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Adenosine A_{2B} receptor agonist improves epidermal barrier integrity in a murine model of epidermal hyperplasia

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ABSTRACT

Adenosine regulates multiple physiological processes through the activation of four receptor subtypes, of which the A_{2B} adenosine receptor (A_{2B}AR) has the lowest affinity for adenosine. Being the adenosine receptor subtype most prominently expressed in epidermis, we recently described the antiproliferative and anti-inflammatory effect of the selective A_{2B}AR agonist BAY60–6583 (BAY) in human keratinocytes stimulated with 12-O-tetradecanoylphorbol-13-acetate (TPA), so we sought to establish the effect of topical application of BAY in a model of murine epidermal hyperplasia.

Topical application of BAY (1 or 10 μg/site) prevented the inflammatory reaction and skin lesions induced by TPA, minimizing hyperproliferation and acanthosis, as well as the expression of specific markers of proliferative keratinocytes. On the other hand, pre-treatment with the selective A_{2B}AR antagonist, PSB-1115 (PSB, 5 or 50 μg/site) reversed these beneficial effects. Additionally, BAY application normalized the expression of epidermal barrier proteins, whose integrity is altered in inflammatory skin diseases, while treatment with the antagonist alone worsened it.

Our results, besides confirming the anti-inflammatory and antiproliferative effects of the A2BAR agonist, further demonstrate a role of A_{2B}AR activation to preserve the epidermal barrier. Therefore, the activation of A_{2B}AR may constitute a possible new pharmacological target for the treatment of skin inflammatory diseases such as psoriasis.

1. Introduction

Adenosine interacts with four G protein-coupled adenosine receptors (A_1, A_{2A}, A_{2B}, and A_3), each with distinct tissue distribution and effector coupling. A_1 and A_3 inhibit adenylyl cyclase, linked to G_i proteins, while A_{2A} and A_{2B} stimulate it, coupled to G_s proteins. A_{2B} and A_3, in various cellular systems, are also coupled to G_q proteins, activating phospholipase C and triggering calcium mobilization [1].

Adenosine signalling has been implicated in many pathophysiological functions in the skin [2–5]. In psoriasis, A_{2A} and A_3 AR exhibit anti-inflammatory and immunosuppressive effects, modulating the activation of different cell types where these receptors are highly expressed, such as neutrophils, macrophage/monocytes, dendritic cells, and lymphocytes. In this sense, the efficacy of methotrexate as standard therapy of psoriatic patients is related to the increase of adenosine production by Treg cells, which can supress CD4+ effector T cell

Abbreviations: AR, adenosine receptors; BAY, BAY60–6583; CK, cytokeratin; DAB, 3,3-diaminobenzidine; NECA, 5'-N-ethylcarboxamidoadenosine; MPO, myeloperoxidase; PSB, PSB-1115; TMB, tetramethylbenzidine; TPA, 12-O-tetradecanoylphorbol- 13-acetate.

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functions via A_{2A} activation [5–7]. Similarly, the selective A_3 agonist piclidenoson mediates immunomodulatory effects by downregulation of NF-κB signaling pathway in peripheral blood mononuclear cells of psoriatic patients [8,9,10]. In this line, a novel photoactivable drug, acting on A_3 receptors, locally downregulated the immune system [11, 12].

Adenosine receptors have been identified in various skin cell types, although their participation in both physiological and pathological processes has not been fully established [2]. It is known that A_{2A} AR is the main receptor subtype in the dermis, and its activation promotes cell proliferation, collagen synthesis, wound healing, and revascularization [13–15]. In contrast, A_{2B} is the major AR in epidermis, and its stimulation, by either adenosine or the selective partial agonist BAY 60–6583 (BAY), inhibits keratinocyte proliferation by the increase of intracellular Ca^{2+} [16]. Interestingly, the expression of A_{2B} AR is reduced in the epidermis of psoriatic patients, suggesting a possible physiological role of this AR as a regulator of normal keratinocyte growth which would be lost in psoriatic skin [12,16]. Since adenosine and the A_{2B} AR partial agonist BAY also reduce inflammatory response in human keratinocytes [16], the activation of this AR could constitute an interesting therapeutic strategy in the control of skin diseases with an inflammatory and hyperproliferative state. However, no *in vivo* studies have been carried out so far at this level.

Thus, the main objective of the present study was to investigate the effect of A_{2B} AR activation in the murine epidermal hyperplasia model induced by 12-O-tetradecanoylphorbol-13-acetate (TPA), which reproduces some histopathological parameters characteristic of inflammatory/hyperproliferative skin, such as oedema, cell infiltration, cytokine production, epidermal hyperplasia and skin barrier alteration [17–20]. Topical application of the A_{2B} AR agonist BAY and the A_{2B} AR antagonist PSB-1115 (PSB) confirmed the beneficial effect of A_{2B} AR activation on skin.

2. Materials and methods

2.1. Animals

The experiments were conducted using 6–8 weeks old female Swiss CD1 mice (Janvier, Le Genest St Isle, France) weighing 25–30 g. Mice were maintained in optimal conditions with unrestricted food and water access. The studies adhered to the EU Directive 2010/63/EU for animal experiments, and the protocol was approved by the Committee of Ethics in Experimentation of the University of Valencia (2016/VSC/PEA/00138).

2.2. TPA-induced epidermal hyperplasia model

On day 0, dorsal area of mice was shaved with an electric clipper and treated with depilatory cream (Deliplus, Barcelona). After 24 hours, 20 μl of vehicle (acetone), the antagonist PSB-1115 (PSB, Tocris, Bristol, UK) at 5 or 50 μg per site, the agonist BAY60–6583 (BAY, Tocris Bristol, UK) at 1 or 10 μg/site, or the combination antagonist followed by agonist in a dose ratio (5:1) (PSB 50 μg + BAY 10 μg) or (PSB 5 μg + BAY 1 μg) were topically applied to 1 cm^2 of the shaved dorsal area. After 1 hour, 20 μl of 12-O-tetradecanoyl phorbol 13-acetate (TPA) (Sigma-Aldrich, St. Louis, MO) (2nmol/site) or vehicle were administered in the same area of the correspondent group. This procedure was repeated during three consecutive days. All groups received the same final volume of vehicle.

Along the treatment, the severity of cutaneous lesions was assessed by an independent observer. The method visually evaluates macroscopic alterations of the skin as erythema, desquamation, and ulceration, according to an established score [21]. The scale graded the lesions between 0 and 4 (0, none; 1, mild; 2, moderate; 3, severe; 4, ulcerated). On fourth day, mice were euthanized and biopsies of 1 cm^2 were collected and weighted.

2.3. Histological, immunohistochemical and immunofluorescence analysis

Formalin-fixed paraffin-embedded tissue sections (7 μm) were mounted on slides, deparaffinised with xylene, rehydrated through graded alcohols, stained with H&E (Sigma, St. Louis, EEUU) and visualized with a Leica DM IL LED Fluo microscope (Leica Microsystems, Wetzlar, Germany). Histological analysis was performed blindly by an independent observer. Epidermal thickness was determined using Leica Application Suite Software (Leica Microsystems, Wetzlar, Germany), by averaging 10–12 measurements per field, 4 fields per section for three mice per group. Quantification of the infiltrating cells was performed averaging four random fields per section for three mice per group.

Immunohistochemical analysis was carried out in deparaffinised sections performed as mentioned above. After antigen retrieval (10 mM sodium citrate buffer, pH 6.0, 10 min at 90–100°C and 30 min of cooling at room temperature), sections were incubated overnight at 4 °C with the primary antibodies anti-cytokeratin 6 Rabbit monoclonal (1:500) (SAB5500131, Sigma-Aldrich, EEUU R&D System, Abingdon, UK), anti-cytokeratin 10 Rabbit monoclonal (1:500) (SAP4501656, lot 310249, Sigma-Aldrich, EEUU R&D System, Abingdon, UK), anti-Ki67 (1:200; Cat. MA5–14520, lot UB2725211) (ThermoFischer Scientific Waltham, USA) and anti-Cytokeratin 10 (1:500) (Sigma-Aldrich. St. Louis, MO, USA.). After washing, samples were incubated for 1 hour with secondary HRP anti-rabbit antibody (1:100) (P0448, lot 20066477, Dako, Glostrup Denmark), followed by a 3,3-diaminobenzidine (DAB) (Vector, Burlingame, EEUU) and counterstaining with Gill haematoxylin #2 (Sigma, St. Louis, EEUU).

The immunofluorescence analysis was performed following the steps of immunochemistry protocol. Filaggrin polyclonal antibody (1:500) (Ref 905801, lot B257576), anti-involucrin antibody (1:500) (Ref 92401, lot B255812) and loricrin polyclonal antibody (1:500) (Ref 905101, lot B258012) all from BioLegend, San Diego, EEUU, were incubated overnight at 4 °C. The secondary antibody Alexa Fluor® 488 goat anti-rabbit (A-11008, Molecular ProbesTM Invitrogen, Paisley, UK) was incubated for 1 h at room temperature. Finally, the mounting medium ProLongTM Gold antifade with DAPI was added to obtain the nucleus counterstain. Samples were evaluated by epifluorescence microscopy with a Leica DM IL LED Fluo microscope (Leica Microsystems, Wetzlar, Germany). As a negative control, sections were incubated with PBS instead of primary antibody. Fluorescence quantification was performed with Image J (National Institutes of Health, Bethesda, Maryland).

2.4. Determination of cytokine release and inflammatory parameters in skin homogenates

Frozen biopsies (-80°C) were pulverized in liquid N_2 using a supercooled mortar and pestle, then resuspended and homogenized in 1 ml of buffer A (10 mM HEPES, 1 mM EDTA, 1 mM EGTA, 10 mM KCl) containing an antiprotease cocktail. Tissue homogenates were sonicated and centrifuged at 2000 g for 10 min at 4 °C and the supernatants were collected. Levels of cytokines in supernatants were analysed by ELISA following the manufacturers' instructions: TNF-α (R&D Systems, Abingdon, UK), IL-6 (Invitrogen,Vienna, Austria), IL-1β and CXCL-1 (R&D systems Abingdon, UK). Myeloperoxidase (MPO) activity was assayed as described previously [22]. Briefly, 5 μl of skin homogenates was incubated at 37 °C for 10 min with PBS buffer, phosphate buffer pH 5.4, hydrogen peroxide solution (0052% v/v), and the substrate tetramethylbenzidine (TMB). Quantification of protein levels or MPO activity were measured by absorption at 450 nm in a spectrophotometer Wallac 1420 VICTOR3 TM (PerkinElmer, Finland).

2.5. Western-Blot analysis

Protein concentration in the homogenates was determined with the DC Bio-Rad Protein assay kit (Bio-Rad, Hercules, CA). Proteins (20 μg

/lane) were separated on a 10% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) and transferred to poly-vinylidene fluoride membrane (GE Healthcare Life Sciences, Barcelona, Spain). Membranes were incubated overnight with the specific antibodies against filaggrin (1:1000) (Ref 905801, lot B257576, BioLegend, San Diego, EEUU) and caspase-14 (1:500) (PA5–72903, lot UE2768569A, ThermoFischer Scientific Waltham, USA). Enhanced chemioluminescence using horseradish peroxidase conjugated secondary antibodies (D P0448, lot 20066477, Dako, Glostrup Denmark) and a standard ECL substrate (GE Healthcare, Wessling, Germany) was captured with the AutoChemi image analyzer (UVP Inc., Upland, CA). β-actin (1:5000) (A2066, lot 106M4770V, Sigma-Aldrich St. Louis, MO, USA), was used as a protein loading control.

2.6. Statistical analysis

Results are expressed as the mean ± SD. Statistical analyses were performed using one-way ANOVA followed by Tukey's pot-hoc comparison, using GraphPad Prism 6 software (GraphPad Software, Inc., San Diego, CA). A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. BAY60-6583 improves macroscopical lesions, oedema, epidermal hyperplasia, and cell infiltration induced by TPA

The dose ratio antagonist / agonist (5:1) was established according to the referenced studies for both molecules [23–27]. Topical application of TPA for three consecutive days induced severe macroscopic skin lesions, which were significantly attenuated by pre-treatment with the A_{2B} AR agonist (BAY) at 1 and 10 μg/site (Fig. 1a,b). When the A_{2B} antagonist PSB (5 μg/site or 50 μg/site) was previously administered to BAY (groups PSB50+BAY10+TPA and PSB5+BAY1+TPA) the beneficial effect caused by the agonist was significantly reversed (Fig. 1a,b). Besides, the higher dose of PSB (50 μg/site) even significantly worsened the score induced by TPA (group PSB50+TPA).

Oedema induced by TPA was determined by the weight of punch biopsies. Topical application of BAY significantly reduced this parameter at both assayed doses, whereas pre-treatment with PSB abrogate this effect in a dose-dependent manner (Fig. 1c). The analysis of haematoxylin and eosin staining indicated that BAY was able to reduce epidermal hyperplasia and cell infiltrate induced by TPA. Again, PSB pre-treatment reversed this beneficial effect, being the results in the group PSB50+BAY10+TPA statistically significant (Fig. 1d-f).

3.2. BAY60-6583 inhibits inflammatory mediators in skin homogenates

Neutrophils are the first cells to migrate towards the inflammation focus, via the chemoattractant CXCL-1 and other chemokines [18,20]. We determined myeloperoxidase (MPO) activity in skin homogenates as marker of leucocyte infiltration, as well as CXCL-1 levels. Both parameters were significantly reduced by applying 1 or 10 μg/area of BAY, effect that was abrogated by pre-treating with the antagonist PSB (Fig. 2a, b). Other cytokines known to play a role in this animal model such as IL-1β, IL-6 and TNF-α were also measured [18,28]. Although the application of the A_{2B} AR agonist significantly reduced these parameters at both assayed doses, the effect was not clearly reversed by pre-treatment with the antagonist PSB (5 μg/site or 50 μg/site) (Fig. 2, c-e). These results are in agreement with previous reports indicating other mechanisms independent of AR which could participate in the anti-inflammatory effect of adenosine and A_{2B} agonists, such as the activation of membrane phosphatases [16,29,30].

Noteworthy, in TPA-treated animals, the higher dose of PSB (group PSB50+TPA) increased the levels of IL-1β even above the control group

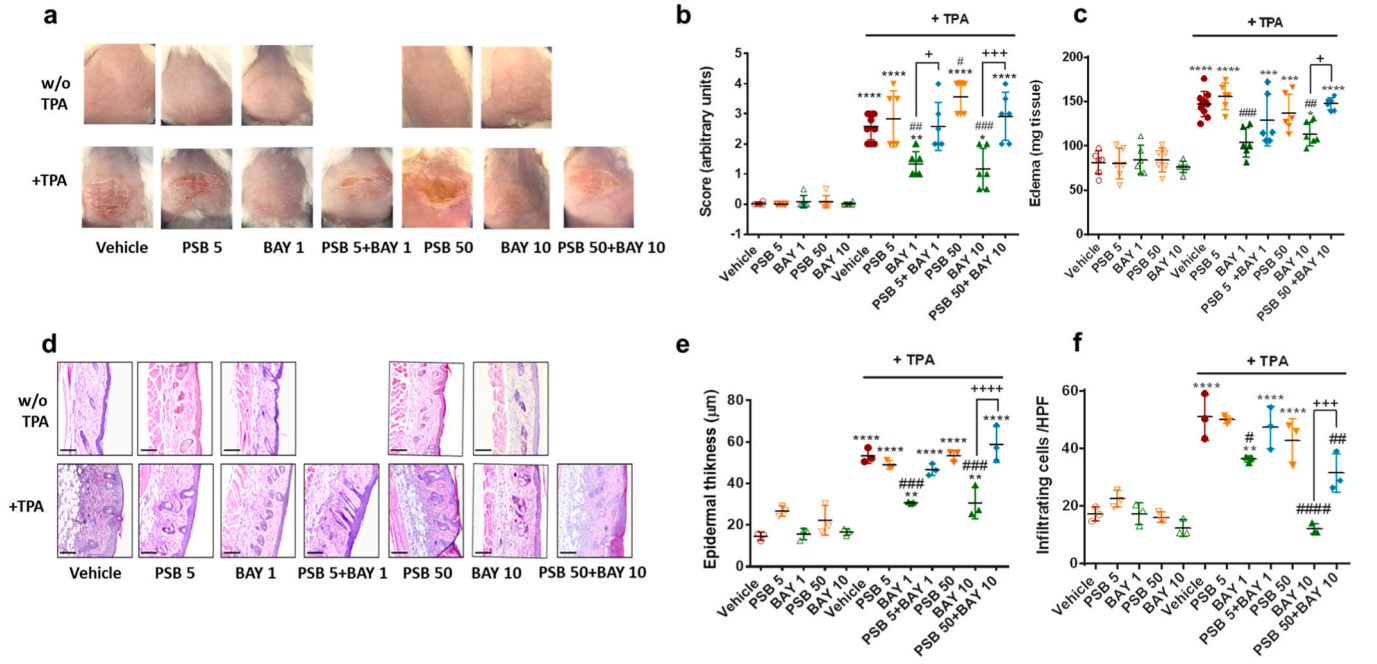

Fig. 1. BAY60-6583 (BAY) improves macroscopic lesions, epidermal hyperplasia, edema and cell infiltration induced by 12-O-tetradecanoylphorbol-13-acetate (TPA). BAY at 1 μg/area (BAY1) and 10 μg/area (BAY10) was topically applied 1 hour before TPA administration during 3 consecutive days. The A_{2B} AR antagonist PSB-1115 (PSB) was applied at 5 μg/area (PSB5) or 50 μg/area (PSB50) alone or previously BAY application. (a) Macroscopic appearance of the skin at the end of the experiment. (b, c) Score of lesions and edema. Data are the mean +/- SD of n = 10 mice in the (Vehicle+TPA) group and n = 6 mice in each of the other groups. **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$, *$P < 0.05$ *vs.* Vehicle. ### $P < 0.001$, ## $P < 0.01$ *vs.* (Vehicle+TPA). +++ $P < 0.001$, + $P < 0.05$ *vs.* (BAY1 +TPA) or (BAY10+TPA). One-way ANOVA with Tukey's post-test. (d) Hematoxylin and eosin (H&E) staining of skin biopsies. Scale bar=100 μm. (e, f) Epidermal thickness of H&E–stained sections and number of infiltrating cells in representative high-power fields (HPF). Four fields per tissue section were analyzed and averaged. Data are the mean +/- SD of 3 mice per group. **** $P < 0.0001$ *vs.* Vehicle. #### $P < 0.0001$, ### $P < 0.001$, ## $P < 0.01$ # $P < 0.05$ *vs.* (Vehicle+TPA). ++++ $P < 0.0001$, +++ $P < 0.001$, *vs.* (BAY1+TPA) or (BAY10+TPA). ANOVA with Tukey's post-test. Vehicle: acetone-treated mice.

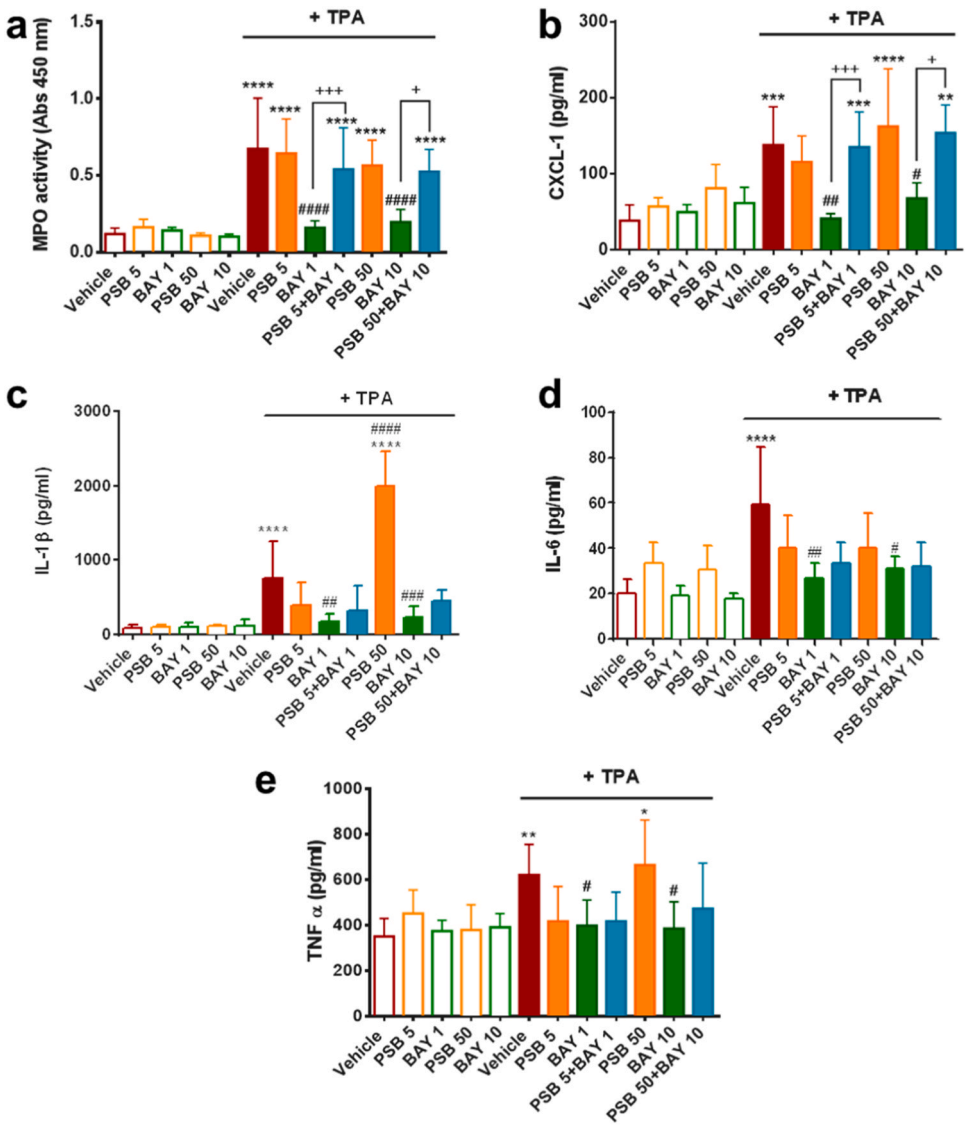

Fig. 2. BAY60-6583 reduces MPO activity, CXCL-1, IL-1β, IL-6 and TNF-α in skin homogenates. BAY at 1 μg/area (BAY1) and 10 μg/area (BAY10) was topically applied 1 hour before TPA administration during 3 consecutive days. The A_{2B}AR antagonist PSB-1115 (PSB) was applied at 5 μg/area (PSB5) or 50 μg/area (PSB50) alone or previously BAY application. Data are the mean +/- SD of n = 10 mice in the (Vehicle+TPA) group and n = 6 mice in each of the other groups. **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ *vs.* Vehicle. #### $P < 0.0001$, ### $P < 0.001$, ## $P < 0.01$, #$P < 0.05$ *vs.* (Vehicle + TPA). +++ $P < 0.001$, + $P < 0.05$ *vs.* (BAY1+TPA) or (BAY10+TPA) ANOVA with Tukey's post-test. Vehicle: acetone-treated mice.

(vehicle +TPA). These results, and the worsening observed in the score for this treatment group (Fig. 1b), suggested an excessive damage induced by the antagonist at higher doses. Thus, we selected the ratio BAY (1 μg/site) / PSB (5 μg/site) for further studies.

3.3. BAY60-6583 enhanced cytokeratin 10 (CK10) expression in epidermis and reduces CK6 and Ki67 positive cells

The inhibitory effect of BAY on TPA-induced epidermal hyperplasia was confirmed by the immunohistochemical detection of Ki67 (a nuclear antigen expressed in proliferating cells), and cytokeratin-6 (CK6), highly expressed in keratinocytes under proliferative pathological conditions [31]. Both proliferation markers where significantly reduced after application of BAY (1 μg/site) in TPA-stimulated animals (Fig. 3a-c). In addition, the A_{2B} agonist was able to restore the basal expression of CK10 (Fig. 3d), a marker of early differentiation stage in keratinocytes which is downregulated during skin injury [32,33]. Pre-treatment with PSB (5 μg/site) abrogated the beneficial effect of BAY on CK6 and CK10 expression (group PSB5+BAY1+TPA). Interestingly, PSB treatment was able to decrease the expression of CK10 even in healthy skin, suggesting a physiological role of A_{2B} AR as regulator of normal epidermal differentiation (Fig. 3d).

3.4. BAY60-6583 preserves the epidermal barrier integrity

The regulatory effect of A_{2B} activation on CK10 expression under both physiological and pathological conditions, led us to delve into the potential role of this AR on epidermal barrier function. Increased keratinocyte proliferation and disturbed differentiation includes changes in keratins and cornified envelope proteins [33,34]. The expression of the early keratinocyte differentiation marker involucrin is upregulated in psoriatic lesions [35,36]; whereas the expression of filaggrin, protein needed to complete terminal differentiation, and loricrin, a structural protein of the cornified layer, is reduced in psoriasis and atopic dermatitis, indicating a defective epidermal barrier in these pathologies [37–39,63].

In our experimental conditions, TPA induced an aberrant overexpression of involucrin and a clear reduction of filaggrin and loricrin,

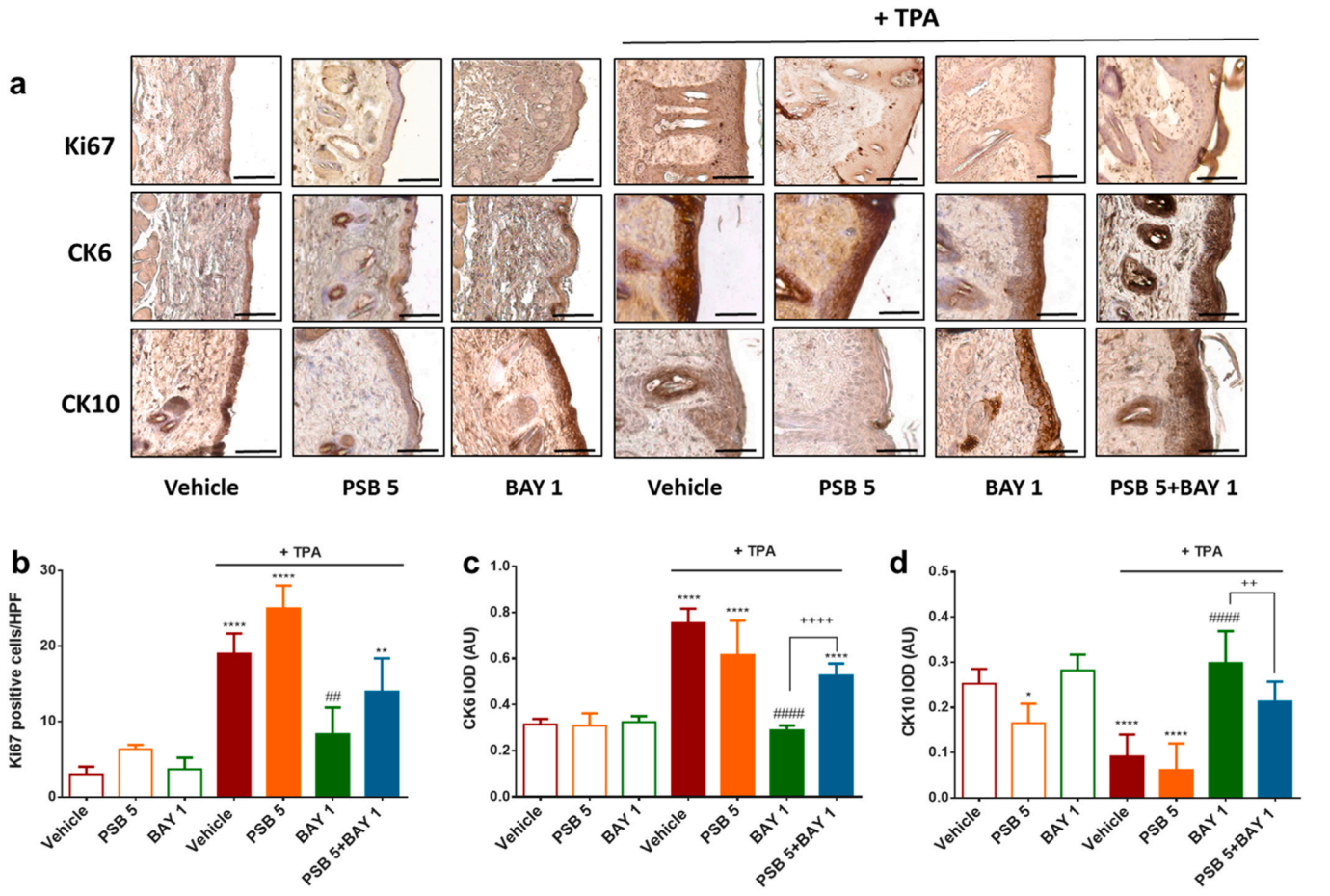

Fig. 3. BAY60-6583 (BAY) enhanced cytokeratin 10 (CK10) expression in epidermis and reduces CK6 and Ki67 positive cells. (**a**) Immunohistochemical detection of Ki67, CK6 and CK10 in tissue sections, marked in brown. Scale bar = 50 μm. (**b**) Number of Ki67 positive cells in representative high-power fields (HPFs). (**c, d**) CK6 and CK10 expression expressed as arbitrary units (AU) of integrated optical density (IOD). Four fields per tissue section were analyzed and averaged. Data represent the mean +/-SD of n=3 mice per group. **** $P < 0.0001$ ** $P < 0.01$, * $P < 0.05$ *vs.* Vehicle, #### $P < 0.0001$, ## $P < 0.01$ *vs.* (Vehicle+TPA), ++++ $P < 0.0001$, ++ $P < 0.01$ *vs.* (BAY1+TPA). ANOVA with Tukey's post-test. BAY was tested at 1 μg/area. PSB-1115 (PSB) was tested at 5 μg/area. Vehicle: acetone-treated mice.

suggesting an immature epidermal cornification and a defective skin barrier function. The application of BAY restored the basal expression pattern of all these proteins (group BAY1+TPA), while pre-treatment with the A_{2B} AR antagonist significantly reversed this beneficial effect (group PSB5+BAY1+TPA) (Fig. 4). It is noteworthy that over-expression of involucrin in inflammatory skin diseases is correlated with cell migration beyond the basal layer [35]. Thus, the inhibition of cell infiltration and involucrin induction after A_{2B} activation in TPA-stimulated skin could be in accordance with the relationship of both parameters. Finally, in a similar manner to CK10, the application of PSB in healthy animals significantly reduced the basal expression of filaggrin, supporting the idea of a physiological role of A_{2B} in maintaining the barrier function (Fig. 4c).

3.5. BAY60-6583 treatment restored the correct filaggrin/caspase 14 expression

Among other factors, the integrity of the barrier skin depends on the presence of natural moisturising factors resulting from cleavage of filaggrin, in which caspase-14 is involved [40]. The precursor profilaggrin is synthesized in the keratohyalin granules of the granular layer, and caspase-14 is in part responsible for the proteolysis of the N-terminal fragment of profilaggrin to obtain filaggrin, thus promoting the correct formation of the stratum corneum [41,42]. Western blot analysis of skin homogenates showed a clear correlation in the expression pattern filaggrin/caspase 14 (Fig. 5). Thus, a high basal expression of both proteins was observed in healthy skin (vehicle group) which was drastically reduced after TPA treatment (vehicle+TPA group). BAY application significantly reverted the detrimental impact of TPA (group BAY1+TPA), whereas pre-treatment with PSB blunted this beneficial effect (group PSB+BAY1+TPA).

4. Discussion

In the present study, the beneficial effect of A_{2B} AR activation in skin has been demonstrated. Topical application of the selective A_{2B} agonist BAY not only improved macroscopical lesions induced by TPA, but also reduced keratinocyte hyperproliferation, confirming previous *in vitro* studies [16,43]. Besides, the inhibition of CK6 expression suggests potential interest of A_{2B} AR activation in conditions like psoriasis, where CK6 is overexpressed [33,44]. Interestingly, and despite its controversial role in pulmonary fibrosis that could be either beneficial or detrimental [45,46], the activation of A_{2B} AR has been proved to be protective and antiproliferative in other cell types in which A_{2B} is the mainly AR expressed, such as intestinal epithelial cells [47], vasculature and retinal pigment epithelium [48], alveolar epithelial cells [49], cardiac fibroblasts [50] or coronary artery smooth muscle cells [51,52]. In this sense, the recent development of new selective and high-affinity agonists and antagonists have revived the interest in the study of A_{2B}AR, which was generally relegated due its low affinity for the endogenous ligand adenosine and the lack of potent and selective ligands [1]. In this regard, BAY60–6583 is considered the only potent and highly selective A_{2B} AR

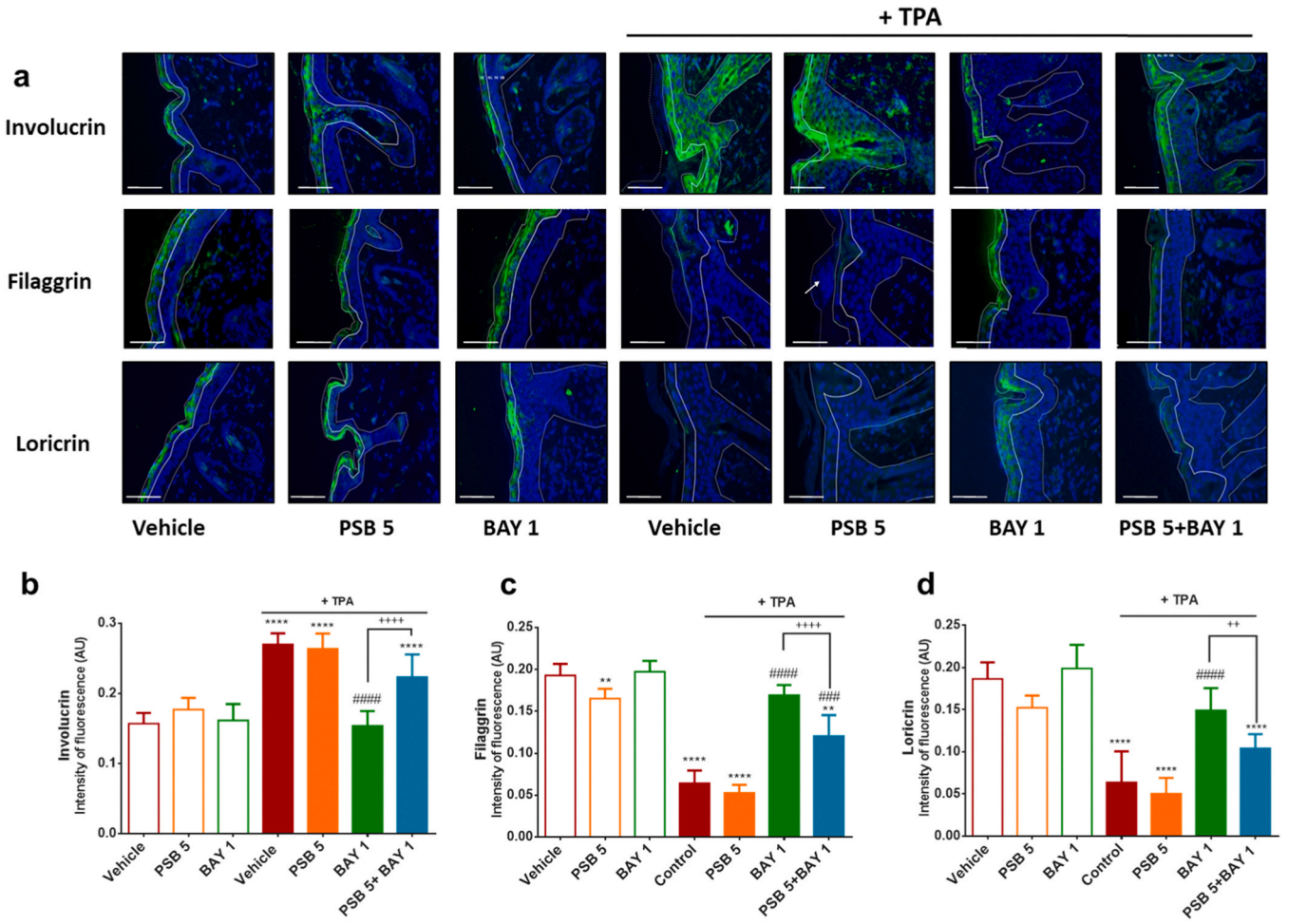

Fig. 4. BAY60-6583 (BAY) normalizes the expression of proteins involved in the epithelial barrier function. **(a)** Immunofluorescence staining of involucrin, filaggrin and loricrin (labeled in green). Cell nuclei were counterstained in blue (DAPI). Scale bar = 50 μm. (**b, c, d**) Intensity of fluorescence of involucrin, filaggrin and loricrin (AU). Data represent the mean ± SD of n=3 mice per group. **** P <0.0001, ** P <0.01 *vs*. Vehicle. #### P < 0.0001, ### P < 0.001 *vs*. (Vehicle+TPA). ++++ P < 0.0001, ++ P < 0.01 *vs*. (BAY1+TPA) ANOVA with Tukey's post-test. BAY60-6583 was tested at 1 μg/area (BAY 1). PSB-1115 (PSB5) was tested at 5 μg/area. Vehicle (acetone-treated mice).

agonist widely used, both in *in vitro* and *in vivo* studies. However, it is important to point out that it shows submaximal efficacy, acting as a partial agonist. As such, BAY may display different pharmacological activity depending on the A_{2B} AR expression level and the local concentration of the endogenous ligand adenosine [53].

Topical application of BAY also reduced the inflammatory response induced by TPA. This result is noteworthy, since the role of the A_{2B} receptor in inflammatory/immune cells is highly controversial, being able to act as anti-inflammatory or pro-inflammatory depending on the cell type, the signaling pathway involved or the pathophysiological environment [45,50,54]. Despite this controversy, various studies indicated that A_{2B} activation reduces neutrophilic migration and microvascular permeability in experimental models of acute inflammation in lung [55, 56] and colon mucosa [47]. In addition, A_{2B}AR can inhibit neutrophilic adhesion and induce a phenotypic change of macrophages from M1 to M2, decreasing the production of TNF-α and IL-1β [45]. In agreement with these studies, our results have also demonstrated the reduction of neutrophilic migration, and inflammatory cytokines after A_{2B} AR activation in skin. Besides, the decrease of CXCL-1 in skin homogenates confirmed previous results showing a concentration-dependent inhibition of this chemokine after BAY treatment in TPA-stimulated human keratinocytes [16]. Since CXCL-1 is produced by keratinocytes during the innate response phase of psoriasis, the application of an A_{2B}AR agonist could reduce infiltration and neutrophilic activation characteristics of this early phase [57].

The present study also has shown that A_{2B}AR activation in skin preserves the correct epidermal barrier function not only under pathological but also under physiological conditions. In this sense, BAY application in TPA-treated animals restored the basal expression pattern of involucrin, CK10, loricrin and filaggrin. But more interesting, A_{2B} blockade with PSB even significantly decreased the normal expression ratio of CK10 and filaggrin in healthy animals, providing evidence for a physiological regulatory role of A_{2B}AR in the proliferation and differentiation of keratinocytes.

Among the proteins involved in the cornification process, the correlation filaggrin/caspase-14, have become especially relevant [40]. In contrast to other caspases, caspase-14 is a nonapoptotic caspase expressed and activated mainly in the epidermis and absent in most other adult cell types [42]. This protease is involved in the transition from profilaggrin to the active form filaggrin and the development of natural moisturising factors of the skin, being dysregulated in an impaired skin barrier [41,58]. Thus, basal expression of caspase-14 is significantly reduced in psoriatic lesions in both humans and mice [59, 60]. It has been reported that proinflammatory cytokines present in inflamed skin can reduce the amount of both filaggrin and caspase 14, suggesting a link between skin inflammation and disturbed epidermal barrier [61,62]. In this sense, our results showed that the inflammatory environment induced by TPA drastically decreased the expression of

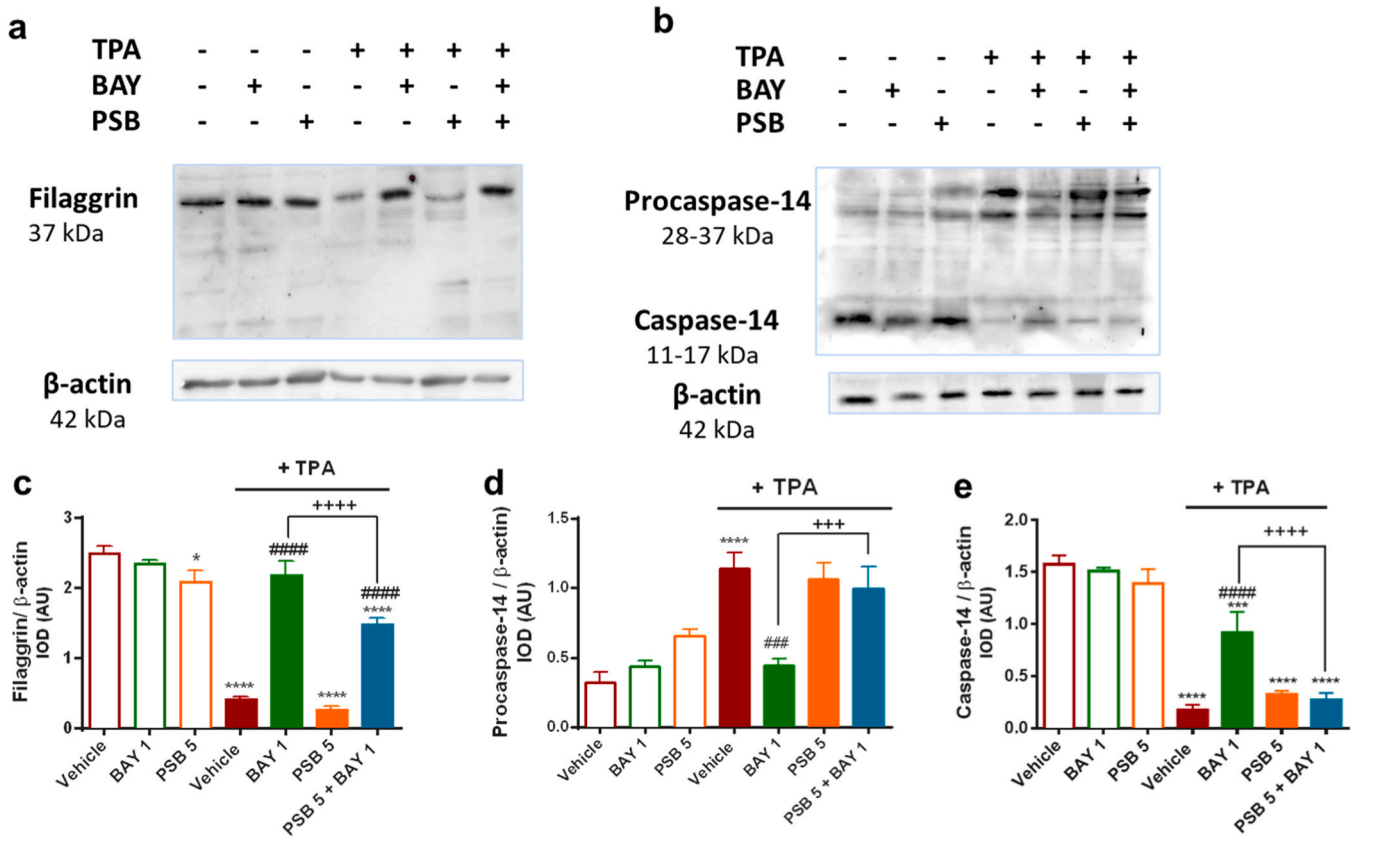

Fig. 5. BAY60-6583 (BAY) restored the physiological expression of filaggrin and caspase-14 in mice treated with TPA (**a,b**) Western blotting analysis of filaggrin and procaspase-14/caspase-14 expression on protein extracts from skin homogenates. One out of three mice investigated is shown. (**c,d,e**) Filaggrin/ β-actin, procaspase 14/β-actin and caspase 14/β-actin are expressed as arbitrary units (AU) of integrated optical density (IOD). Data represent the mean +/- SD of n=3 mice per group. **** P <0.0001 *vs.* Vehicle. #### P < 0.0001 *vs.* (Vehicle+TPA). ++++ P < 0.0001 *vs.* (BAY1+TPA). ANOVA with Tukey's post-test. BAY60-6583 tested at 1 μg/area (BAY 1). PSB-1115 was tested at 5 μg/area (PSB5). Vehicle: acetone-treated mice.

both filaggrin and caspase-14, while treatment with BAY recovered their expression until physiological basal levels.

It is known, that Ca^{2+} gradient in the epidermis is essential in regulating many skin functions, including keratinocyte differentiation, skin barrier formation, and permeability barrier homeostasis [63,64]. In fact, treating the parakeratotic plaques of patients with a vitamin D3 analogue results in the up-regulation of caspase-14 and coincides with amelioration of the lesions [60]. Thus, we could hypothesize that the protective and regulatory effect of A_{2B} activation on epidermal proliferation and skin barrier function could be related to the Ca^{2+} increase via Gq protein stimulation in keratinocytes [16]. However, due to the multiple signalling pathways participating after the activation of this AR, and the different cell type involved, further studies are needed to stablish the specific mechanism involved in the beneficial role of A_{2B} AR in the skin.

5. Conclusions

The beneficial effect of A_{2B} activation in this experimental model supports its protective role in various tissues where it is mainly expressed, such as the colon, preserving gastrointestinal epithelial barrier and regulating inflammation [47]. A_{2B}AR has also demonstrated protective effects in promoting endothelial barrier function [27], and reducing myocardial damage in ischemia-reperfusion processes [50]. A_{2B} signalling in alveolar epithelial cells is also protective in an acute model of lung inflammation in which aerosolized BAY attenuated pulmonary oedema and histologic injury [49]. Thus, in a similar manner, our results reveal, in an *in vivo* model, the protective role of A_{2B} AR activation in skin after topical application of an A_{2B} agonist, opening an interesting way for the development of new topical therapies in skin pathologies characterized by epidermal hyperproliferation, alteration of epidermal barrier function and inflammation, such as psoriasis in which AR expression is altered.

CRediT authorship contribution statement

M. Carmen Terencio: Writing – original draft, Visualization, Supervision, Methodology, Investigation, Formal analysis. **Miguel Marco-Bonilla:** Visualization, Investigation, Formal analysis. **Asunción Marín-Castejón:** Writing – original draft, Visualization, Methodology, Investigation, Formal analysis. **M. Carmen Montesinos:** Writing – review & editing, Supervision, Resources, Methodology, Investigation, Funding acquisition, Conceptualization. **Rosa Andrés-Ejarque:** Supervision, Methodology, Conceptualization. **María Antonia Noguera:** Writing – review & editing, Visualization, Investigation. **M. Luisa Ferrandiz:** Writing – review & editing, Resources, Funding acquisition. **M. Carmen Carceller:** Writing – review & editing, Visualization, Investigation. **Jorge Arasa:** Writing – review & editing, Supervision, Methodology.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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