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ORIGINAL ARTICLE

Adenosine A_{2A} and A_{2B} Receptors Differentially Modulate Keratinocyte Proliferation: Possible Deregulation in Psoriatic Epidermis

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Adenosine is a potent regulator of inflammation and immunity, but the role of adenosine receptors in keratinocytes remains controversial. We determined that in addition to A_{2B} receptors, human epidermal keratinocytes also express A_{2A} receptors, although to a lower extent. Through the use of selective adenosine receptor agonists and antagonists, we showed that physiological concentrations of adenosine activate A_{2B} receptors in normal human keratinocytes, inducing cell cycle arrest through the increase of intracellular calcium but not through cAMP signaling. In contrast, the selective activation of A_{2A} receptors by CGS-21680 induces keratinocyte proliferation via p38–mitogen-activated protein kinase activation. Adenosine and selective A_{2A} and A_{2B} agonists presented anti-inflammatory profiles independent of adenosine receptors but mediated by membrane phosphatase activation. Finally, keratinocyte exposure to diverse inflammatory cytokines altered adenosine receptor expression by reducing A_{2B} and increasing A_{2A}, a pattern also observed in psoriatic epidermis. Because increased epidermal turnover and inflammatory response are characteristics of psoriatic disease, further studies are needed to assess the role and consequences of the altered adenosine receptor expression in lesional and nonlesional psoriatic keratinocytes.

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INTRODUCTION

In the era of biologic therapies, the classic immunomodulator methotrexate is still considered a first-line, inexpensive systemic treatment of psoriasis with a very well-established safety profile (Yelamos and Puig, 2015). Methotrexate is a competitive inhibitor of dihydrofolate reductase, which blocks DNA synthesis and cell mitosis of rapidly dividing cells. However, folic acid supplements are co-administered to reduce its toxicity and adverse effects without compromising its anti-inflammatory efficacy, suggesting that other mechanisms of action might exist (Bangert and Costner, 2007). Although no single mechanism is sufficient to account for all the anti-inflammatory activities of methotrexate, several studies have shown the involvement of adenosine (Chan and Cronstein, 2010; Yelamos and Puig, 2015).

Adenosine is an endogenous purine nucleoside that can be released or formed by enzymatic dephosphorylation of adenosine triphosphate, adenosine diphosphate, and adenosine monophosphate during inflammation, wounding, and other pathological states. Once in the extracellular compartment, adenosine acts through four subtypes of adenosine receptors (ARs), A_1, A_{2A}, A_{2B}, and A_3, all belonging to the large family of G protein-associated receptors (Fredholm et al., 2011). Numerous studies indicate that adenosine is a potent regulator of inflammation and immunity (Antonioli et al., 2014; Ernst et al., 2010). In contrast, little is known about adenosine effects on epidermal cells, and reports are often contradictory. Thus, activation of adenosine A_{2B} receptors promotes murine keratinocyte proliferation (Braun et al., 2006), whereas adenosine inhibits human keratinocyte proliferation through uptake by the cell membrane transporter hENT1 (Brown et al., 2000).

Purinergic signaling in healthy and diseased skin has emerged as a renewed area of interest (Burnstock et al., 2012). In particular, activation of adenosine A_{2A} receptor plays an important role in promotion of wound healing (Montesinos et al., 2015), being especially relevant in the formation of new granulation tissue and revascularization of the wound bed (Montesinos and Valls, 2010; Valls et al., 2009). Although mice with a targeted disruption of this receptor subtype showed a defect in granulation tissue formation, wound re-epithelialization was hardly compromised

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Abbreviations: AR, adenosine receptors; BAY, BAY60–6583; CGS, CGS-21680; iCa^{2+}, intracellular calcium; MRS, MRS-1706; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NECA, 5′-N-ethylcarboxamidoadenosine; NHEK, normal human epidermal keratinocyte; SCH, SCH-442416; TNF, tumor necrosis factor; TPA, 12-O-tetradecanoylphorbol-13-acetate

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(Montesinos et al., 2002). Moreover, topical application of an A_{2A} agonist has proved to be beneficial, preventing epidermal hyperplasia induced by repeated 12-*O*-tetradecanoylphorbol-13-acetate (TPA) treatment, showing an anti-inflammatory effect (Arasa et al., 2014). In addition, this receptor subtype could be involved in the murine imiquimod-induced model of psoriasis, because imiquimod has been also characterized as an A_{2A} AR antagonist (Flutter and Nestle, 2013; Schon et al., 2006). Furthermore, an A_3 AR agonist is currently being evaluated in phase II and III clinical trials in patients with moderate to severe plaque psoriasis because of its regulatory effect on T-cell function (David et al., 2012; Kofoed et al., 2015).

In this study we have assessed the expression and signal transduction mechanisms of AR in normal human epidermal keratinocytes (NHEK). In addition, through the use of selective AR agonists and antagonists, we have shown a differential effect of A_{2B} and A_{2A} AR on cell proliferation and a receptor-independent anti-inflammatory effect. Finally, we have determined the possible influence of the inflammatory milieu on AR expression in NHEK, which mimics the expression of AR in psoriatic epidermis.

RESULTS

Adenosine A_{2B} but not A_{2A} receptor stimulation increases intracellular cAMP and iCa^{2+} in NHEK

Earlier reports indicated that A_{2B} was the only AR subtype expressed by NHEK (Brown et al., 2000), whereas, in murine keratinocytes, lower expressions of A_{2A} and A_3 AR have also been described (Braun et al., 2006). We analyzed the expression of AR in NHEK by reverse transcriptase PCR and confirmed that, although A_{2B} is the major AR subtype expressed by these cells (ΔCt against glyceraldehyde-3-phosphate dehydrogenase $= 7.02 \pm 0.074$, $n = 10$), A_{2A} receptors are also expressed to a lesser extent (ΔCt against glyceraldehyde-3-phosphate dehydrogenase $= 12.50 \pm 0.16$, $n = 12$, $P < 0.001$ vs. A_{2B}), whereas subtypes A_1 and A_3 were undetectable (Figure 1a).

Both A_{2A} and A_{2B} ARs are supposedly coupled to G_s proteins and, thus, their activation increases intracellular cAMP, which has been mainly associated with the anti-inflammatory response of adenosine (Ernst et al., 2010). We incubated NHEK with the selective A_{2A} agonist CGS-21680 (CGS), the selective A_{2B} agonist BAY60−6583 (BAY), or the nonselective agonist 5′-*N*-ethylcarboxamidoadenosine (NECA) alone or in combination with the selective antagonists SCH-442416 (SCH) and MRS-1706 (MRS) (see Supplementary Table S1 online). After 15 minutes, NECA and BAY induced a significant increase in the intracellular cAMP concentration that was inhibited by the selective A_{2B} antagonist MRS, indicating signaling through G_s protein (Figure 1b). In contrast, CGS failed to induce cAMP elevation, and the selective A_{2A} antagonist SCH did not affect NECA-induced production of cAMP, ruling out this second messenger downstream of the A_{2A} AR activation in NHEK.

Because A_{2B} AR has also been reported to signal through intracellular calcium (iCa^{2+}) via G_q protein activation in certain cellular types (Aherne et al., 2011; Haskó et al., 2008), we incubated NHEK with AR agonists and antagonists and determined iCa^{2+} levels by coupling with a fluorescent dye. As shown in Figure 1c, stimulation with either the nonselective agonist NECA or the selective A_{2B} agonist BAY led to an increase in iCa^{2+} that was reversed by co-incubation with the A_{2B} antagonist MRS but not by the A_{2A} selective antagonist SCH; the A_{2A} agonist CGS had no effect at this level. These results are consistent with A_{2B} AR signaling through G_q in NHEK as well.

Adenosine A_{2A} and A_{2B} receptor subtypes differentially modulate keratinocyte proliferation

Using selective AR agonists and antagonists, we found that activation of A_{2A} and A_{2B} AR had opposing effects on NHEK proliferation, determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction. Thus, incubation with the selective A_{2B} agonist BAY decreased keratinocyte proliferation, whereas the selective A_{2A} agonist CGS increased cell growth (Figure 2a), effects that were inhibited by the selective antagonists MRS and SCH, respectively.

The nonselective agonist NECA, widely used in research as an adenosine equivalent that does not suffer enzymatic deamination, enhanced keratinocyte proliferation. This effect was abrogated by the selective antagonist SCH, indicating the involvement of A_{2A} AR. Because it has been shown that adenosine inhibited proliferation of NHEK (Brown et al., 2000), we determined its effect in the presence or absence of the selective antagonists. As shown in Figure 2a, the antiproliferative effect of adenosine was primarily mediated by A_{2B} AR, because it was abrogated by MRS. The opposing effects of the nonselective agonist NECA and the endogenous ligand adenosine are probably due to the higher affinity of NECA for the A_{2A} AR (see Supplementary Table S1) (Alnouri et al., 2015), thus explaining the previously reported divergences regarding the effect of AR activation on NHEK proliferation.

Cell proliferation results were confirmed by cell cycle analysis. As shown in Figure 2b, A_{2A} stimulation by CGS significantly increased the fraction of cells in G2/M in accordance with the increased cell proliferation observed by MTT assay, even though there was no appreciable change in the S phase. In contrast, A_{2B} activation decreased S and G_2/M fraction while increasing G_1, showing an antiproliferative effect. These results were validated with selective AR antagonists (see Supplementary Figure S1 online). Similar to the MTT assay, NECA and adenosine evidenced opposing effects in the cell cycle mediated by A_{2A} and A_{2B} receptors, respectively (Figure 2b, and see Supplementary Figure S1).

Opposite effects of A_{2A} and A_{2B} AR on the cell cycle are mediated by p38 mitogen-activated protein kinase and iCa^{2+}, respectively

Because A_{2B} AR signaled through cAMP in NHEK, we evaluated the effect of the adenylyl cyclase activator forskolin on keratinocyte cell growth. In contrast to the effect of adenosine and BAY, forskolin highly increased proliferation (Figure 2a and b), suggesting that other signaling pathways rather than cAMP mediate the cell cycle arrest induced by A_{2B} activation in NHEK. To determine the possible participation of downstream targets to the cAMP signaling cascade, cells were incubated with the protein kinase A inhibitor H-89 (10 μmol/L) and the exchange protein activated by cAMP (EPAC) ESI-09 (10 μmol/L). Unfortunately, both inhibitors showed toxicity, determined by lactate dehydrogenase and trypan blue exclusion (data not shown) to warrant reliable results.

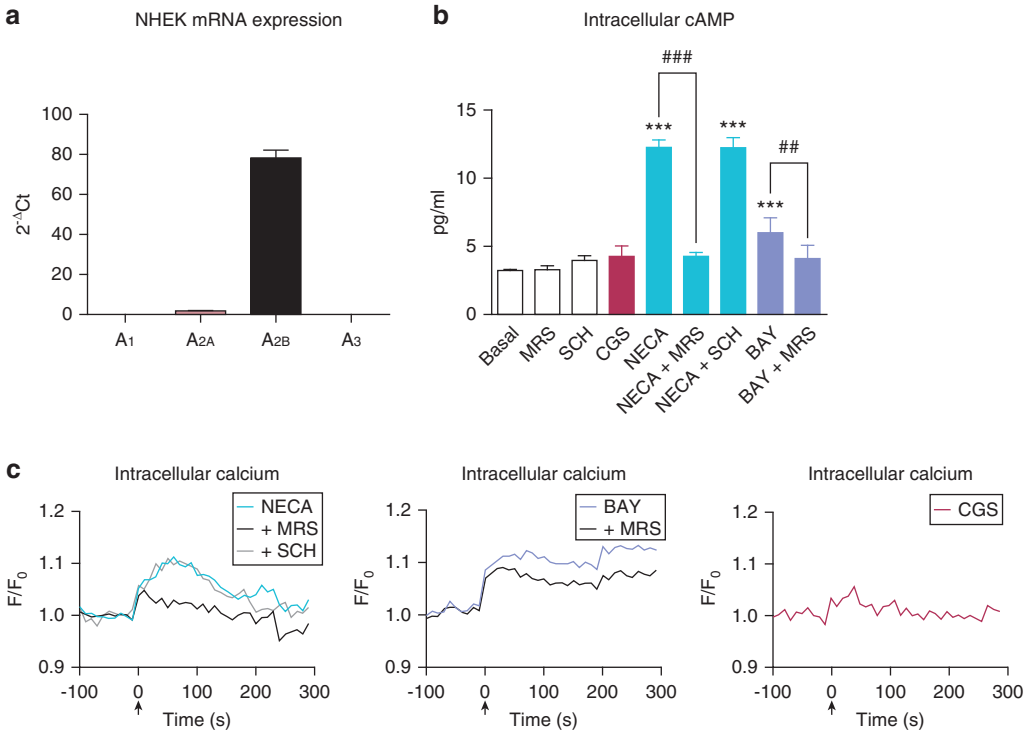

Figure 1. Basal mRNA expression of ARs in NHEK and intracellular cAMP and calcium levels after AR stimulation. (**a**) Quantitative real-time reverse transcriptase-PCR determination of AR levels in NHEK. Data are mean ± standard error of the mean (n = 10–12). Values were normalized to the housekeeping gene GAPDH. (**b**) cAMP levels after 15-minute stimulation with CGS-21680 (A_{2A} selective), BAY60−6583 (A_{2B} selective), or NECA (nonselective), alone or in combination with the antagonists SCH-442416 (A_{2A} selective) and MRS-1706 (A_{2B} selective). Data are mean ± standard error of the mean (n = 6–9). $^{***}P < 0.001$ versus basal (nonstimulated NHEK) using Dunnett *t* test. $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ using Student *t* test. (**c**) Representative measurement (n = 10) of intracellular calcium release after stimulation with AR agonists (arrows) with or without antagonists. CGS, BAY, and NECA were tested at 10 μmol/L. SCH and MRS were pre-incubated for 30 minutes at 1 μmol/L. AR, adenosine receptor; BAY, BAY60−6583; CGS, CGS-21680; F/F_0, fluorescence signal relative to starting signal; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MRS, MRS-1706; NECA, 5′-*N*-ethylcarboxamidoadenosine; NHEK, normal human epidermal keratinocyte; s, seconds; SCH, SCH-442416.

As stated before, AR activate additional signaling pathways apart from G_s proteins, such as extracellular signal-regulated kinases and p38 mitogen-activated protein kinase or G_q-mediated increase of iCa^{2+} (Aherne et al., 2011). Given the pivotal role of these signaling pathways in the control of the cell cycle, we pre-incubated NHEK with the selective inhibitors PD98059 (extracellular signal-regulated kinase 1/2), SB202190 (p38), or the calcium chelator 1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis(acetoxymethyl ester) (BAPTA-AM) and determined the cell cycle distribution after 24-hour stimulation with AR agonists. All these inhibitors did not affect cell cycle at the concentrations assayed (see Supplementary Figure S2 online). BAPTA-AM significantly reversed the antiproliferative effect of adenosine (Figure 3a) and BAY (Figure 3b), decreasing the proportion of cells in G_1 and suggesting that calcium mediates, at least in part, the partial cell cycle arrest induced by A_{2B} activation. On the other hand, the p38 inhibitor SB202190 partially blocked the effect of A_{2A} AR activation, reversing the increased fraction of cells in the G_2/M phase induced by CGS (Figure 3c).

Adenosine and AR agonists inhibit the inflammatory response of NHEK

Epidermal keratinocytes are key participants in innate or adaptive immune responses through production of cytokines and chemokines such as tumor necrosis factor (TNF)-α or IL-8 (Lowes et al., 2014) and adenosine is a well-established regulator of inflammation (Haskó and Cronstein, 2013). Therefore, we determined the effect of AR activation on cytokine release by NHEK stimulated with the protein kinase C activator TPA, which can reproduce certain inflammatory parameters of psoriatic skin in animal models (Andres et al., 2013). Adenosine, as well as the selective A_{2A} and A_{2B} agonists, inhibited the levels of TNF-α and IL-8 in a concentration-dependent manner; however, this effect was not reversed by any of the selective antagonists (Figure 4). These results suggested an unspecific anti-inflammatory mechanism independent of ARs. This controversial point has been previously reported in other cell types such as neutrophils and macrophages, indicating that adenosine can suppress cell activation and TNF-α release by other independent mechanisms such as the activation of membrane phosphatases (Fotheringham et al., 2004; Haskó and Cronstein, 2004; Kreckler et al., 2009). In this sense, preincubation of NHEK with the serine/threonine protein phosphatase inhibitor okadaic acid enhanced TNF-α release induced by the protein kinase C activator TPA as previously described (Fujiki et al., 2013). Nevertheless, in the presence of okadaic acid, all tested AR agonists failed to inhibit cytokine release after TPA stimulation (see Supplementary Figure S3 online).

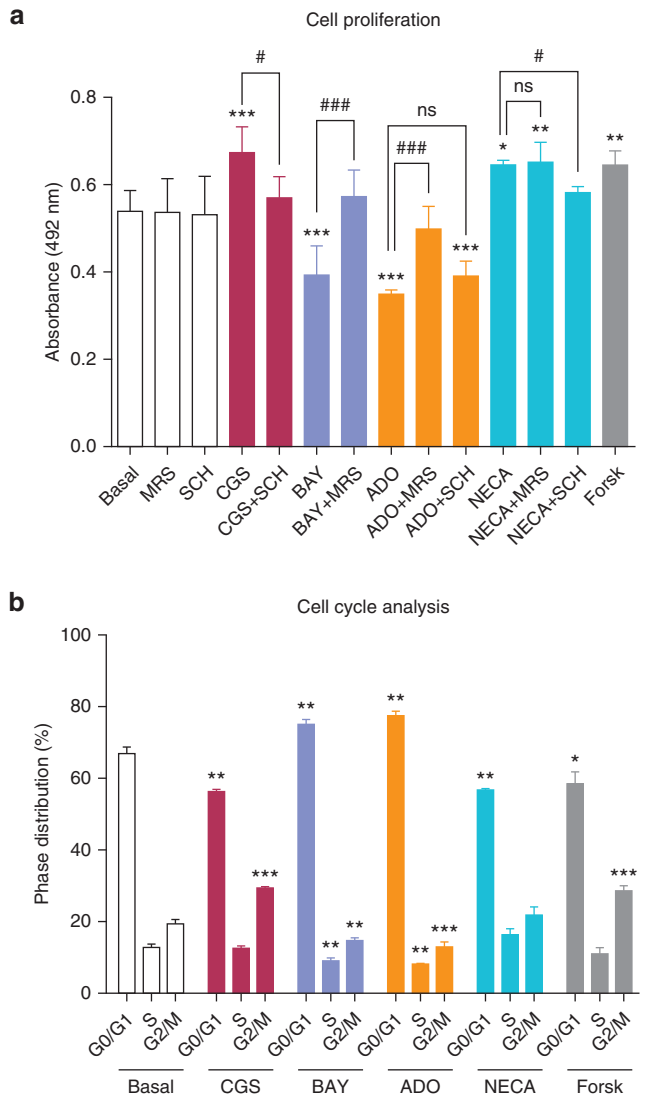

Figure 2. Effect of adenosine receptor stimulation on NHEK cell growth. (**a**) Proliferation was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay after 48 hours of treatment. Data are mean absorbance at 490 nm ± standard error of the mean (n = 8–10). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus basal cells (nonstimulated) using Dunnett *t* test. $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ using Student *t* test. (**b**) Results of cell-cycle analysis by flow cytometry after 24 hours of treatment. Data are mean ± standard error of the mean (n = 10–12). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus the same distribution phase in the basal cell cycle (Dunnett *t* test). CGS-21680, BAY60–6583, adenosine, and NECA were tested at 1 μmol/L. The antagonists SCH-442416 (A_{2A} selective) and MRS-1706 (A_{2B} selective) were pre-incubated for 30 minutes at 1 μmol/L. The adenylyl cyclase activator forskolin was tested at 50 μmol/L. ADO, adenosine; BAY, BAY60–6583; CGS, CGS-21680; Forsk, forskolin; MRS, MRS-1706; NECA, 5′-*N*-ethylcarboxamidoadenosine; NHEK, normal human epidermal keratinocyte; ns, not significant; SCH, SCH-442416.

Inflammatory milieu could alter AR expression in NHEK and psoriatic epidermis

Several reports have described that the constant exposure of inflammatory cytokines modulate AR expression in both infiltrating and resident cells (Khoa et al., 2001; Morello et al., 2006; Nguyen et al., 2003; Xaus et al., 1999). Consequently, we hypothesized that the inflammatory milieu characteristic of psoriatic plaques could alter AR expression in NHEK. To assess this hypothesis, we determined mRNA levels of A_{2A} and A_{2B} receptors in NHEK preincubated with an array of pro-inflammatory cytokines known to be up-regulated in psoriasis (TNF-α, IL-1β, IL-6, IL-17, IL-23, IFN-α, or IFN-γ) or with the protein kinase C activator TPA. We observed that IFN-γ, and the reference stimulus TPA, altered the expression of both ARs, decreasing A_{2B} and increasing A_{2A}. On the other hand, IFN-α significantly decreased A_{2B} AR expression, whereas TNF-α and IL-1β increased A_{2A} AR. Other psoriatic mediators such as IL-6, IL-17, and IL-23 had no effect (Figure 5a). In view of these results, we next compared the pattern of AR expression in epidermis obtained from biopsy samples of plaque-type psoriasis versus epidermis from surgical foreskin resections of healthy donors, used throughout this study as the source of NHEK. In healthy epidermis, AR expression was similar to the one described earlier for NHEK (Figure 5b). However, even though A_{2B} continued to be more expressed than A_{2A}, and A_1 and A_3 were undetectable in psoriatic epidermis, we observed a significantly different level of expression of both receptor subtypes compared with normal epidermis: A_{2B} receptor expression was reduced, whereas A_{2A} receptor mRNA was increased (Figure 5b). Accordingly, immunoblotting analysis confirmed that psoriatic epidermis showed higher expression of A_{2A} and lower expression of A_{2B} ARs than foreskin epidermis at the protein level, despite a certain degree of interindividual variability (Figure 5c), possibly because of receptor glycosylation, as previously reported (Linden et al., 1999). Therefore, these results suggest that some inflammatory mediators generated in psoriatic skin could deregulate the normal ratio of AR expression in epidermis and consequently alter the physiological role of AR in keratinocytes.

DISCUSSION

In this study we have elucidated the contribution of AR in controlling keratinocyte proliferation, resolving a long-lasting controversy. We have determined that adenosine promotes cell-cycle arrest, in agreement with older reports (Brown et al., 2000; Cook et al., 1995); however, using much lower concentrations and selective antagonists, we have shown the involvement of A_{2B} AR. On the other hand, we have shown that A_{2A} AR enhances keratinocyte proliferation, mediating the proliferative effect of the nonselective agonist NECA described by Braun et al. (2006). Therefore, our study underlines the importance of the selection of the right pharmacological tools.

It is generally believed that A_{2B} AR is a low-affinity receptor, requiring pathophysiological concentrations of adenosine for activation (Haskó et al., 2009). However, in many cell types, potency or affinity is markedly influenced by receptor number and the measured response (Fredholm et al., 2011). Our results suggest a predominant role for the highly expressed A_{2B} AR subtype in the physiological control of cell growth in NHEK, in contrast to other skin resident cells such as fibroblasts with higher expressions of A_{2A} AR, whose activation increases cell proliferation and collagen synthesis (Perez-Aso et al., 2014; Perez-Aso et al., 2013; Rathbone et al., 1992). Similar results have been obtained in other cell types where A_{2B} is the most expressed AR (Dubey et al., 2015; Jackson, 2011; Mayer et al., 2011). In particular, epithelial-specific

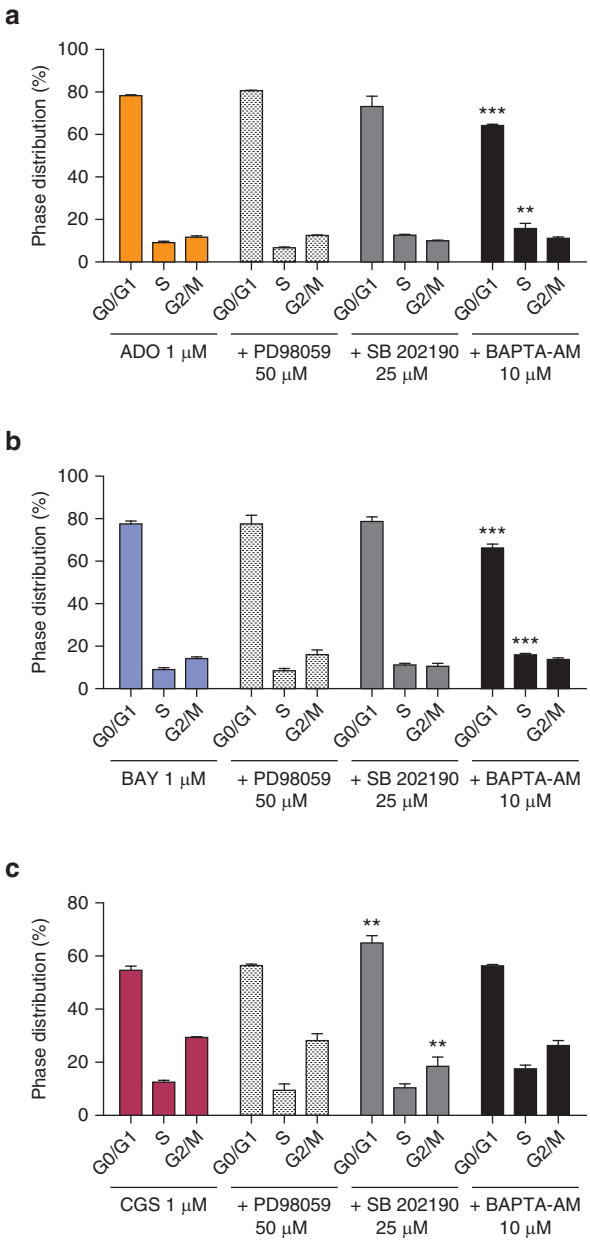

Figure 3. Effect of mitogen-activated protein kinase inhibition or intracellular calcium chelation on cell cycle phase distribution after treatment of normal human epidermal keratinocytes with adenosine receptor agonists. After a 30-minute pretreatment with the extracellular signal-regulated kinase 1/2 mitogen-activated protein kinase inhibitor PD98059, the p38 mitogen-activated protein kinase inhibitor SB202190, or the calcium chelator BAPTA-AM, NHEK were incubated for 24 hours with (**a**) the physiological agonist adenosine, (**b**) the selective A_{2B} agonist BAY60−6583, or (**c**) the selective A_{2A} agonist CGS-21680. Data are mean ± standard error of the mean (n = 10−12). $^{**}P < 0.01$, $^{***}P < 0.001$ versus the same distribution phase in the corresponding control agonist treatment (Dunnett *t* test). ADO, adenosine; BAY, BAY60−6583; CGS, CGS-21680; BAPTA-AM, 1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis(acetoxymethyl ester); M, mol/L.

A_{2B} AR mediated the protective effect of adenosine in intestinal inflammation by promoting epithelial barrier (Aherne et al., 2015).

Our results also indicate that intracellular calcium mediates the antiproliferative effect of A_{2B} AR activation on NHEK, with no involvement of cAMP. Calcium regulates the growth, differentiation, and apoptosis of many cell types, including epidermal keratinocytes, in which it induces G_1/G_0 cell cycle arrest (Bikle et al., 2012). Thus, some antipsoriatic drugs such as 1α,25-dihydroxyvitamin D_3 or fumaric acid exert an antiproliferative effect mediated by iCa^{2+} elevation (Dascalu et al., 2000). In contrast to the A_{2B}-mediated antiproliferative effect of adenosine, activation of A_{2A} AR induces a proliferative response in NHEK dependent on p38 and independent of cAMP. This observation is in agreement with several reports stating that the peculiar long C-terminus of A_{2A} AR is involved in independently signaling through either mitogen-activated protein kinase or G_s, depending on the membrane lipid microenvironment (Charalambous et al., 2008; Klinger et al., 2002).

We have recently shown that topical application of CGS increased keratinocyte and fibroblast proliferation when applied topically on uninvolved mouse skin but abrogated TPA-induced epidermal hyperplasia, mainly because of its marked anti-inflammatory effect (Arasa et al., 2014). In this study, we have shown that CGS and the other AR agonists show anti-inflammatory properties in NHEK. However, this effect appears to be independent of receptor activation, because the reduction of cytokine release by both selective and nonselective AR agonists could not be reversed by their corresponding AR antagonist. In this regard, we have identified a possible mechanism related to serine/threonine protein phosphatase activation, as previously reported for adenosine in other cellular lineages (Fotheringham et al., 2004; Kreckler et al., 2009).

Some observations suggest that purine metabolism could be defective in skin autoimmune diseases such as psoriasis (Kiehl and Ionescu, 1992; Kose et al., 2001); overexpression of A_{2A} and A_3 AR in inflammatory cells has been found in different autoimmune disorders (Ochaion et al., 2009; Varani et al., 2011). In this study, we have observed a reduction in A_{2B} AR expression besides the increased expression of A_{2A} AR in psoriatic epidermis. Furthermore, some inflammatory mediators released during the psoriatic process could be in part responsible for these variations, particularly cytokines such as IL-1β, TNF-α, IFN-α, and IFN-γ, which are mainly involved in the initiation of the psoriatic plaque (Perera et al., 2012). Moreover, such cytokines as IL-1β and TNF-α have been shown to regulate A_{2A} AR function as well by preventing its desensitization (Khoa et al., 2006).

On the other hand, the IL-17/IL-23 axis cytokines, characteristic of the established psoriatic plaque, had no effect. Therefore, it seems reasonable to think that the variations in AR expression could occur during the early stages of lesion development. In this regard, the up-regulation of A_{2A} AR by TNF-α and Il-1β could play a protective anti-inflammatory role, as other researchers have suggested (Borea et al., 2016). Otherwise, IFN-α produced by plasmacytoid dendritic cells is one of the key and initial steps in psoriasis pathogenesis contributing to keratinocyte proliferation (Farkas and Kemeny, 2012). Thus, the reduction of A_{2B} expression after stimulation with IFN-α could cause the loss of the antiproliferative control elicited by this receptor subtype in NHEK. However, further experiments comparing

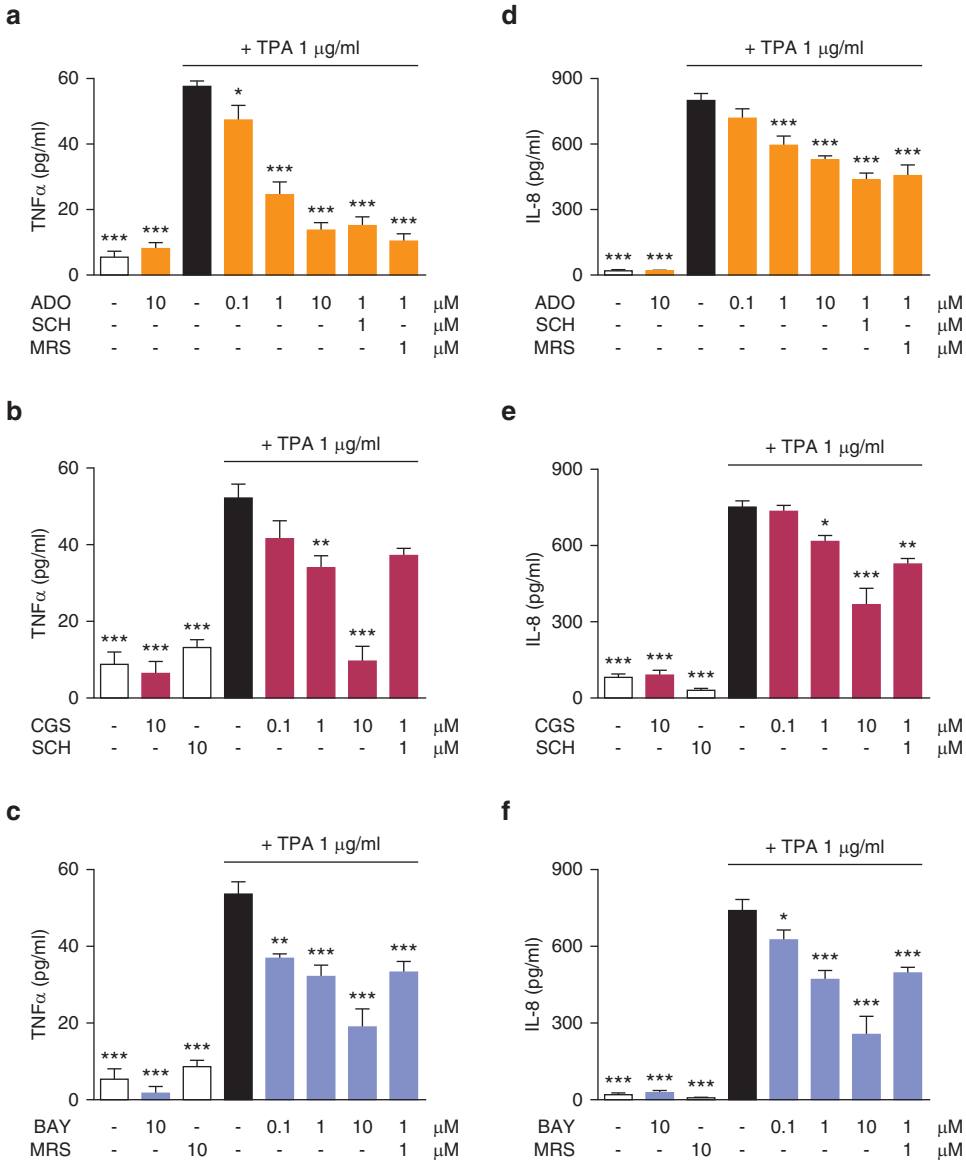

Figure 4. Inhibitory effect of adenosine receptor agonists on TNF-α and IL-8 release in normal human epidermal keratinocytes. Cells were incubated for 30 minutes with adenosine, the selective A_{2A} agonist CGS-21680, or the selective A_{2B} agonist BAY60–6583 before stimulation for 7 hours with TPA (1 μg/ml). Pretreatment with the A_{2A} antagonist SCH-442416 or the A_{2B} antagonist MRS-1706 was performed for 30 minutes before the addition of the agonist. (**a**–**c**) TNF-α and (**d**–**f**) IL-8 levels were determined in cell supernatants by ELISA. Values are expressed as mean ± standard error of the mean (n = 8–14). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus the untreated TPA-stimulated cells using Dunnett *t* test. ADO, adenosine; BAY, BAY60–6583; CGS, CGS-21680; M, mol/L; MRS, MRS-1706; SCH, SCH-442416; TNF, tumor necrosis factor; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

lesional to nonlesional psoriatic skin will need to be conducted to confirm this hypothesis.

This study has shown an intriguing dual role of A_{2A} and A_{2B} ARs on keratinocyte proliferation and their possible deregulation in psoriatic epidermis. The consequences of the altered AR expression on adenosine effects in psoriatic keratinocytes will need to be assessed in further studies, given the high potential of this endogenous mediator to ameliorate inflammatory diseases. Our results suggest that adenosine plays an important role in regulating epidermal inflammation and keratinocyte function and thus may constitute an interesting therapeutic strategy for inflammatory hyperproliferative skin diseases such as psoriasis.

MATERIALS AND METHODS

Statement on use of human materials

All protocols and procedures were approved by the University of Valencia Ethical Committee and conformed to the Helsinki guidelines. Patient consent for experiments was not required, because Spanish laws consider human tissue left from surgery as discarded material.

Isolation, culture, and stimulation of primary human keratinocytes

Primary human keratinocytes were isolated from foreskins of healthy young donors as described previously (Andres et al., 2013). Briefly, skin samples were treated with a dispase solution and trypsinized. Keratinocytes were grown (37° C/5% CO_2) in a serum-free low-Ca^{2+} (<0.1 mol/L) Defined Keratinocyte-SFM (Invitrogen, Carlsbad, CA). For all experiments, cells were seeded at passage numbers 1–3 and treated upon reaching 60–80% confluence. The day before the experiments, medium was replaced to growth factor-free keratinocyte medium.

Adenosine agonists and antagonists

AR agonists and antagonists (see Supplementary Table S1) were purchased from Tocris (Bristol, UK) and dissolved in DMSO. The concentration of DMSO in all experiments was less than 0.1%. In all the experiments, antagonists were added 30 minutes before agonists.

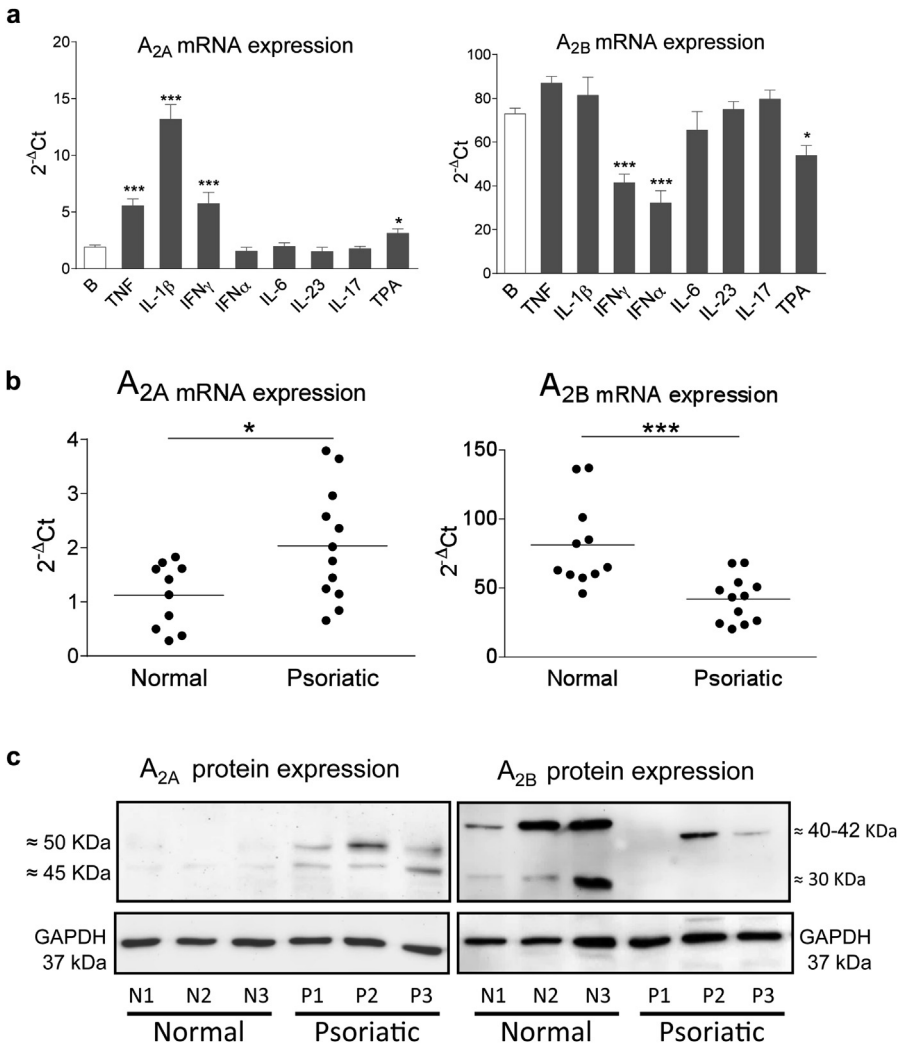

Figure 5. Differential expression of adenosine receptors in normal and psoriatic epidermis. (**a**) Normal human epidermal keratinocytes were incubated with TNF-α, IL-23, IL-17, IL-1β (10 ng/ml), IFN-γ, IFN-α, IL-6 (50 ng/ml), or TPA (1 μg/ml) for 3 hours. Subsequently, mRNA levels of A_{2A} and A_{2B} adenosine receptors were evaluated by quantitative real-time reverse transcriptase-PCR. Data are mean ± standard error of the mean (n = 6–8). $^{*}P < 0.05$, $^{***}P < 0.001$ versus nontreated normal human epidermal keratinocytes using Dunnett *t* test. All values were normalized to the housekeeping gene GAPDH. (**b**) Variation of A_{2A} and A_{2B} AR mRNA expression in psoriatic versus normal epidermis. Data are mean ± standard error of the mean (n = 10–12). $^{*}P < 0.05$, $^{***}P < 0.001$ using the Mann-Whitney test. (**c**) A_{2A} and A_{2B} AR inmmunoblotting of whole tissue homogenates of normal (N1–N3) and psoriatic epidermis (P1–P3). B, nontreated normal human epidermal keratinocytes; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TNF, tumor necrosis factor; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

Intracellular cAMP determination

NHEK seeded in 24-well plates were stimulated with AR agonist for 15 minutes. Cells were harvested, and intracellular cAMP was assessed using Cyclic AMP Direct EIA Kit (Arbor Assays, Ann Arbor, MI).

iCa^{2+} determination

iCa^{2+} was assessed using Fluo-4 NW Calcium assay kit (Invitrogen, Carlsbad, CA), following the manufacturer's recommendations. Briefly, NHEK seeded in 96-well plates were incubated for 1 hour with the dye loading solution and were stimulated with AR agonists (10 μmol/L). Fluorescence was measured at different time points using a Wallac 1420 VICTOR2 (PerkinElmer, Waltham, MA).

Proliferation assay

NHEK were seeded in a 12-well plate and incubated for 48 hours with AR agonists (1 μmol/L) in growth factor-free keratinocyte medium. Cell density was determined by MTT reduction, and cytotoxicity was assessed by measuring lactate dehydrogenase release in the supernatants (Andres et al., 2013).

Cell cycle assay

AR agonists (1μmol/L) were added to NHEK cultured in 25 cm^2-flasks with growth factor-free keratinocyte medium. After 24 hours, cells were trypsinized, and cell cycle was measured using BD Cycletes Plus DNA Reagent Kit (BD Biosciences, San Jose, CA). Results were analyzed using a BD FACS VERSE cytometer (BD Biosciences).

Determination of cytokine release

NHEK seeded in 24-well plates were incubated with AR agonists for 30 minutes before stimulation with TPA from Sigma-Aldrich (St. Louis, MO). After 7-hour supernatants were collected. TNF-α, (R&D Systems, Abingdon, UK) and IL-8 (eBioscience, San Diego, CA) levels were assessed in cell supernatants using ELISA assays, following standard manufacturer protocols.

Determination of AR expression

Lesional psoriatic skin was obtained from punch biopsy from the center of a plaque-type psoriatic lesion at time of diagnosis. For the quantitative real-time reverse transcriptase-PCR determination, 12 patients (mean age ± standard deviation = 42.5 ± 6.5 years, white, 8 men and 4 women) and, for the immunoblotting analysis, 8 patients (mean age ± standard deviation = 39.4 ± 5.4 years, white men) were included in this study.

RNA isolation, and quantitative real-time reverse transcriptase–PCR were performed in isolated NHEK cells and normal and psoriatic epidermis following the manufacturer's specifications. NHEK cells were stimulated with TNF-α, IL-1β, IL-17, IL-23

(10 ng/ml), IL-6, IFN-α, IFN-γ (50 ng/ml), or TPA (1 μg/ml) for 3 hours. All cytokines were from PeproTech (London, UK) except IL-6 (R&D Systems, Abingdon, UK). Tripure Isolation Reagent (Roche, Mannheim, Germany) and ImRrom-II Reverse Transcription System (Promega, Madison, WI) were used for construction of cDNA, including random hexamers as primers. Subsequently, mRNA expressions of A_1, A_{2A}, A_{2B}, A_3, and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase were determined using TaqMan MGB probes, FAM dye-labeled gene expression assay mix (assay ID: Hs00379752_m1, Hs00169123_m1, Hs00386497_m1, Hs00181232_m1, and Hs99999905_m1, respectively) and Taqman Universal Master Mix II (Applied Biosystems, Foster City, CA). All samples were analyzed in doublets on a StepOnePlus (Applied Biosystems, Foster City, CA). Relative gene expression levels were determined by using the quantitative C_T method.

Western blot of homogenized skin biopsy samples (20 μg of protein) was performed as previously described (Arasa et al., 2014). Specific antibodies against A_{2A} and A_{2B} AR were from Alomone labs (Jerusalem, Israel). Secondary staining was obtained by incubating with goat anti-rabbit antibody (Dako, Glostrup, Denmark). Glyceraldehyde-3-phosphate dehydrogenase was used as the reference protein for loading control.

Statistical analysis

Results are presented as mean ± standard error of the mean; n represents the number of experiments. The level of statistical significance was determined by analysis of variance, followed by the Dunnett *t* test for multiple comparisons, Student *t* test, or Mann-Whitney test for dual comparisons. All statistical analyses were performed using GraphPad Prism 6 software (GraphPad Software, Inc., San Diego, CA). Significance was assumed at a *P*-value of 0.05 or less.

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CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at http://dx.doi.org/10.1016/j.jid.2016.07.028.

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