Purinergic Receptors Are Part of a Functional Signaling System for Proliferation and Differentiation of Human Epidermal Keratinocytes

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We investigated the expression of $P2X_5$, $P2X_7$, $P2Y_1$ and $P2Y_2$ receptor subtypes in normal human epidermis and in relation to markers of proliferation (PCNA and Ki-67), keratinocyte differentiation (cytokeratin K10 and involucrin) and markers of apoptosis (TUNEL and anticaspase-3). Using immunohistochemistry, we showed that each of the four receptors was expressed in a spatially distinct zone of the epidermis, suggesting different functional roles for these receptors. Functional studies were performed on primary cultures of human keratinocytes and on explanted rat skin, where different P2 receptor subtype agonists and antagonists were applied to cultured keratinocytes or injected subcutaneously into the skin, respectively. An increase in cell number was caused by low doses of the nonspecific P2 receptor agonist ATP, the $P2Y_2$ receptor agonist UTP ($p < 0.001$), and the $P2Y_1$ receptor agonist 2MeSADP ($p < 0.05$). There was a significant decrease in cell number as a result of treatment with the $P2X_5$ receptor agonist ATPγS ($p < 0.001$) and the $P2X_7$ receptor agonist BzATP ($p < 0.001$). Suramin caused a significant block in the effect of 100 μM ATP ($p < 0.01$) and 1000 μM ATP ($p < 0.001$) on cell number. These results imply that different purinergic receptors have different functional roles in the human epidermis with $P2Y_1$ and $P2Y_2$ receptors controlling proliferation, while $P2X_5$ and $P2X_7$ receptors control early differentiation, terminal differentiation and death of keratinocytes, respectively. ***Keywords: skin/purinergic receptors/apoptosis/proliferation. J Invest Dermatol 120:1007–1015, 2003***

Adenosine triphosphate (ATP) is now recognized as an important messenger molecule for cell-cell communication, with ATP binding specifically to purinergic receptors (Ralevic and Burnstock, 1998). The majority of studies have been concerned with the short-term events that occur in neurotransmission and secretion, but there is increasing evidence that purinergic signaling can have long-term, trophic effects in cell proliferation, differentiation and death (Abbracchio and Burnstock, 1998).

Purinergic receptors are classified into two groups: P1 receptors are selective for adenosine and P2 receptors are selective for adenosine 5′-triphosphate (ATP) and adenosine 5′-diphosphate (ADP), which act as extracellular signaling molecules (Burnstock, 1978). P2 receptors are divided into two main families: P2X (ligand–gated ion channels) and P2Y (G protein-coupled receptors), based on molecular structure, transduction mechanisms and pharmacological properties (Abbracchio and Burnstock, 1994). Seven subtypes of P2Y receptors have been described (King and Burnstock, 2002), and seven subtypes of P2X receptors are recognized (Khakh *et al*, 2001).

There is growing evidence that ATP may act as an important local messenger in the skin, particularly the epidermis. Functional roles in the regulation of proliferation and differentiation of cutaneous keratinocytes have been proposed (Pillai and Bikle, 1992; Cook *et al*, 1995). In particular, $P2X_5$ receptors are expressed on cells undergoing proliferation and differentiation, while $P2X_7$ receptors are associated with keratinized dead cells (Gröschel-Stewart *et al*, 1999). $P2Y_2$ receptors, found in the basal layer of normal epidermis, are claimed to be involved in keratinocyte proliferation (Dixon *et al*, 1999). $P2Y_1$ receptors are thought to be mitogenic in vascular smooth muscle cells and endothelial cells (Burnstock, 2002).

The epidermis is a multilayered squamous epithelium in which dividing basal cells withdraw from the cell cycle and progressively differentiate as they are displaced towards the skin surface. Eventually the cells lose their nuclei and other organelles to become flattened squames, which are finally shed from the surface as bags of cross-linked keratin filaments enclosed in a cornified envelope (Leigh *et al*, 1994). Keratinocytes can undergo apoptosis when stimulated by a variety of agents (McCall and Cohen, 1991).

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Abbreviations: ABC, avidin biotin complex; ATP, adenosine 5′-triphosphate; ATPγS, adenosine 5′-0-(3-thiotriphosphate); ADP, adenosine 5′-diphosphate; BzATP, 2′- & 3′-0-(4-benzoyl-benzoyl) adenosine 5′-triphosphate; DAB, nickel-diaminobenzidine; 2MeSADP, 2-methylthio-adenosine 5′-diphosphate; NaCl, sodium chloride; NGS, normal goat serum; NHS, normal horse serum; PBS, phosphate buffered saline; PCNA, proliferating cell nuclear antigen; TUNEL, TdT-mediated dUTP nick end labelling; UTP, uridine 5′-triphosphate

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There is evidence that the normal differentiation programme uses components of the apoptotic biochemical machinery to produce a cornified cell and that these mechanisms are required for the normal loss of the nucleus. Indeed, differentiating keratinocytes and apoptotic cells share features, such as DNA fragmentation (Polakowska *et al*, 1994) and caspase-3 expression (Weil *et al*, 1999).

This study demonstrates the distribution of P2X and P2Y receptor subtypes in human epidermis for the first time. Functional studies were performed on primary cultures of human keratinocytes and on explanted rat skin, where P2 receptor subtype agonists and antagonists were applied to cultured keratinocytes or injected subcutaneously into the skin, respectively. We show that purinergic receptors on keratinocytes are functional and propose that these receptors are part of the normal homeostatic mechanisms controlling keratinocyte proliferation, differentiation and death.

MATERIALS AND METHODS

Tissues Twenty-six samples of normal human skin were examined immunohistochemically in this study. Ethical Committee Approval was obtained to harvest human skin. Samples of postoperatively redundant skin from reduction mammoplasty, abdominoplasty, and from the leg were obtained. Neonatal skin samples came from preauricular skin tags and accessory digits. Tissue was frozen in isopentane precooled in liquid nitrogen. Blocks were sectioned at 10 μm on a cryostat (Reichert Jung CM1800), collected on gelatin-coated slides and air-dried at room temperature. The slides were stored at −20°C.

Antibodies The immunogens used for production of polyclonal $P2X_5$ and $P2X_7$ antibodies were synthetic peptides corresponding to 15 receptor-type-specific amino acids (AA) in the intracellular C-termini of the cloned rat and human P2X receptors, as described previously (Gröschel-Stewart *et al*, 1999, Oglesby *et al*, 1999). $P2X_5$ and $P2X_7$ antibodies (provided by Roche Bioscience, Palo Alto, California) were kept frozen at a stock concentration of 1 mg/ml. Polyclonal anti-$P2Y_1$ and $P2Y_2$ antibodies were obtained from Alomone Laboratories (Jerusalem, Israel), and corresponded to the third extracellular loop of the $P2Y_1$ (AA 242–258) and $P2Y_2$ receptor (AA 227–244) and were kept frozen at a stock concentration of 0.6 mg/ml. Proliferating cell nuclear antigen (PCNA) is a marker for proliferation in human keratinocytes (Miyagawa *et al*, 1989). Cytokeratin K10 and involucrin are markers for keratinocyte differentiation (Eckert *et al*, 1997). PCNA (monoclonal antiproliferating cell nuclear antigen, clone PC10, raised in mouse ascites fluid; Sigma, Poole, UK), cytokeratin K10 (BioGenex, San Ramon, California) and involucrin (Sigma) antibodies were raised in mouse. Ki-67 antigen is a marker for cell proliferation in normal human keratinocytes (Gerdes *et al*, 1991). Active caspase-3 is part of the apoptotic machinery of the cell and is expressed in terminally differentiating keratinocytes (Weil *et al*, 1999). Ki-67 (DAKO, Denmark) and active caspase-3 (Abcam, Cambridge, UK) antibodies were both raised in rabbit.

Immunohistochemistry For immunostaining of cryostat sections, the avidin-biotin technique was used according to a revised protocol (Llewellyn-Smith *et al*, 1993). Sections were fixed for 2 min in 4% formaldehyde in 0.1 M phosphate buffer, containing 0.2% of a saturated solution of picric acid (pH 7.4). Sections were immersed for 10 min in 50% methanol containing 0.4% hydrogen peroxide, followed by a 20 minute preincubation in 10% normal horse serum (NHS) in 0.1 M phosphate buffer, containing 0.05% merthiolate (Sigma). Sections were incubated with the primary antibodies diluted to 1:100 or 1:200 (in 10% NHS in PBS + 2.5% NaCl) at 4°C overnight. Sections were then incubated with biotinylated donkey antirabbit IgG (Jackson ImmunoResearch Laboratory, West Grove, Pennsylvania) diluted to 1:500 in 1% NHS in PBS for 30 min, followed by ExtrAvidin peroxidase conjugate (Sigma) diluted to 1:1000 in PBS for 30 min. After a wash step, a nickel-diaminobenzidine (DAB) enhancement technique was used to visualize the reaction product. Sections were washed three times with PBS after each of the above steps except for after preincubation with 10% NHS. After the last wash, sections were dehydrated twice in isopropanol and mounted with EUKITT (BDH Laboratory Supplies, Poole, UK).

For immunofluorescent staining, sections were fixed, incubated with primary antibodies overnight, and then incubated with the secondary antibody as above. This was followed by Streptavidin-FITC (Amersham Life Science) diluted 1:200 in PBS-merthiolate for 1 h. Sections were washed in PBS and mounted in Citifluor (Citifluor Ltd, London, UK).

Control experiments were carried out with primary, secondary and tertiary antibodies omitted from the staining procedure or the primary antibodies preabsorbed with the corresponding peptides.

Double-labeling techniques

Double-labeling with P2X or P2Y receptor antibodies and either PCNA, cytokeratin K10, or involucrin. Sections were fixed, incubated with primary antibodies overnight and the secondary antibody as described above. 1:200 Streptavidin Texas red (Amersham International plc.) was applied for 1 h. Sections were preincubated for 30 min with 10% normal goat serum (NGS) and then incubated for 2 h with either: 1:1000 PCNA (Sigma); 1:50 mouse antihuman cytokeratin K10 (BioGenex); 1:50 mouse monoclonal anti-involucrin (Sigma). After a wash step, 1:200 goat antimouse FITC (Nordic Immunological Laboratories, Tilburg, the Netherlands) was applied for 1 h; sections were washed and mounted in Citifluor.

Double-labeling with P2X or P2Y receptor antibodies and either Ki-67 antigen or antihuman caspase-3 Sections were incubated with primary and secondary antibodies as described above. 1:1500 ExtrAvidin peroxidase conjugate (Sigma) was applied for 1 h, tyramide amplification for 8 min (Tyramide Amplification Kit, NEN Life Science Products, Boston, MA) and then 1:200 Streptavidin Texas red for 10 min. Sections were washed three times in PBS after each of the above steps. Sections were preincubated for 20 min in 10% NGS and then incubated for 2 h with either 1:50 rabbit antihuman Ki-67 antigen (DAKO) or 1:50 rabbit antihuman active caspase-3 (Abcam). Sections were washed and incubated with 1:1000 Oregon-green-labeled goat-antirabbit IgG (Jackson ImmunoResearch Laboratory) for 45 min. Sections were washed and mounted in Citifluor.

Double-labeling with $P2X_7$ receptor antibodies and TdT-mediated dUTP nick end labeling (TUNEL) TUNEL identifies cells undergoing apoptosis by labeling nuclear DNA fragments that have been cleaved during apoptosis (Gavrieli *et al*, 1992). TUNEL labeling was performed using a kit (Boehringer Mannheim, Germany). After overnight incubation with $P2X_7$ receptor antibody diluted to 1:200 as above, sections were washed in PBS and then incubated with the TUNEL reaction mixture for 1 h at 37°C. As a negative control, sections were incubated with the TUNEL Label solution only. After further washes in PBS, sections were incubated for 1 h with 1:500 biotinylated donkey antirabbit antibody. Sections were washed in PBS and then incubated for 1 h with 1:200 Streptavidin Texas red. Sections were washed in PBS and mounted in Citifluor.

Photography The results were photographed using a Zeiss Axioplan, high definition light microscope (Oberkochen, Germany) mounted with a Leica DC 200 digital camera (Heerbrugg, Switzerland).

Human primary cultures of keratinocytes in low calcium medium Human keratinocyte primary cultures were grown from redundant skin of consenting patients under age 30, who had undergone prominent ear correction, breast reduction and abdominoplasty. Cultures were grown in the presence of irradiated 3T3 feeder cells (Rheinwald, 1980). Keratinocytes at passage 0 were disaggregated via trypsin at approximately 70% confluency and seeded (passage 1) into low calcium keratinocyte medium (200 ml MCDB 153 medium, 10 ml chelated fetal calf serum, 2 ml RM^+medium, 2 ml penicillin/streptomycin mixture, 2 ml L-glutamine and filter sterilized (0.2 μm) before use). Cells were media-changed at 24 h after passage to remove the nonadherent, dead keratinocytes and remaining feeder cells. At 48 h after passage, cells were disaggregated with trypsin/EDTA and seeded into 96 well plates at a density of 4×10^5 cells/well in low calcium medium for use in the proliferation assay.

Proliferation assay Twenty-four hours after seeding into 96 well plates, medium was gently aspirated from culture wells and concentration ranges of P2 receptor subtype agonists and antagonists (obtained from Sigma) were applied to the keratinocytes, diluted in low calcium keratinocyte medium. These included ATP; uridine 5′-triphosphate (UTP), $P2Y_2$ receptor agonist (von Kügelgen and Wetter, 2000); adenosine 5′-0-(3-thiotriphosphate) (ATPγS), $P2X_5$ receptor agonist (Khakh *et al*, 2000)); 2′- & 3′-0-(4-Benzoyl-benzoyl) adenosine 5′-triphosphate (BzATP), $P2X_7$ receptor agonist (Khakh *et al*, 2001), 2-Methylthioadenosine 5′-diphosphate (2MeSADP), $P2Y_1$ receptor agonist (von Kügelgen and Wetter, 2000) and Suramin, a nonselective P2X and P2Y receptor antagonist (Ralevic and Burnstock, 1998). The pH of the drug solutions made up in culture medium was 7.0.

Changes in cell number were quantified via a colorimetric assay using crystal violet (Gillies *et al*, 1986; Kueng *et al*, 1989) and read using a spectrophotometric plate reader (Model 550, Microplate Manager® 4.0 Bio-Rad Laboratories, Inc. Hercules, CA, USA) at 0, 24, 48 and 72 h after

addition of drugs. For the colorimetric assay, a solution of 0.5 g crystal violet, 0.85 g NaCl, 5 ml 10% formal saline, 50 ml absolute ethanol, 45 ml distilled H_20 was used. Medium was gently aspirated from wells of a 96 well plate and 100 µl of the colorimetric assay mixture was added to each well and incubated at room temperature for 10 min. This mixture allowed simultaneous fixation of cells and penetration of the crystal violet dye into the cells. After washing three times in PBS, 33% acetic acid was used to elute color from cells and optical density was read at 595 nm using the spectrophotometric plate reader. Changes in cell number measured via the assay were validated at least once for each drug set by doing actual cell counts with a hemocytometer. To confirm that the optical density of the wells correlated with cell numbers, a control assay was performed for each experiment, where known numbers of cells were seeded in ascending seeding densities and the plate read as soon as cells had attached. Cell number *versus*. optical density was plotted. The R^2 value of the trend line was always >0.98 (data not shown).

Statistical analysis Each proliferation assay experiment was repeated an average of eight times, each with triplicate samples. Data analysis was performed using Microsoft Excel 97 and GraphPad Prism 3.0 software. One-way analysis of variance (ANOVA) and Bonferroni's multiple comparison test were carried out between groups.

Subcutaneous injection experiment Skin was harvested from the back of adult male Wistar rats and 0.1 ml of ATP, ATPγS, BzATP, UTP or 2MeSADP was injected subcutaneously into the dermis, using a 1-ml syringe and a 27G needle, at concentrations from 1 to 500 µM. Controls had 0.1 ml of saline injected in the same way. The area of the injection site was marked on the skin with a marker pen and the solution infiltrated subcutaneously to a diameter of about 0.5 cm around the injection site. The skin was then placed in Hank's balanced salt solution and kept at 4°C for one hour, after which the tissue was frozen in isopentane precooled in liquid nitrogen. Blocks were sectioned at 10 µm on a cryostat (Reichert Jung CM1800) until the marked area indicating the injection site was visible. Sections were collected on gelatin-coated slides and air-dried at room temperature. The injection site was indicated by blue ink on unstained slides. The slides were stained with hematoxylin and eosin and compared to unstained serial sections to reveal the injection site. From previous work (Terenghi *et al*, 1994), $n=6$ was established as the minimum size per group to avoid interanimal and intersample variation influencing the outcome of the results. This group size ensured that the statistical significance is at the 5% level, with an 80% statistical power. Each drug set was therefore repeated in six different animals.

RESULTS

$P2X_5$ and $P2X_7$ receptors are expressed in human skin $P2X_5$ and $P2X_7$ receptor immunoreactivity was observed in the epidermal keratinocytes of all human skin samples. $P2X_5$ receptor staining was restricted to the first two viable layers of the epidermis, with very few stained cells in the stratum granulosum. No $P2X_5$ receptor staining was found in the stratum corneum. Higher magnification revealed differences in staining for this receptor between the stratum basale and the stratum spinosum in both intensity and subcellular distribution, with staining of the stratum basale appearing slightly less intense than that of the stratum spinosum (**Fig 1*a***). In addition, staining within the stratum spinosum was mainly in the cytoplasm and possibly the plasma membrane, but not in the nucleus, whereas in the stratum basale the majority of staining appeared to be in the plasma membrane. Furthermore, this staining of the stratum basale cell membranes appeared to be polarized, being mainly concentrated on the basal aspects of the cells that were associated with the basement membrane. $P2X_7$ receptor immunoreactivity was solely associated with cells and cell fragments within the stratum corneum (**Fig 1*b***), and were often seen to be nuclear or perinuclear.

Some differences were noted in the pattern and intensity of staining among the different skin areas. In lower leg skin, there was strong immunostaining for $P2X_5$ receptors in all the cells of the stratum basale and stratum spinosum (**Fig 1*a***). In breast and abdominal skin, $P2X_5$ immunoreactivity was similar to that in the lower leg with strong $P2X_5$ receptor staining within the stratum basale and spinosum. However, in these tissues, the stratum granulosum showed more intense $P2X_5$ immunoreactivity and almost all of the cells in the stratum granulosum stained positive for the $P2X_5$ antibody.

In lower leg skin (**Fig 1*b***), corneocytes and cell fragments within the stratum corneum stained positively for $P2X_7$. The staining was of high intensity compared to that seen in the other areas examined. For example, in breast skin, which is thin, the staining for $P2X_7$ receptors was at the junction between the stratum granulosum and the stratum corneum in an almost continuous thin line. There was little staining of the stratum corneum in breast skin compared to that seen in the lower leg, abdomen and thigh skin. In abdominal skin, the $P2X_7$ receptor staining was in an almost continuous line, and staining of the stratum corneum was patchy. In thigh skin, membrane fragments within the stratum corneum stained positively for $P2X_7$ receptors, which were also sometimes expressed on the outer surface of the stratum corneum. In leg skin, the epidermis was thicker and the zones of the epidermis were better defined than in other regions of the skin; therefore this was used for further studies.

$P2Y_1$ and $P2Y_2$ receptors are expressed in human skin $P2Y_1$ (**Fig 1*e***) and $P2Y_2$ receptors (**Fig 1*f***) were expressed in normal human skin. $P2Y_1$ receptors were found in the basal layer of the epidermis. $P2Y_2$ receptors were also found in the basal layer of the epidermis, with some expression in the stratum spinosum.

Control immunostaining experiments Both the omission of the primary antibody and preabsorption with corresponding peptides were performed as controls. With immunofluorescence staining, there was some nonspecific immunostaining of nuclei within the stratum spinosum and granulosum when the primary antibody was omitted, but there was no immunoreaction when the secondary and tertiary antibodies were omitted. The immunoreaction was abolished after absorption of the $P2X_5$ (**Fig 1*c***) or $P2X_7$ receptor antibodies (**Fig 1*d***) with the corresponding peptides, confirming the specificity of the immunoreaction. The immunoreaction was significantly reduced after absorption of the $P2Y_1$ (**Fig 1*g***) or $P2Y_2$ antibody (**Fig 1*h***) with the corresponding peptide, with some nonspecific staining of nuclei within the stratum spinosum and granulosum, confirming the specificity of the findings.

Cells positive for $P2Y_1$ and $P2Y_2$ receptors also express markers for cellular proliferation Neonatal skin was dual stained for Ki-67 and $P2Y_1$ receptors (**Fig 2*a***) and PCNA and $P2Y_2$ receptors (**Fig 2*b***). The proliferation markers identified a proliferating subpopulation of basal and parabasal keratinocytes. Cells positive for these two markers were also positive for $P2Y_1$ and $P2Y_2$ receptors.

Cells positive for $P2X_5$ receptors also express markers for keratinocyte differentiation Double-labeling of $P2X_5$ receptors with cytokeratin K10 (**Fig 2*c***) or involucrin (**Fig 2*d***) showed that $P2X_5$ receptors were expressed in differentiating keratinocytes within the epidermis. Cytokeratin K10, an early marker of keratinocyte differentiation was found in most suprabasal keratinocytes. The stratum basale stained only for $P2X_5$ receptors, indicating that no differentiation was taking place in these cells. The colocalization of $P2X_5$ receptors and cytokeratin K10 appeared mainly in the cytoplasm of differentiating cells within the stratum spinosum and partly in the stratum granulosum. Involucrin, a late marker of keratinocyte differentiation was expressed from the upper stratum spinosum up to the stratum corneum. There was colocalization of $P2X_5$ receptors with involucrin within the cytoplasm of cells within the upper stratum spinosum, with a few cells double-labeling within the stratum granulosum.

Double-labeling of $P2X_7$ receptors with markers for apoptosis in human skin show colocalization The cell

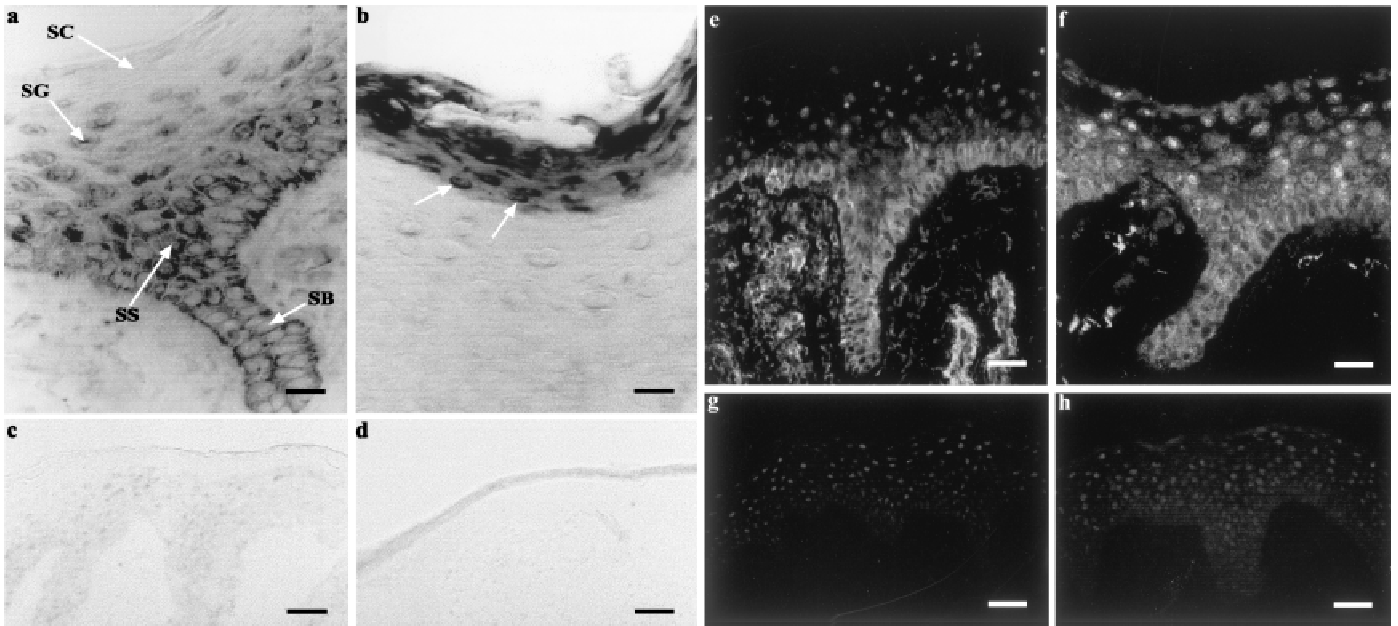

Figure 1. Human epidermal keratinocytes in frozen sections of leg skin express $P2X_5$, $P2X_7$, $P2Y_1$ and $P2Y_2$ receptors. (*a*) $P2X_5$ receptor immunostaining was present in the stratum spinosum (SS) and to a lesser extent in the stratum basale (SB). Only a few cells in the stratum granulosum (SG) had positive staining, which was of much lower intensity. There was no staining in the stratum corneum (SC). *Scale bar* 15 μm. (*b*) $P2X_7$ receptor immunostaining was present in whole cell remnants and cell fragments within the stratum corneum and was often seen to be nuclear or peri-nuclear (*arrows*). *Scale bar* 15 μm. (*c*) Control: the immunoreaction was abolished after preabsorption of the $P2X_5$ antibody with an excess of the corresponding peptide used for immunization. *Scale bar* 60 μm. (*d*) Control: the immunoreaction was significantly reduced after preabsorption of the $P2X_7$ antibody with an excess of $P2X_7$ peptide. *Scale bar* 60 μm. (*e*) $P2Y_1$ receptors were found in the basal layer of the epidermis. *Scale bar* 20 μm. (*f*) $P2Y_2$ receptors were found in the basal layer of the epidermis, with some expression in the stratum spinosum. *Scale bar* 20 μm. (*g*) Control: the immunoreaction was significantly reduced after absorption of the $P2Y_1$ antibody with $P2Y_1$ peptide. *Scale bar* 60 μm. (*h*) Control: the immunoreaction was significantly reduced after absorption of the $P2Y_2$ antibody with $P2Y_2$ peptide. *Scale bar* 60 μm.

nuclei in the uppermost level of the stratum granulosum stained strongly positive for TUNEL (**Fig 2*e***) and Caspase-3 (**Fig 2*f***). Double-staining of the $P2X_7$ receptor and TUNEL showed that there was some overlap of staining, but that TUNEL largely stained the cell nuclei of living cells undergoing apoptosis in the uppermost layer of the stratum granulosum, while $P2X_7$ immunostaining also stained the dead cells in the stratum corneum above. The negative control for TUNEL showed no reaction. Double labeling of $P2X_7$ receptors with anti-Caspase-3 showed colocalization within the stratum corneum (**Fig 2*f***).

Functional studies of primary keratinocyte cultures show that P2 agonists can alter cell number Readings were taken of changes in optical density of cells grown in 96 well plates at 0, 24, 48 and 72 h after drug application. The peak effect of the drugs was seen at 48 h after application, after which the effect decreased.

At 48 h after application of drugs to primary cultures of human keratinocytes, ATP (1–10 μM), UTP (100 μM) ($p < 0.001$) (**Fig 3*a***), and 2MeSADP (500 μM) ($p < 0.05$) (**Fig 3*b***) caused an increase in keratinocyte cell number. Whereas ATP (100 μM) ($p < 0.001$), ATPγS 100–500 μM ($p < 0.001$) (**Fig 3*a***) and BzATP (100–500 μM) ($p < 0.001$) (**Fig 3*c***) caused a significant decrease in cell number. Suramin (**Fig 3*d***) caused a significant block in the effect of high dose ATP at 100 μM ($p < 0.01$) and 1 mM ($p < 0.001$).

Direct subcutaneous injection of purinergic receptor agonists results in changes in the morphology of the rat epidermis No change in the morphology or thickness of the epidermis was seen when 0.1 ml saline was injected subcutaneously into control skin (**Fig 4*a,b***). Subcutaneous injection of either 0.1 ml 10 μM ATP (**Fig 4*c,d***), or 100 μM UTP (**Fig 4*e***) resulted in a thickening of the rat epidermis compared to control skin, in 6 out of 6 rats studied. Subcutaneous injection of 500 μM 2MeSADP (**Fig 4*f***) caused an increase in thickness of the epidermis in 3 out of 6 rats, with 3 showing no change. 10 μM ATP caused a 3-fold increase in the thickness of the epidermis (**Fig 4*d***), whereas 100 μM UTP caused a 2-fold increase in thickness (**Fig 4*g***). Both ATP and UTP increased the number of viable cell layers, with UTP causing a smaller increase in the stratum granulosum and stratum corneum. 2MeSADP also caused an increase in the number of viable cell layers (**Fig 4*h***).

Subcutaneous injection of 300 μM ATPγS (**Fig 4*i,k***) caused a slight decrease in thickness of the epidermis in 3 out of 6 rats, with the remaining 3 preparations showing no change compared to control. Subcutaneous injection of 300 μM BzATP (**Fig 4*j***) caused a decrease in thickness of the viable cell layers of the epidermis in 4 out of 6 rats studied (in 2 out of 6 there was no change). The thickness of the epidermis was almost halved and the cells of the stratum granulosum were flattened and thinner than in control skin (**Fig 4*l***). The concentration of 300 μM was chosen for ATPγS and BzATP because this represented the midpoint of the range of effective concentrations used within human primary keratinocyte cultures.

DISCUSSION

Early papers suggested that extracellular ATP may be an important physiological regulator of epidermal growth and differentiation acting through G protein-coupled P2Y receptors (Pillai and Bikle, 1992; Cook *et al*, 1995). However, these studies were not in a position to detect the P2 receptor subtypes involved or to show their differential localization within the epidermis. In this study, we have obtained direct evidence for the expression of $P2X_5$, $P2X_7$, $P2Y_1$ and $P2Y_2$ receptors in human keratinocytes, using immunohistochemistry and functional studies *in vitro*.

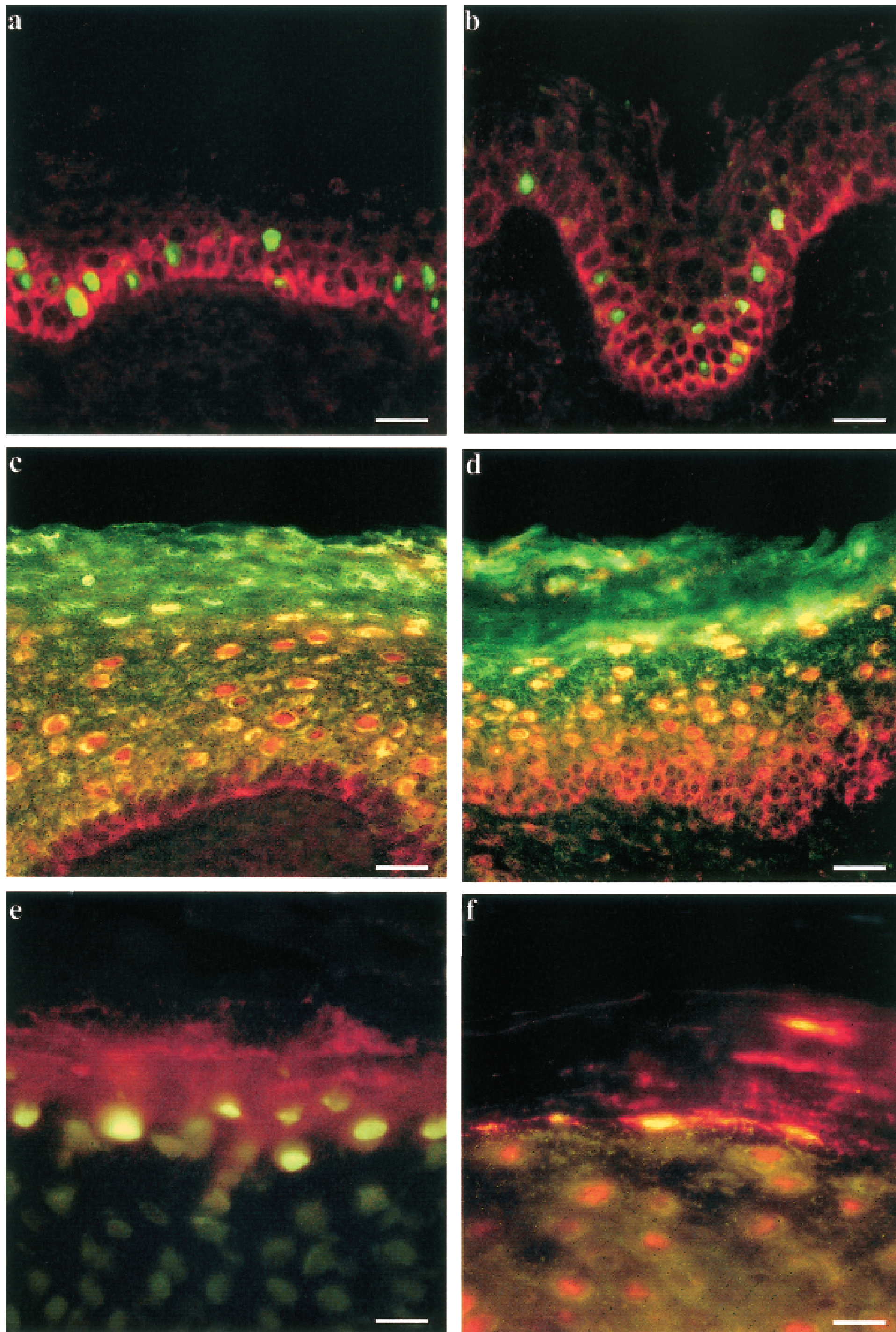

Figure 2. Double-labeling of $P2Y_1$ and $P2Y_2$ receptors with markers of proliferation show colocalization within a subpopulation of basal and parabasal keratinocytes. Double-labeling of $P2X_5$ receptors with markers of differentiated keratinocytes show colocalization within the stratum spinosum, and double-labeling of $P2X_7$ receptors with markers of apoptosis in human leg skin show colocalization within the stratum corneum. (*a*) Ki-67 immuno-labeling (a marker for proliferation) stained the nuclei (*green*) of a subpopulation of keratinocytes in the basal and parabasal layers of the epidermis. $P2Y_1$ receptor immunostaining (*red*) was found in the basal layer on cells also staining for Ki67. *Scale bar* 30 μm. (*b*) PCNA immuno-labeling (a marker for proliferation) stained the nuclei (*green*) of a subpopulation of keratinocytes. These nuclei were often distributed in clusters and found in the basal and parabasal layers of the epidermis. $P2Y_2$ receptor immunostaining (*red*) was also expressed in basal and parabasal epidermal cells. *Scale bar* 30 μm. (*c*) $P2X_5$ receptor immunostaining (*red*) showed overlap (*yellow*) with cytokeratin K10 (*green*), an early marker of keratinocyte differentiation. $P2X_5$ receptors were present in the basal layer of the epidermis up to the mid-granular layer. Cytokeratin K10 was distributed in most suprabasal keratinocytes. The stratum basale stained only for $P2X_5$ receptors, indicating that no differentiation was taking place in these cells. The colocalization of $P2X_5$ receptors and cytokeratin K10 appeared mainly in the cytoplasm of differentiating cells within the stratum spinosum and partly in the stratum granulosum. Note that the stratum corneum also stained for cytokeratin K10, which labeled differentiated keratinocytes, even in dying cells. *Scale bar* 30 μm. (*d*) $P2X_5$ receptor immunostaining (*red*) showed overlap (*yellow*) with involucrin (*green*). $P2X_5$ receptors were present in the basal layer of the epidermis up to the mid-granular layer. Note that the pattern of staining with involucrin was similar to that seen with cytokeratin K10, except that cells from the stratum basale up to the mid-stratum spinosum were not labeled with involucrin, which is a late marker of keratinocyte differentiation. *Scale bar* 30 μm. (*e*) TUNEL (*green*) labeled the nuclei of cells at the uppermost level of the stratum granulosum and $P2X_7$ antibody (*red*) mainly stained cell fragments within the stratum corneum. *Scale bar* 15 μm. (*f*) Anti-Caspase-3 (*green*) colocalized with areas of $P2X_7$ receptor immunostaining (*red*) both at the junction of the stratum granulosum and within the stratum corneum. Areas of colocalization were yellow. Note that the differentiating keratinocytes in the upper stratum granulosum were also positive for anti-Caspase-3. *Scale bar* 15 μm.

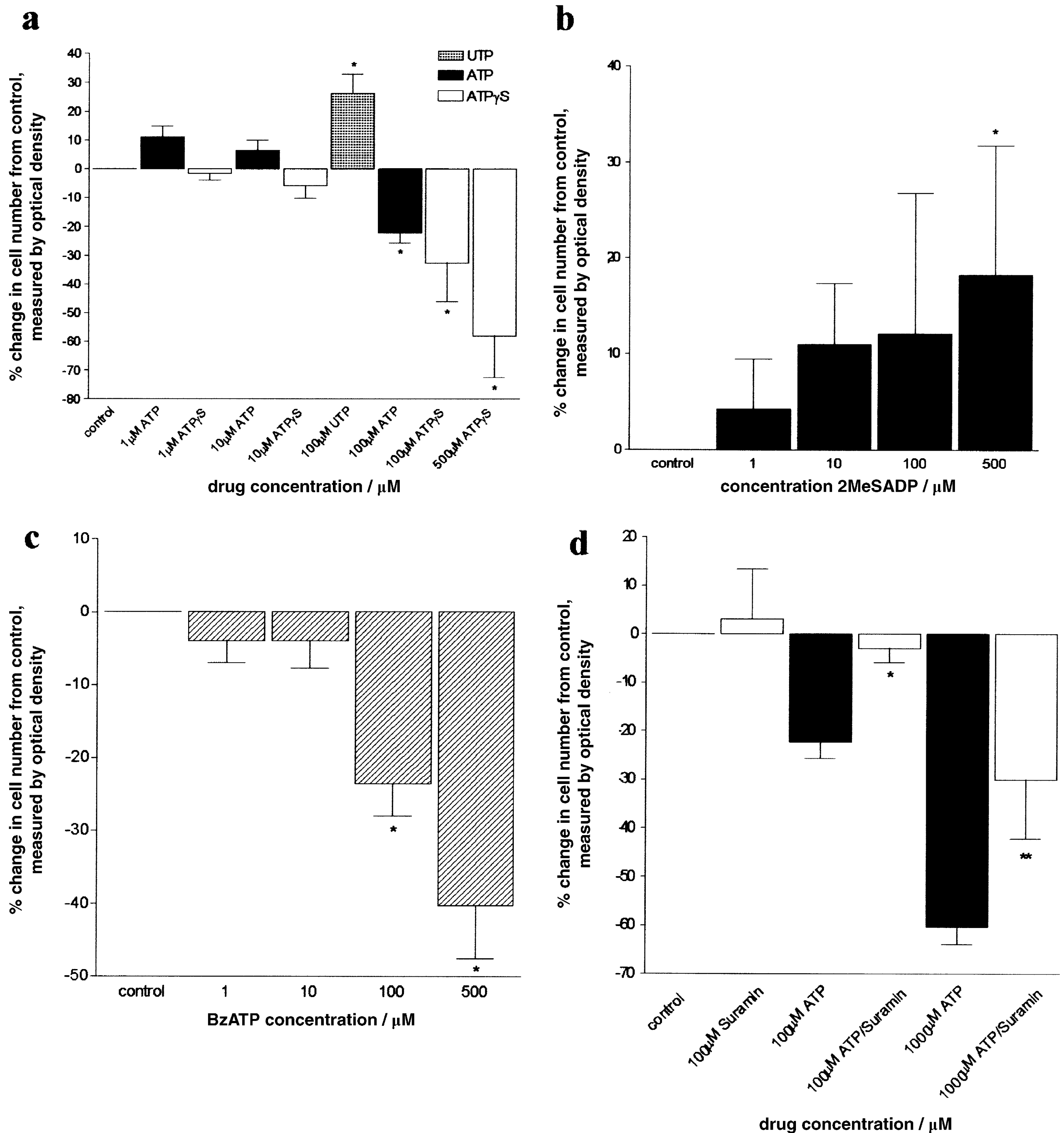

Figure 3. At 48 h after application of drugs to primary human keratinocyte cultures: (*a*) ATP (1–10 μM) and UTP (100 μM) cause an increase in cell number, whereas ATPγS (100–500 μM) and ATP (100 μM) cause a significant decrease. Results represent the mean of 8 experiments. *$p < 0.001$ compared to control. (*b*) 2MeSADP (500 μM) causes a significant increase in cell number. Results represent the mean of 8 experiments. *$p < 0.05$ compared to control. (*c*) BzATP (100–500 μM) causes a significant decrease in cell number. Results represent the mean of 9 experiments. *$p < 0.001$ compared to control. (*d*) Suramin causes a significant block in the effect of 100 μM ATP (*$p < 0.01$) and 1000 μM ATP (**$p < 0.001$) on cell number. Results represent the mean of 4 experiments. Error bars represent mean ± SEM.

$P2Y_1$ and $P2Y_2$ receptors show a strong immuno-positive signal in the basal layer of the epidermis. Some basal cells are stem cells, others are transit-amplifying cells (which can undergo a variable number of rounds of division before becoming postmitotic) and some are postmitotic cells that are poised to move into the suprabasal layers. We performed double-labeling of $P2Y_1$ and $P2Y_2$ receptors and keratinocyte proliferation markers Ki-67 and PCNA. This confirmed the presence of these $P2Y_1$ and $P2Y_2$ receptors in proliferating cells. We have shown that the $P2Y_1$ and $P2Y_2$ receptor selective agonists, 2MeSADP and UTP, respectively, can cause an increase in human keratinocyte number *in vitro*. Direct subcutaneous injection of UTP into rat skin also causes a two-fold increase in thickness of the epidermis. This suggests that these receptors are involved in keratinocyte

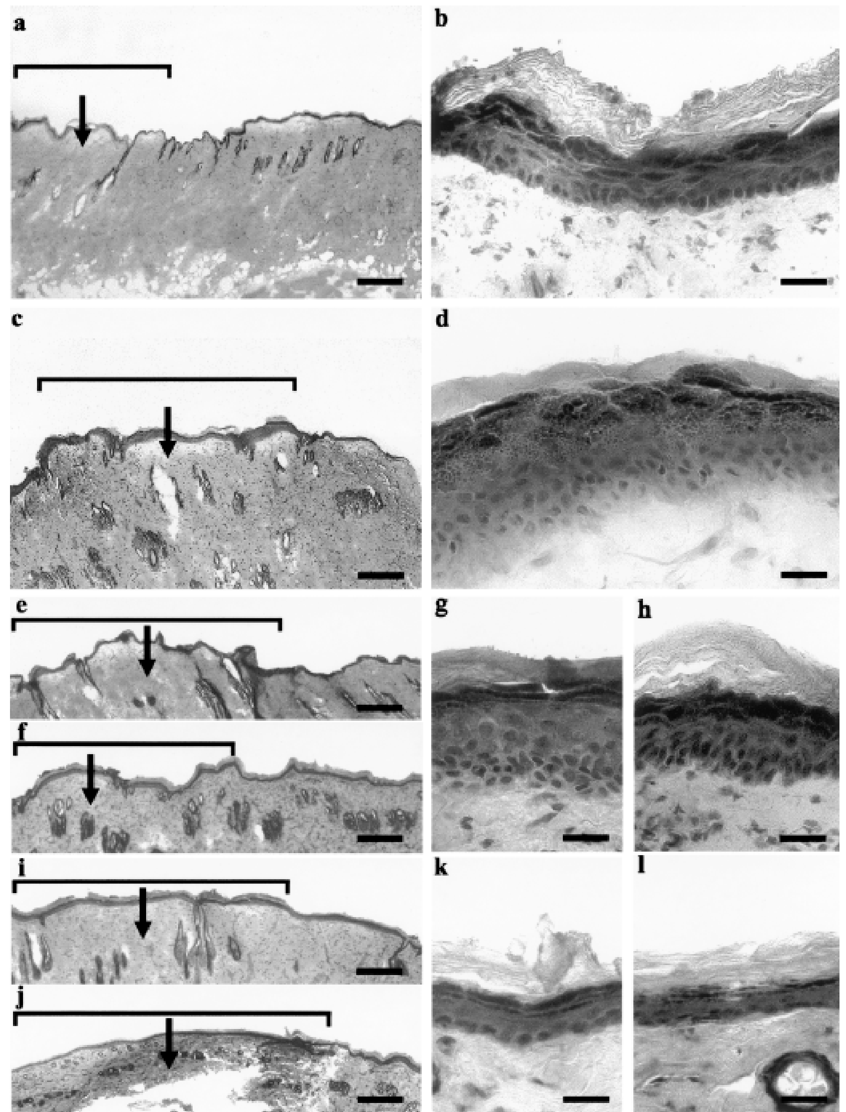

Figure 4. Subcutaneous injection of purinergic receptor agonists cause changes in the thickness of the epidermis of rat skin. (*a*) Low power view of saline injection site (*arrow*) in control section. Bar represents region of subcutaneous infiltration of saline. *Scale bar* 375 μm. (*b*) High power view of (*a*) taken from the region where saline was injected subcutaneously in control sections. Saline injection has not affected the thickness of the epidermis. *Scale bar* 20 μm (*c*) The epidermis was significantly thickened in the area of the injection site (*arrow*) of 10 μM ATP. The bar represents the area of the epidermis affected by low dose ATP, which is much thicker than the surrounding unaffected epidermis. *Scale bar* 375 μm. (*d*) High power view of (*c*) taken from the region where 10 μM ATP was injected subcutaneously. Note how the epidermis has increased in thickness three-fold compared to control. *Scale bar* 20 μm (*e*, *f*) Low power view of injection site of 100 μM UTP, and 500 μM 2MeSADP, respectively, showing that UTP and 2MeSADP caused an increase in thickness of the epidermis at the injection site (*arrow*). *Scale bar* 375 μm (*g*) 100 μM UTP caused a two-fold increase in epidermis thickness compared to control. *Scale bar* 20 μm. (*h*) 500 μM 2MeSADP caused an increase in thickness of epidermis compared to control. (*i*, *j*) Low power view of injection site of 300 μM ATPãS and 300 μM BzATP, respectively, showing that the epidermis was reduced in thickness in the area of the injection site (*arrow*). *Scale bar* 375 μm. (*k*) 300 μM ATPãS reduced the thickness of the epidermis. *Scale bar* 20 μm. (*l*) 300 μM BzATP almost halved the thickness of the epidermis and the morphology of stratum granulosum cells was changed such that cells were much flatter and thinner compared to control. *Scale bar* 20 μm.

proliferation. Since the effect of 2MeSADP is weaker than that of UTP, it also suggests that $P2Y_2$ receptors may have a more significant role in this process. Previous work has localized $P2Y_2$ receptor mRNA in human epidermal basal cells via *in situ* hybridization (Dixon *et al*, 1999). UTP has also been shown to cause proliferation in keratinocytes (Dixon *et al*, 1999) and HaCaT cells (Lee *et al*, 2001).

In this study, we have also shown the distribution of $P2X_5$ receptors within human epidermis for the first time. These receptors are also expressed in the basal layer, but are expressed more strongly in the stratum spinosum and variably into the stratum granulosum. The position of the keratinocyte within the epidermis correlates with its state of differentiation. Differentiation of the basal keratinocyte results in a permanent loss of growth potential and the subsequent sequential expression of differentiation markers. The first markers expressed are the two suprabasal keratins K1 and K10 (Schweizer and Winter, 1983; Eichner *et al*, 1986) followed by other proteins such as involucrin (Watt, 1983), which is detected in the late stratum spinosum and in the stratum granulosum. Double-labeling of $P2X_5$ receptors with either cytokeratin K10 or involucrin was performed to demonstrate that $P2X_5$ receptors are expressed on differentiating keratinocytes. There is a striking overlap of expression of $P2X_5$ receptors with keratin K10, an early differentiation marker but much less overlap with involucrin, which is expressed later. $P2X_5$ receptors may be more likely to be involved in keratinocyte differentiation because ATPγS, a potent $P2X_5$ receptor agonist, causes a significant decrease in cell number in culture. This may be because keratinocytes are withdrawing from the cell cycle and differentiating. ATPãS may cause premature differentiation of the upper layers of the epidermis in rat skin, reducing the number of viable cell layers after subcutaneous injection. There is also evidence from other tissues regarding the role of $P2X_5$ receptors. In fetal rat skeletal muscle, $P2X_5$ receptors are sequentially expressed during development (Ryten *et al*, 2001) and associated with differentiating cells (Ryten *et al*, 2002). $P2X_5$ receptors have also been implicated in the regulation of osteoblastic differentiation and proliferation (Hoebertz *et al*, 2000).

The colocalization of $P2X_7$ receptors with two different apoptosis markers, TUNEL, and anticaspase-3 in the stratum corneum is of particular interest. The $P2X_7$ receptor is unlike other P2X receptors because it is a bifunctional molecule that can be triggered to act as a channel permeable to small cations, or, on prolonged stimulation, form a cytolytic pore permeable to large hydrophilic molecules up to 900 Da (Surprenant *et al*, 1996). The opening of this pore results in the increase in intracellular cytosolic free calcium ions. The $P2X_7$ receptor is involved in the induction of cell death (Zheng *et al*, 1991; Ferrari *et al*, 1996). $P2X_7$ receptors are also found on dendritic cells, macrophages and microglial cells, where extracellular ATP can trigger apoptosis via these receptors and there is increasing evidence

that this process is dependent on the caspase signaling cascade (Coutinho-Silva *et al*, 1999; Ferrari *et al*, 1999).

Caspase-3 is considered an executioner caspase, which once activated will cleave a number of cellular substrates whose limited proteolysis is definitive of apoptosis (Nicholson and Thornberry, 1997). Caspase-3 is expressed in terminally differentiating keratinocytes (Weil *et al*, 1999) and colocalized with $P2X_7$ receptors in the stratum corneum. During apoptosis, the nucleus condenses and DNA is fragmented by endonucleases, which can be detected by TdT-mediated dUTP nick end labeling (TUNEL). TUNEL-positive keratinocytes have been found in the upper regions of the granular layer of the epidermis, before cornification (Polakowska *et al*, 1994; Tamada *et al*, 1994; Gandarillas *et al*, 1999) and were also associated with $P2X_7$ receptors. These markers suggest that part of the apoptotic machinery of the cell is activated during keratinocyte terminal differentiation and that this activation may be required for the normal loss of the nucleus in corneocyte formation. Our study suggests that $P2X_7$ receptors are also likely to be part of that machinery.

We have shown that $P2X_7$ receptors on keratinocytes are functional, cell numbers are significantly reduced when stimulated with BzATP in culture, and subcutaneous injection of BzATP appears to cause premature differentiation or death of the upper layers of the epidermis in rat, leaving behind one to two viable cell layers. If $P2X_7$ receptors are involved in the terminal differentiation programme, which ends in "cell death" and formation of the keratinized anucleate corneocyte, then cells grown in culture may also die by this mechanism. ATP in high doses has a similar effect to that of BzATP in culture and it is likely that ATP is stimulating $P2X_7$ receptors on keratinocytes. This has been demonstrated in other cell systems (North and Surprenant, 2000), and we have shown that this effect can be significantly blocked by suramin in culture. Suramin is a nonspecific antagonist of P2X and P2Y receptors.

In summary, P2 purinergic receptor agonists alter keratinocyte cell numbers via several mechanisms, namely UTP and low concentrations of ATP cause an increase in cell number, probably via a direct proliferative effect on basal cells via $P2Y_2$ receptors. 2MeSADP also leads to an increase in cell number, probably via $P2Y_1$ receptors in the basal layer. ATPγS results in a decrease in cell number because cells are lost from the cell cycle by being made to differentiate via activation of $P2X_5$ receptors. BzATP and high concentrations of ATP decrease cell numbers via a direct effect on $P2X_7$ receptors, which are known to be involved in mediating apoptosis and are likely to be part of the machinery of end-stage terminal differentiation of keratinocytes.

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