option in this disease.

Materials and Methods

Cell culture and test reagents

As previously described (16), the spontaneously immortalized keratinocyte cell line HaCaT was grown in DMEM (Invitrogen Life Technologies, Karlsruhe, Germany) containing 1.8 mM Ca^{2+} supplemented with 10% FCS (Biochrom, Berlin, Germany), 50 U/ml penicillin G, and 50 μg streptomycin. Cells were maintained in a humidified atmosphere containing 5% CO_2 at 37°C. Confluent monolayers 3 d after seeding were used throughout all experiments. Both forskolin and rolipram were from Sigma-Aldrich (Munich, Germany) and used at 5 and 10 μM, respectively. Isoproterenol (Iso) (Sigma-Aldrich) was used at 100 μM in vitro and 10 μM for injection in neonatal mice. H89 (Sigma-Aldrich) was applied at 10 μM. Propranolol (Prop) (Sigma-Aldrich) was used at 10 μM.

Purification of PV-IgG fractions

Sera were obtained from PV patients and purified by protein A-agarose chromatography as described earlier (7). IgG from four different PV patients was used in this study. The disease was confirmed by 1) the typical clinical appearance, 2) direct immunofluorescence microscopy of a perilesional biopsy, and 3) the detection of anti-Dsg3 and/or anti-Dsg1 Abs by ELISA (Euroimmun, Lübeck, Germany). ELISA values of the four PV-IgG fractions used are shown in Table I.

Measurement of cAMP levels

Cellular cAMP levels were measured following PV-IgG treatment for indicated time periods and after addition of cAMP elevating agents. cAMP levels were detected using an ELISA-based cAMP enzyme immunoassay kit (Sigma-Aldrich), according to the manufacturer's instructions.

Electrophoresis and Western blotting

Cells were grown in 12-well plates for 3 d, incubated with PV-IgG or PV-IgG and forskolin/rolipram (F/R) for 24 h, and lysed in Laemmli buffer for 10 min on ice. Skin samples derived from the neonatal mouse model were grinded with 10 strokes of a homogenizer in Laemmli buffer. Lysates were then subjected to gel electrophoresis and Western blotting according to standard procedures as described elsewhere (5). For immunodetection of Dsg3, a polyclonal Ab (clone H-145; Santa Cruz Biotechnology, Heidelberg, Germany) was applied in a 1:1000 dilution. Total and phosphorylated p38MAPK (phospho-Y180/182) was detected using mAbs (New England Biolabs, Frankfurt, Germany) in a 1:1000 dilution.

Immunofluorescence

HaCaT cells were grown to confluence and incubated with PV-IgG and various agents as described previously. Finally, cells were fixed with ice-cold acetone for 2 min. Following several rounds of washing with PBS, cells were incubated with PBS containing 10% BSA and 3% normal goat serum for 30 min. A monoclonal Dsg3 Ab (Invitrogen, Karlsruhe, Germany) was used as primary Ab in a 1:100 dilution overnight at 4°C, followed by several rinses with PBS. Finally, cells were incubated with a Cy3-labeled goat-anti-mouse secondary Ab (1 h, 1:600; Dianova, Heidelberg, Germany) and mounted on glass slides with 60% glycerol in PBS containing 1.5% *n*-propyl gallate (Serva, Heidelberg, Germany) as anti-fading compound. Coverslips were imaged with a laser confocal setup (MRC-1024; Bio-Rad, Munich, Germany) via a Plan-Apochromat ×63/1.40 oil objective attached to a Axiovert 135 TV microscope (both Carl Zeiss Microimaging, Goettingen, Germany).

Dispase-based dissociation assay

The assay was performed essentially as described earlier (6). Briefly, cells were seeded in 12-well plates, cultured until confluence, and incubated for various times and different conditions as indicated. Following a brief rinse in HBSS (Sigma-Aldrich), dispase II (Sigma-Aldrich) was added for 30 min to release monolayer from well bottom. Dispase solution was exchanged to HBSS, and mechanical stress was applied by five times of pipetting with a 1-ml pipette. Finally, resulting fragments per well were counted under a binocular microscope.

In vivo pemphigus mouse model

All animal experiments were approved by the Regierung von Unterfranken (Az 55.2-2531.01-4/10). Neonatal mice (from 1 to 2 d of age weighing <2 g) were injected intradermally into the backskin with 50 μl IgG of a healthy volunteer (5 mg/g body weight), PV1-IgG (3 mg/g body weight), or PV4-IgG (6 mg/g body weight). Mice were preinjected with 50 μl PBS, Iso, or Prop 2 h before receiving the IgG fractions. Twenty hours later, mice were sacrificed, and skin samples were collected. Samples were either fixed in 10% formalin in PBS and subjected to H&E staining, according to standard procedures, or embedded in cryomounting medium (Reichert-Jung, Nussloch, Germany) and snap frozen. Additional skin samples were harvested for detection of p38MAPK phosphorylation by Western blotting. The frozen samples were cut with a cryostat (Frigo-Cut 2800; Reichert-Jung, Nussloch, Germany). Every 400 μm, one section was stained in 1% toluidine blue solution and examined for presence or absence of intraepidermal cleavage until the entire sample was processed. Representative cryosections were subjected to immunostaining as outlined above. The monoclonal AK23 Ab, which recognizes murine Dsg3 (Biozol, Eching, Germany), was used as primary Ab (1:100 in PBS) together with Cy3-labeled goat-anti-mouse as secondary Ab (1:600 in PBS; Dianova). Cy3-labeled goat-anti–human Ab was applied to visualize bound PV-IgG within the epidermis (1:600 in PBS; Dianova). Immunostained sections were imaged with a laser confocal setup (MRC-1024; Bio-Rad) via a Plan-Apochromat ×20/0.50

objective attached to a Axiovert 135TV microscope (both Carl Zeiss Microimaging). H&E-stained sections were photographed using a digital camera (HRP-100; Diagnostic Instruments, Sterling Heights, MI) attached to a Axiomot 2 plus microscope equipped with a Plan Neofluar ×20/0.50 objective (both Carl Zeiss Microimaging).

Statistics

Data were processed with SigmaStat (Systat Software, Erkrath, Germany). Data values were compared using one-way ANOVA, followed by Bonferroni correction or ANOVA on ranks for non-Gaussian variables followed by Student-Newman-Keuls post hoc test. Statistical significance was assumed for $p < 0.05$. Error bars in graphs represent SEM.

Results

Increase of cAMP levels prevented intraepidermal blistering in vivo

To assess the effect of cAMP on pemphigus skin blistering in vivo, we used the neonatal passive transfer model. Injection of IgG fractions of PV patients (PV-IgG) into neonatal mice induces the typical PV phenotype with flaccid skin blisters and microscopically visible suprabasal split formation (17). Newborn BALB/c mice were injected with PBS, followed by injection with PV1-IgG (Table I) or with IgG fractions from a healthy volunteer 2 h later, and formation of intraepidermal blisters was evaluated after another 20 h. The results of four independent experiments are shown in Table II. Eight of eight injected animals developed suprabasal blistering (Fig. 1*A*, 1*E–G*), whereas animals injected with control IgG showed normal histology as demonstrated by H&E staining and Dsg3 immunostaining (Fig. 1*A*, 1*B–D*). Immunostaining of Dsg3 confirmed the strictly suprabasal cleavage formation (Fig. 1*F*) in response to injection of PV-IgG. Incubation of representative sections with Cy3-labeled anti–human-Fc Ab demonstrated binding of PV-IgG within the epidermis (Fig. 1*G*), whereas control-IgG deposits were localized unspecifically within the dermis only but did not bind to keratinocytes (Fig. 1*D*, asterisk).

We next applied the β receptor agonist Iso to elevate cellular cAMP levels and as a control the β receptor antagonist Prop. Blister formation was not perturbed by preinjection with 10 μM Prop, followed by coinjection of 10 μM Prop and PV1-IgG 2 h later (Fig. 1*A*, 1*H–J*). In striking contrast, blister formation was completely inhibited by simultaneous treatment with Iso (zero of eight mice developed blistering; Fig. 1A, 1*K–M*). In this group, 10 μM Iso was preinjected 2 h before injection of another dose of 10 μM Iso together with PV1-IgG. Binding of PV1-IgG to keratinocytes was not perturbed by Iso as visualized by staining against anti–human-Fc (Fig. 1*M*). These experiments were repeated with another IgG fraction (PV4-IgG) in two independent experiments (data not shown).

Taken together, Iso-mediated cAMP increase completely blocked blister formation induced by Ab fractions of PV patients in a neonatal mouse model.

Increased cAMP prevented PV-IgG–induced cell dissociation, Dsg3 reorganization, and Dsg3 depletion in cultured keratinocytes

We then characterized the mechanisms underlying cAMP-mediated protection against PV-IgG in cultured HaCaT keratinocytes. In addition to Iso, we also applied a combination of adenylyl cyclase activator forskolin and phosphodiesterase IV inhibitor rolipram (F/R). Immunostaining of PV Ag Dsg3 was performed to visualize the morphologic changes induced by PV-IgG. In confluent HaCaT monolayers, Dsg3 was distributed linearly along cell borders (Fig. 2*A*). Incubation of PV-IgG for 24 h resulted in fragmentation and loss of Dsg3 staining (Fig. 2*B*), which was largely prevented by treatment with Iso or F/R (Fig. 2*D*,

2*F*), respectively. Incubation with Iso or F/R alone had no pronounced effect on Dsg3 staining (Fig. 2*C*, 2*E*).

Next, we applied dissociation assays to investigate whether loss of cell adhesion caused by PV-IgG was inhibited by F/R or Iso (Fig. 3*A*). Confluent HaCaT were incubated with dispase to release monolayers from well bottom. After application of mechanical stress, resulting fragment numbers per well were counted and used as measure for intercellular adhesion. Compared with controls (1.6 ± 0.7), PV2-IgG drastically increased the amount of fragments and thus reduced intercellular adhesion (165.1 ± 30.5). The loss of keratinocyte cohesion induced by PV2-IgG was significantly ameliorated by simultaneous treatment with F/R or Iso (20.5 ± 5.1 and 76.0 ± 16.4 fragments, respectively). Similar effects were observed for incubation with PV3-IgG (data not shown).

Internalization and subsequent depletion of Dsg3 levels have been attributed to mediate loss of keratinocyte cohesion in response to PV-IgG (18, 19). Thus, we investigated the effect of increased cAMP on PV-IgG–induced depletion of Dsg3 levels. PV3-IgG significantly reduced the total amount of cellular Dsg3 levels to 72 ± 5% of controls after 24 h. Simultaneous incubation with F/R completely abrogated Dsg3 depletion (97 ± 10%).

To confirm the effect of the mediators applied, we measured cellular cAMP levels in HaCaT keratinocytes (Fig. 4C). Both F/R and Iso drastically increased keratinocyte cAMP levels (8.8 ± 0.3- and 8.6 ± 0.3-fold, respectively) after 24 h compared with controls.

Taken together, these experiments demonstrate that increased cAMP is sufficient to largely prevent cell dissociation as well Dsg3 redistribution and depletion in response to PV-IgG treatment in vitro.

PV-IgG–induced elevation of cAMP levels

The experiments from above clearly demonstrate a protective effect of cAMP elevation on pemphigus autoantibody-induced loss of keratinocyte cohesion. Thus, we asked whether PV-IgG interferes with cAMP signaling and reduces cAMP levels to weaken intercellular adhesion. However, keratinocyte cAMP levels were augmented at the time points 1, 6, and 24 h after addition of two PV-IgG to the culture media (Fig. 4A). PV2-IgG augmented cAMP 1.3 ± 0.3, 1.8 ± 0.3, and 1.6 ± 0.3 compared with controls, respectively. PV3-IgG was similarly effective leading to an increase to 1.8 ± 0.4, 2.2 ± 0.2, and 1.4 ± 0.1). To confirm that PV-IgG was effectively reducing keratinocyte adhesion at time points of elevated cAMP levels, we applied dissociation assays that yielded an increase of fragments to 50.8 ± 12.0, 127.2 ± 19.1, and 165.1 ± 30.5 at 1, 6, and 24 h, respectively, when using PV2-IgG (Fig. 4*B*). Taken together, because elevation of cAMP levels on one hand was protective against PV-IgG–induced loss of cell cohesion and on the other hand PV-IgG incubation alone increased keratinocyte cAMP, we hypothesized that a cellular rescue pathway exists involving cAMP signaling.

cAMP promotes recovery from pathogenic pemphigus effects

To further strengthen our hypothesis that cAMP signaling serves as a cellular rescue pathway, we investigated whether cAMP signaling may be involved in the recovery of keratinocytes following PV-IgG treatment. Subsequently, we incubated cultured keratinocytes with PV-IgG for 24 h, exchanged media to remove excess PV-IgG, and evaluated recovery to the normal adhesive state over time by dissociation assays. Monolayers incubated for 24 h with PV2-IgG demonstrated 165 ± 20 fragments (Fig. 5*A*). As early as 1 h after medium exchange, the amount of fragments was reduced to 83 ± 9. Fragment numbers further decreased by time to 66 ± 21 at 2 h, 44 ± 6 at 4 h, and 18 ± 6 at 6 h. Twelve hours after medium exchange, fragment numbers were similar to those in controls

(2 ± 0.3 versus 5 ± 3). To investigate whether cAMP signaling enhances recovery, we chose the time point of 2 h after medium exchange, because at this time a considerable amount of fragments (i.e., loss of cell adhesion) was still present. Addition of either F/R or Iso to fresh culture media for 2 h after 24 h of PV2-IgG incubation significantly reduced the number of fragments to 0.6 ± 0.1-fold of controls that received medium without cAMP-increasing agents (Fig. 5*B*). This indicates that elevated cAMP signaling promotes recovery of keratinocytes from pemphigus autoantibodies.

cAMP is known to bind and activate the serine/threonine kinase protein kinase A (PKA). To evaluate whether PKA is involved in the recovery of keratinocytes, we incubated cells together with the PKA-specific inhibitor H89 for 2 h after 24 h of PV2-IgG incubation and medium exchange. Addition of H89 led to a significant increase in fragments (1.5 ± 0.2-fold) compared with controls, which received fresh media only (Fig. 5*B*). Thus, the effect of cAMP on keratinocyte recovery is at least in part mediated by PKA signaling.

Increased cAMP blocked PV-IgG–induced p38MAPK activation in vitro and in vivo

One of the best characterized signaling molecules involved in pemphigus is p38MAPK. Pemphigus-IgG increase phosphorylation of p38MAPK and inhibition of p38MAPK blocks autoantibody-induced pathogenic effects both in vitro and in vivo (9, 19–21). In this paper, we investigated whether cAMP interferes with p38MAPK activation. Because p38MAPK activation in vitro occurs early in response to pemphigus-IgG, the phosphorylation state of p38MAPK was measured in HaCaT by Western blotting after 15 min of PV3-IgG treatment (Fig. 6*A*, 6*B*). PV3-IgG increased p38MAPK phosphorylation 2.0 ± 0.3-fold compared with controls. Indeed, activation of p38MAPK was blocked by incubation both with F/R (1.0 ± 0.1-fold and Iso (1.2 ± 0.1-fold). Finally, we investigated p38MAPK phosphorylation levels in homogenates of mice skin (Fig. 6*C*, 6*D*). Similar to the situation in vitro, PV1-IgG–induced p38MAPK phosphorylation (1.7 ± 0.1-fold compared with controls) was blocked by pretreatment with Iso (1.1 ± 0.2-fold of controls). Thus, cAMP signaling may prevent loss of cell adhesion by interference with PV-IgG–induced p38MAPK activation.

Discussion

In this study, we provide evidence for cAMP-mediated protection against PV-IgG–induced blister formation in a neonatal mouse model. In cultured keratinocytes in vitro, cAMP elevation abrogated changes induced by PV-IgG on a morphological, functional, and mechanistical level, because redistribution of Dsg3, loss of cell cohesion, and depletion of Dsg3 levels were reduced. Moreover, we show that an increase of cAMP likely represents a cellular response pathway following PV-IgG challenge, because PV-IgG treatment alone significantly augmented cAMP levels. Consistent with the concept of a protective cellular rescue pathway, increased cAMP also accelerated recovery of keratinocytes from PV-IgG–induced loss of cell adhesion. This protective cAMP-mediated pathway seemed to involve PKA signaling, and cAMP elevation blocked p38MAPK activation both in vitro and in vivo. The latter mechanism links cAMP elevation to a well established pathway in pemphigus pathogenesis and provides an explanation for the protective effect of cAMP on cell cohesion.

cAMP signaling prevents loss of keratinocyte adhesion in response to PV-IgG

Desmosomal adhesion is required to provide mechanical strength to various tissues (2). Despite this highly significant function of desmosomes both in physiological as well as pathological conditions, little is known about regulatory pathways of desmosome-mediated adhesion. Our data demonstrate that pharmacological increase of cellular cAMP levels prevents PV-IgG–induced loss of intercellular cohesion in vitro as revealed by dispase-based

dissociation assays. Most significantly, mice treated with β receptor agonist Iso were protected against autoantibody-induced blister formation. Although to our knowledge the role of cAMP in keratinocyte adhesion has not been addressed so far, several studies implicated cAMP signaling in other processes of keratinocyte cell biology, such as migration or epidermal homeostasis. Reduction of cAMP has been demonstrated to promote directional migration (22) and epidermal barrier recovery (23), and vice versa, increased cAMP blocked TGF-β–mediated cell scattering and reduction of membrane-localized E-cadherin (24). Because migratory or invasive cells downregulate adhesion molecules (25), these data are in line with a cAMP-induced increase of cell adhesion as shown in our study. Similarly within the endothelium, it is well established that cAMP increases blood-tissue barrier functions at least in part via augmented VE-cadherin adhesion (14, 15). Consistently, β receptor agonists such as Iso or epinephrine are known to elevate cellular cAMP levels and to increase endothelial barrier properties (26–28). Interestingly, cAMP increase mediated by both β receptor agonist Iso as well as F/R-mediated interference with cAMP turnover were similarly effective to block PV-IgG–induced cell adhesion. It has been demonstrated that keratinocytes express β2 adrenergic receptors and the enzymes required for catecholamine synthesis and do indeed produce epinephrine to modulate cellular behavior via autocrine or paracrine signaling (29). Therefore, it is tempting to speculate that cell adhesion may at least in part be modulated by locally produced catecholamines and subsequent β receptor signaling in response to environmental cues.

cAMP signaling may serve as cellular rescue mechanism in response to PV-IgG

Pemphigus-IgG trigger alterations of a plethora of signaling pathways (4, 30). Typically, the alterations seem to be causally involved in pemphigus pathogenesis because preventing the changes in signaling blocks the effect of pemphigus autoantibodies. Such a relationship has been demonstrated for p38MAPK, RhoA, plakoglobin, and several other signaling pathways (8–10, 21, 31, 32). In contrast, in this paper, we demonstrate a protective cellular signaling pathway that is triggered by PV-IgG themselves. PV-IgG induced a ~1.5-fold increase of cAMP in a 24-h time course. Although pharmacologic cAMP increase under conditions where it was sufficient to protect against PV-IgG–induced loss of cell cohesion was ~9-fold, the effect of PV-IgG on cellular cAMP levels should not be regarded to be minor because a 50% increase of total cAMP levels is a drastic response if one keeps in mind that cAMP signaling is regarded to be strictly compartmentalized (33). These data can be explained by the existence of a cellular rescue mechanism, which is triggered by PV-IgG. This rescue pathway, however, seems not effective to overcome pathogenic effects when triggered by PV-IgG but may at least blunt these effects.

Protective signaling pathways in response to changes of the environment are well established, for instance, in adaption and survival of cells via hypoxia-inducible factors under conditions of decreased oxygen supply (34, 35). Also, loss of cell adhesion of epithelial cells has been demonstrated to result in protective signaling. In intestinal and mammary epithelial cells, Src family kinases are activated by detachment from the basement membrane and delay anoikis in enterocytes, which is the final fate following loss of adhesion (36, 37). Thus, it is conceivable that alterations in crucial cellular functions such as intercellular adhesion promote initiation of protective signaling pathways. Up to now, the direct trigger for the protective signaling observed in this study is unclear. The most obvious explanation is that loss of intercellular adhesion is the stimulus leading to cAMP increase. This may be mediated by Dsgs, which have been reported to serve as receptors in addition to their adhesive functions (38, 39), but may also be induced by other well established adhesion receptors such as E-cadherin or integrins. In contrast, cAMP elevation may specifically be triggered by binding of PV-IgG to keratinocytes. This may not necessarily be restricted to binding of autoantibodies to Dsgs, because in addition, Ags other than Dsg1 and

Dsg3 have been implicated in PV pathogenesis (40, 41). Especially, auto-antibodies against G protein-coupled acetylcholine receptors have been demonstrated (42) and therefore may result in alterations of cAMP levels. Therefore, it will be a matter of future studies to investigate the precise mechanisms leading to cAMP elevation following application of PV-IgG.

cAMP signaling is protective by interference with PV-IgG–induced p38MAPK activation

Our data demonstrate that the protective cAMP increase reduces activation of p38MAPK induced by PV-IgG and involves PKA signaling. These data are in line with other studies in keratinocytes demonstrating a reduced chemokine production via cAMP/PKA-mediated reduction of p38MAPK activity in a model of atopic dermatitis (43). In a variety of studies, activation of p38MAPK kinase has been demonstrated to be crucial for pathogenic effects of pemphigus-IgG and inhibition of p38MAPK signaling prevented loss of adhesion in vivo and in vitro (6, 9, 19–21). Moreover, our group has shown in previous studies that pemphigus-IgG interfere with RhoA signaling in a p38MAPK-dependent manner (8). Thus, a rescue pathway is conceivable in which cAMP-mediated signaling interferes with PV-IgG–induced p38MAPK activation and RhoA inhibition. This protective pathway may outbalance minor alterations of cellular adhesion; however it is obviously unable to prevent massive loss of cell cohesion induced by PV-IgG. Nevertheless, the pharmacologic support of an existent protective pathway as a novel therapeutic approach in pemphigus is particularly attractive because it may be accompanied by less severe side effects. Moreover, because keratinocytes predominantly express β2 receptors (44), only this adrenergic receptor subtype needs to be targeted pharmacologically. A variety of β2 receptor-specific agonists are available and in daily use, for instance, in therapy of asthma (45). Thus, the roles for cAMP signaling demonstrated in this study may increase the portfolio of therapeutic approaches in pemphigus therapy.

Acknowledgments

This work was supported by Deutsche Forschungsgemeinschaft Grants SFB 688 TP A4 and SFB 487 TP B5 and US NIH RO1 R01 AI049427-10 (DSR).

Abbreviations used in this paper

Dsg	desmoglein
F/R	forskolin/rolipram
Iso	isoproterenol
PKA	protein kinase A
Prop	propranolol
PV	pemphigus vulgaris
PV-IgG	pemphigus vulgaris IgG
VE-cadherin	vascular endothelial cadherin

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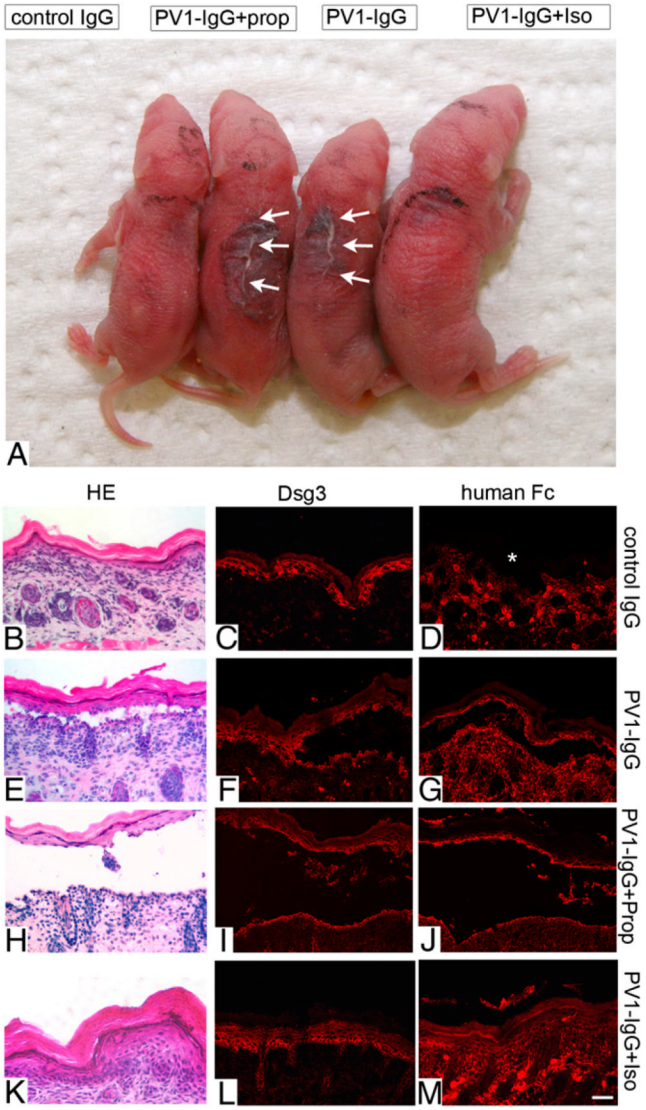

FIGURE 1.

cAMP elevation by Iso blocks PV-IgG–induced blister formation. Compared with controls (*A–D*), neonatal mice injected for 20 h with PV1-IgG or preinjected with 10 mM b receptor antagonist Prop and PV-IgG1 developed macroscopically (*A*, arrows) and histologically (*E–G*, *H–J*) detectable blisters. In contrast, cleavage formation was blocked by pre-injection with 10 μM β receptor agonist Iso (*A*, *K–M*). Immunostaining for Dsg3 confirmed strictly suprabasal cleavage formation (*F*, *I*) and staining for the Fc part of human IgG demonstrated epidermal Ab binding under PV-IgG, PV1-IgG + Prop, andPV-IgG + Isotreatment (*G*, *J*, *M*) but notunder control-IgG injection (*D*, asterisk). *n* = 5–8. Original magnification ×200; scale bar 50 μm.

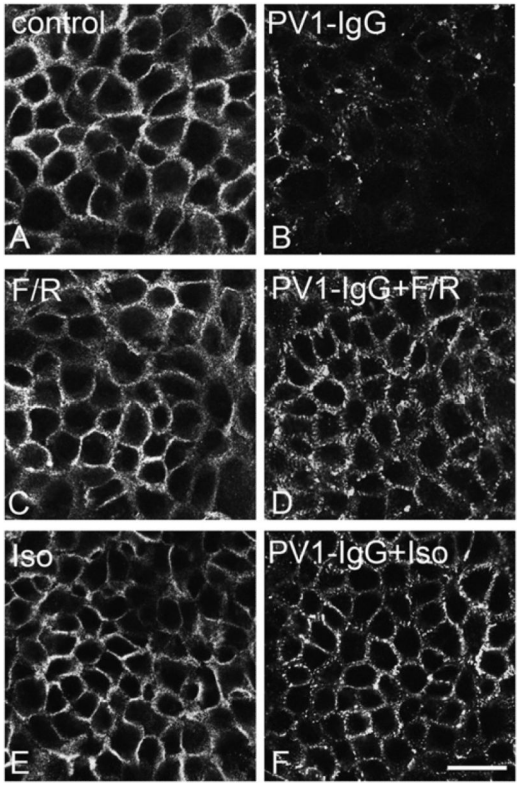

FIGURE 2.

Effects of cAMP elevation on PV-IgG–induced Dsg3 reorganization. Under control conditions (*A*), Dsg3 immunostaining of confluent HaCaT keratinocytes revealed a linear distribution at cell membranes. In contrast, following 24 h of PV2-IgG incubation, Dsg3 was drastically reorganized, and staining intensity was reduced (*B*). Simultaneous incubation with F/R (*D*) or Iso (*F*) largely prevented loss of Dsg3 membrane staining. Incubation with F/R (*C*) and Iso (*E*) alone had no pronounced effect on Dsg3 distribution. $n = 5$. Original magnification ×630; scale bar 20 μm.

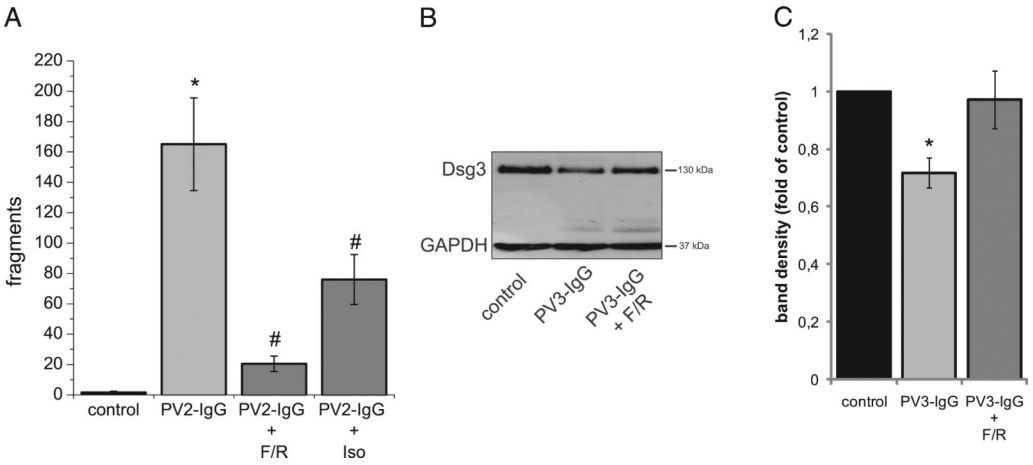

FIGURE 3.

cAMP increase prevents PV-IgG–mediated loss of cell adhesion and Dsg3 depletion. A, Compared with controls, application of mechanical stress to HaCaT monolayers in dispase-based dissociation assays resulted in pronounced fragment formation following 24 h of incubation with PV2-IgG and thus reduced cell adhesion. Fragment formation was largely prevented by simultaneous incubation with F/R or Iso ($n = 8$). *$p < 0.05$ versus controls; #$p < 0.05$ versus PV-IgG treatment. *B* and *C*, Changes of Dsg3 levels were evaluated by Western blotting analyses. Twenty-four-hour treatment of confluent HaCaT with PV3-IgG resulted in depletion of cellular Dsg3 levels, which was inhibited by simultaneous incubation with F/R. GAPDH served as loading control. A representative immunoblot and densitometric quantification of Dsg3 band intensity from five independent experiments are shown in *B* and *C*, respectively. *$p < 0.05$ compared with controls.

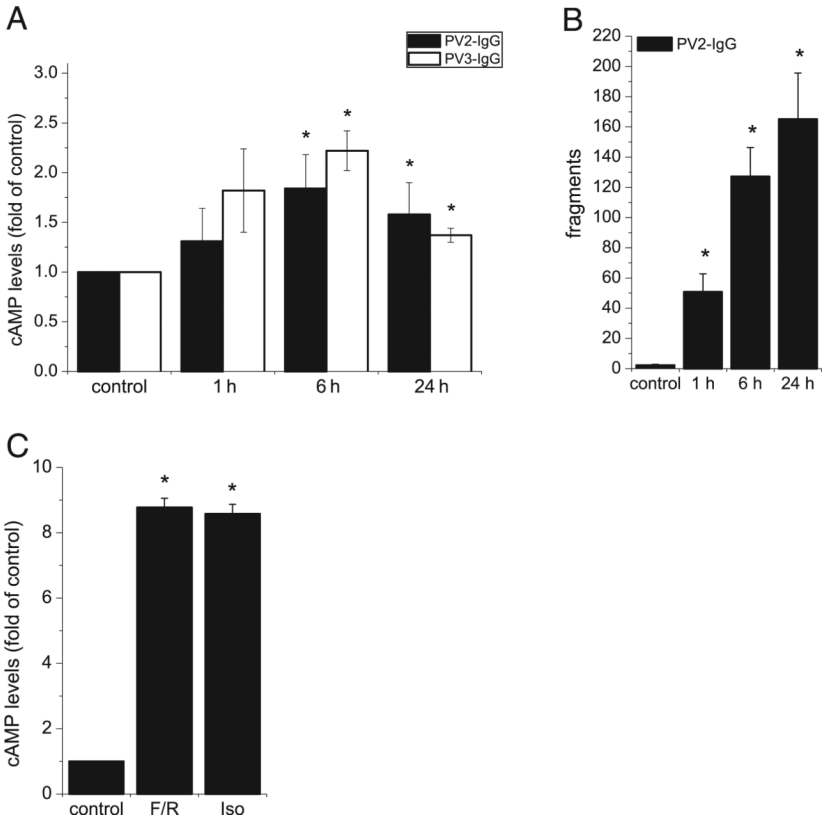

FIGURE 4.

PV-IgG elevate cAMP levels. cAMP levels were assessed in confluent HaCaT (*A*). Following incubation with PV2-IgG and PV3-IgG for 1, 6, and 24 h, cAMP levels were elevated compared with controls ($n = 4$). *$p < 0.05$ compared with controls. Under the same conditions, PV2-IgG effectively increased fragment formation (*B*, $n = 6$). *$p < 0.05$. *C* demonstrates cAMP increase by incubation with F/R and Iso for 24 h in confluent HaCaT ($n = 5$). *$p < 0.05$ versus control.

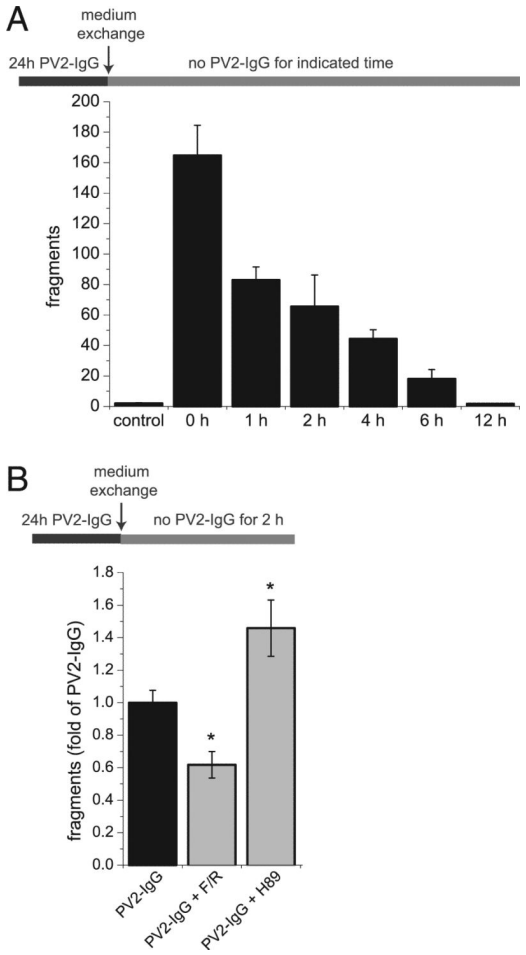

FIGURE 5.

Increased cAMP levels enhance recovery from PV-IgG–induced reduce of cell adhesion in part via PKA signaling. Confluent HaCaT monolayers were incubated with PV2-IgG for 24 h followed by medium exchange. Cell adhesion was quantified by dissociation assays at several time points after medium exchange (*A*). Fragment numbers were already decreased after 1 h of medium exchange and reached control (no PV2-IgG) levels after 12 h ($n = 5$). Effects of cAMP increase and PKA inhibition were studied at time point of 2 h after medium exchange (*B*). F/R significantly decreased fragment numbers, whereas H89 led to elevated number of fragments, when applied for 2 h ($n = 5$). *$p < 0.05$ versus PV2-IgG.

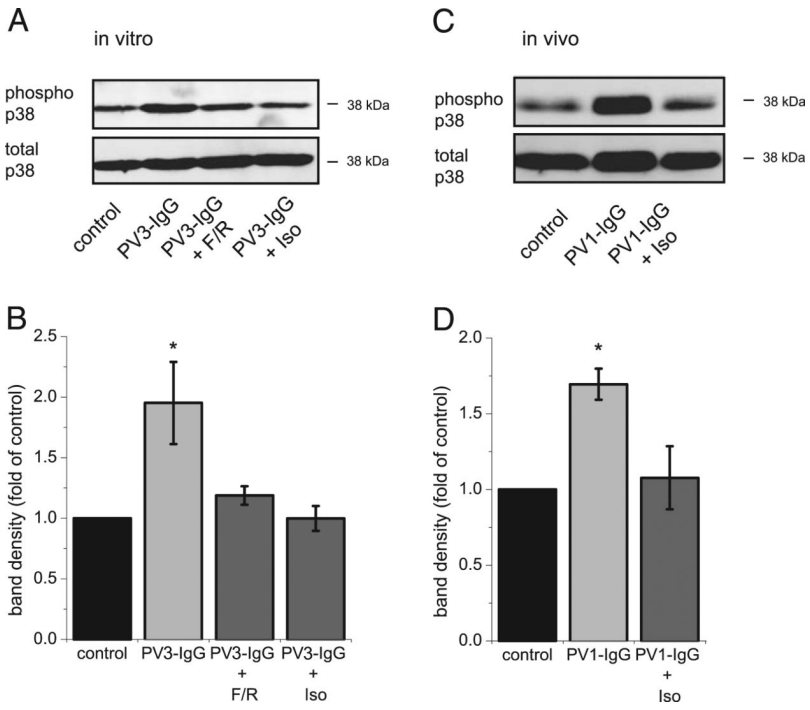

FIGURE 6.

cAMP elevation blocks PV-IgG–induced p38MAPK activation both in vitro and in vivo. The phosphorylation state of p38MAPK was determined by a phospho-specific p38MAPK Ab both in vitro (*A*, *B*) and in vivo (*C, D*). Fifteen-minute PV-IgG incubation increased p38MAPK phosphorylation in cultured keratinocytes, which was completely inhibited by simultaneous treatment with F/R or Iso. Similarly in homogenates of mice skin, Iso pretreatment blocked PV-IgG–mediated p38MAPK phosphorylation. *A* and *C* show representative immunoblots, and in *B* and *D*, densitometric evaluation of p38MAPK phosphorylation was performed. Total p38MAPK levels served as loading control (*B*, $n = 5$; *D*, $n = 6$). *$p < 0.05$.

Table I

Autoantibody profile of patients' IgG

ELISA Values	Dsg3 (U/ml)	Dsg1 (U/ml)
PV1-IgG	1414	1194
PV2-IgG	6586	Negative
PV3-IgG	1239	60
PV4-IgG	1185	977

Table II

Iso blocks blister formation in neonatal mice

Injection of	Mice with Blisters	Mice without Blisters
Control IgG	0	8
PV1-IgG	8	0
PV1-IgG + Prop	5	0
PV1-IgG + Iso	0	8