Human Keratinocytes Release ATP and Utilize Three Mechanisms for Nucleotide Interconversion at the Cell Surface*

Helen E. Burrell‡§, Brenda Wlodarski‡, Brian J. Foster‡, Katherine A. Buckley‡, Graham R. Sharpe¶, John M. Quayle‡, Alec W. M. Simpson‡, and James A. Gallagher‡

From the ‡Department of Human Anatomy & Cell Biology, School of Biomedical Sciences, University of Liverpool, The Sherrington Buildings, Ashton Street, Liverpool L69 3GÉ and the §Dermatology Unit, Department of Medicine, UCD Building, University of Liverpool, Liverpool, L69 3GA, United Kingdom

Funding for the confocal microscope was obtained from the Biotechnology and Biological Sciences and Research Council. The costs of publication of this article were defrayed in part by the payment of page charges. This paper is available on line at http://www.jbc.org

Nucleotide activation of P2 receptors is important in autocrine and paracrine regulation in many tissues. In the epidermis, nucleotides are involved in proliferation, differentiation, and apoptosis. In this study, we have used a combination of luciferin-luciferase luminescence, pharmacological inhibitors, and confocal microscopy to demonstrate that HaCaT keratinocytes release ATP into the culture medium, and that there are three mechanisms for nucleotide interconversion, resulting in ATP generation at the cell surface. Addition of ADP, GTP, or UTP to culture medium elevated the ATP concentration. ADP to ATP conversion was inhibited by diadenosine pentaphosphate, oligomycin, and UDP, suggesting the involvement of cell surface adenylate kinase, F₁F₀ ATP synthase, and nucleoside diphosphokinase (NDPK), respectively, which was supported by immunohistochemistry. Simultaneous addition of ADP and GTP elevated ATP above that for each nucleotide alone indicating that GTP acts as a phosphate donor. However, the activity of NDPK, F₁F₀ ATP synthase or the forward reaction of adenylate kinase could not fully account for the culture medium ATP content. We postulate that this discrepancy is due to the reverse reaction of adenylate kinase utilizing AMP. In normal human skin, F₁F₀ ATP synthase and NDPK were differentially localized, with mitochondrial expression in the basal layer, and cell surface expression in the differentiated layers. We and others have previously demonstrated that keratinocytes express multiple P2 receptors. In this study we now identify the potential sources of extracellular ATP required to activate these receptors and provide better understanding of the role of nucleotides in normal epidermal homeostasis and wound healing.

Extracellular nucleotides, such as ATP and ADP, are now recognized as important autocrine and paracrine factors involved in the regulation of many cellular processes in a wide range of tissues. They act via activation of the P2 family of receptors of which there are two subgroups: the P2X receptors (ligand-gated ion channels) and the P2Y receptors (G protein-coupled receptors) as determined by their molecular structure, transduction pathways, and pharmacological properties (1). There are currently seven members of the P2X subgroup (P2X₁–₇) (2) and eight members of the P2Y subgroup (P2Y₁,₂,₄,₆,₁₁–₁₄) identified in mammalian cell types (3).

In the epidermis, P2 receptors are involved in the regulation of proliferation, differentiation, and apoptosis, and thus subtypes are expressed in different regions. Differential expression of multiple P2 receptor subtypes is species- and cell type-dependent (4–7). Although P2Y₅ receptor expression is almost entirely confined to the proliferative basal layer (7, 8), P2X₅ receptors are expressed in keratinocytes in the early stages of differentiation, i.e. the spinous layer, and P2X₇ receptors are confined to the terminally differentiated cells of the cornified layer (8, 9). In vitro, normal human keratinocytes express mRNA for P2Y₁, P2Y₂, P2Y₄, and P2Y₆ receptors, although from [Ca²⁺]i, and proliferation studies only the P2Y₂ receptor appears to be functional (10). Furthermore, expression of P2Y₂ and P2Y₄ receptor mRNA is down-regulated during Ca²⁺-induced differentiation (10) in agreement with immunohistochemical reports. In contrast, in the HaCaT cell line (human keratinocyte cell line) (11), multiple P2Y receptors are functional, including P2Y₁, P2Y₂, and P2Y₄ subtypes, with the P2Y₄ receptor being the most prominent (10) and possibly reflecting on the differences in culture conditions. Because the upper cornified layers of the epidermis are constantly abraded, nucleotides are important in normal epidermal homeostasis where changes in the rates of proliferation and differentiation are necessary to maintain the epidermal barrier. Furthermore, during wounding, nucleotides are released by platelets as part of the aggregation reaction associated with clotting (12) and by dead or damaged cells at sites of trauma. Collectively, these increase the rate of proliferation above that of normal homeostasis, thus enhancing healing.

Nucleotides are not only released from platelets or by dead or damaged cells, but they are also constitutively released by keratinocytes under static conditions (7). Although the mechanism for ATP release is well established in platelets (13) and neurons/neuroendocrine cells (14), the mechanism by which nucleotides are released by non-neuronal cell types, including keratinocytes, remains controversial. Several postulated mechanisms exist, including ATP-binding cassette proteins (ABC proteins) (15–20), which are in close proximity to P2 receptors (21), exocytotic vesicles (22–24), and cytolysis (25). Once released from the cell surface, nucleotides are quickly processed, providing a turnover of potential stimuli at P2 receptors. Enzymes such as E-type ecto-ATPases/ATP-diphosphohydrolases (26–28), nucleotide pyrophosphatases/phosphodiesterases (29–30), and 5’-nucleotidases (27, 31, 32) all degrade ATP to adenosine, thus removing the potential to activate P2 receptors (27). This process of ATP hydrolysis to ADP, AMP, and subsequently adenosine is balanced by the production of ATP from other ecto-enzymes. Recently, in a number of different cell
types, several mechanisms by which ATP can be synthesized from ADP in the extracellular environment have been identified. These include the use of ecto-adenylyl kinase (33–35), which catalyzes the reaction, 2ADP ↔ ATP + AMP, ecto-FTase, ATP synthase (also known as H⁺-ATPase), which usually catalyzes the mitochondrial synthesis of ATP from ADP and orthophosphate using proton-motive force (36–39), and ecto-nucleoside diphosphokinase (ecto-NDK), which catalyzes the reaction, N₈TP + N₉DP ↔ N₈DP + N₉TP, via a covalent intermediate where the enzyme is phosphorylated at a histidine residue (33, 40–42). Although changes in nucleotide concentrations will be initially localized, diffusion and/or convective flow can be used to facilitate changes in the entire extracellular space. The presence of these enzymes has huge consequences when studying P2 receptors, because subsequent responses are unlikely to be due to the nucleotide added, unless nucleotide conversion is accounted for. More importantly, this will also have repercussions for P2 receptor identification, because agonist potency series are one method by which P2Y receptors are classified.

The presence of interconversion enzymes has not previously been studied in keratinocytes or the skin, nor have all the mechanisms for nucleotide conversion at the cell surface been studied in a single cell type. Furthermore, little is known about the presence of interconversion enzymes has not previously been studied in keratinocytes or the skin, nor have all the mechanisms for nucleotide conversion at the cell surface been studied in a single cell type. Furthermore, little is known about the kinetics of conversion by cells. This makes it difficult to determine a time point at which effects of extracellular nucleotides can be investigated, without the concern that conversion has already taken place. In this study, we have shown evidence for ATP synthesis via ecto-adenylyl kinase, ecto-FTase, and ecto-NDKP in the keratinocyte cell line, HaCaT.

**EXPERIMENTAL PROCEDURES**

**Materials—**Dulbecco’s modified Eagle’s medium (DMEM), fetal calf serum, penicillin, streptomycin, and 0.05% trypsin in 0.02% EDTA were purchased from Invitrogen. EDTA (0.02%), nucleotides, diadenosine pentaphosphate (ApA), apprase type VII, ammonium chloride, BSA, and oligomycin B were purchased from Sigma. VectaShield was purchased from Vector Laboratories Ltd. (Peterborough, UK). Anti-mouse and anti-rabbit immunoglobulin FITC conjugates were purchased from Dako Ltd. (Ely, UK). Mouse monoclonal primary antibody raised to the β subunit of ATP synthase and MitoTracker Red CMX Ros were purchased from Molecular Probes (via Cambridge BioScience Ltd., Cambridge, UK). Epidermal growth factor receptor antibody was purchased from Cell Signaling Technology. Nm23-H1 antibody was purchased from Cell Signaling Technology (Division of Differentiation and Carcinogenesis in vitro, Institute of Biochemistry, German Carcinogenesis Research Centre). ATP monitoring reagent (AMR) was purchased from Cambrex (Wokingham, UK) in the form of a ViaLight™ HS kit and was reconstituted according to the manufacturer’s guidelines. ApA was used in an untreated and treated form. Treated ApA was produced by incubation for 2 h with 20 units/ml grade VII apprase. Apprase activity was removed by heating, treating it at 100 °C for 10 min. Samples of ATP and ADP were then added, and bioluminescence was measured using AMR to ensure that apprase activity had been destroyed. Background ATP and ADP concentrations from the treated-ApA were measured to confirm purity.

**Cell Culture—**HaCaT cells were cultured in DMEM with additional supplements of 5% fetal calf serum, 50 IU/ml penicillin, and 50 μg/ml streptomycin. The osteoblast-like cell line, SaOS-2, was cultivated in DMEM supplemented with 10% fetal calf serum, 50 IU/ml penicillin, and 50 μg/ml streptomycin. Cells were incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO₂. HaCaT cells were passaged at ~70% confluence and were preincubated with 0.02% EDTA for 5–10 min, before incubation with 0.05% trypsin in 0.02% EDTA for 5 min.

1 The abbreviations used are: ecto-NDKP, ecto-nucleoside diphosphokinase; AMR, ATP-monitoring reagent; ApA, diadenosine pentaphosphate; DMEM, Dulbecco’s modified Eagle’s medium; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; BSA, phosphate-buffed saline; EGFR, epidermal growth factor receptor; TRITC, tetramethyl rhodamine isothiocyanate.

**Measurement of Extracellular ATP—**Complete DMEM was removed from confluent HaCaT cells grown in 12-well plates. Cells were then washed twice in warm PBS prior to addition of 0.5 ml of nucleoside-free DMEM. Plates were then incubated for 1 h at 37 °C to ensure that ATP released through mechanical stimulation when changing the medium had degraded. Nucleotides were then added to the cells, and 200-μl samples of medium were collected immediately (after ~5 s) into 5-ml round-bottomed polystyrene tubes. Where more than one nucleotide was added, this was carried out simultaneously. When inhibitors were added, these were introduced to the cells 10 min prior to addition of nucleotides and were incubated at 37 °C with the cells. To each tube, 20 μl of AMR containing luciferin and luciferase was added, and the tubes were placed into a Berthold tube luminometer (LB 9505 Berthold, Wildbad, Germany). Relative light unit readings were taken every second for a period of 5 s. The light emitted was detected by a photon counter covering the spectral range from 380 to 630 nm. Mean values were then calculated, and measurements from controls were subtracted from these samples. In the controls of nucleotide-free medium (vehicle) alone (with the exception of Fig. 5, A and B). For Fig. 5 (A and B), 40-μl samples of medium were removed immediately and then every 10 min for a total of 90 min. To each of these samples, 4 μl of AMR was added, and the ATP concentration was measured as outlined above. Finally, ATP standard curves were produced using known concentrations of ATP, and from these, measurements of relative light units were calculated as ATP concentrations.

**Measurement of ADP—**To measure ADP, 2 μl of each sample was carefully pipetted into the bottom of clean luminometer tubes, and the ATP content was measured by addition of 20 μl of AMR. The amount of light emitted (relative light units) was immediately recorded. To each tube, 6 μl of 100 μM phosphoenolpyruvate (to give final concentration of 10 μM) and 10 units of pyruvate kinase (33.5 μl of 100 units/ml or 1 mg of pyruvate kinase) were added to each sample and the bioluminescence was read every second over a 5-s period. The mean bioluminescence was calculated, and the ATP measurement for the corresponding tube was subtracted. As a control, the amount of light produced for the tube containing 20 μl of AMR, 6 μl of phosphoenolpyruvate, and 33.5 μl of pyruvate kinase was also recorded per second over a 5-s period, and the mean value was calculated. This was then subtracted from the tubes containing ADP, and the final readings were converted into ADP concentrations using a calibration curve.

**Confocal Microscopy—**HaCaT cells were seeded onto 22-mm glass coverslips at a density of 1 × 10⁵ cells/well and were allowed to grow overnight. Cells were washed with PBS three times for 10 min each to remove culture medium. After staining, coverslips were mounted with VectaShield and sealed with nail polish slides before visualization using a Leica (SP2 AOBBS) confocal microscope.

**Control Cells (Intracellular Staining)—**In Fig. 9, cells were incubated for 30 min with MitoTracker Red CMXRos (final concentration 100 nM) and washed in PBS prior to fixation. Cells in all experiments were fixed in 4% paraformaldehyde for 30 min and quenched in 50 mM ammonium chloride for 10 min. Cells were then permeabilized in 0.1% Triton X-100 for 10 min, blocked for 10 min in blocking buffer (0.1% BSA in PBS), and washed in PBS for a further 10 min. Cells were incubated for 5 h in the presence of primary antibodies (3 μg/ml mouse monoclonal antibody raised to the β subunit of ATP synthase and 1:200 rabbit polyclonal antibody raised to nm23-H1) in 0.1% sodium azide and 1% BSA in PBS, before washing five times in PBS for 10 min each to remove excess primary antibody and incubation in the dark with secondary antibodies (3 μg/ml rabbit anti-mouse IgG FITC conjugate for ATP synthase or 3 μg/ml pig anti-rabbit IgG FITC for nm23-H1) in 0.1% sodium azide in PBS for 5 h at 4 °C. Coverslips were then washed five times in PBS for 10 min each to remove excess secondary antibody.

**Cell Surface Staining—**Cells were washed in PBS prior to fixation in 4% paraformaldehyde for 30 min and quenching in 50 mM ammonium chloride for 10 min. The unpermeabilized cells were then washed for 5 h with primary antibodies (1:200 rabbit polyclonal antibody raised to epidermal growth factor receptor (EGFR) simultaneously with 3 μg/ml mouse monoclonal antibody raised to the β subunit of ATP synthase) in 0.1% sodium azide and 1% BSA in PBS. Cells were then washed five times in PBS for 10 min each to remove excess primary antibody and incubated in the dark with 3 μl/mg pig anti-rabbit IgG FITC conjugate (in 0.1% sodium azide in PBS) for 5 h at 4 °C. Cells were then washed...
five times in PBS for 10 min each to remove excess secondary antibody. This was then repeated with 3 μl/ml rabbit anti-mouse TRITC conjugate (in 0.1% sodium azide in PBS) and a further series of 5 washes with PBS as already outlined. To observe NDPK distribution, this procedure was repeated using 1:200 rabbit polyclonal antibody raised to nm23-H1 (in 0.1% sodium azide and 1% BSA in PBS) and 3 μl/ml pig anti-rabbit IgG FITC conjugate (secondary antibody).

**Immunohistochemistry for Paraffin Sections**—Normal human skin was fixed in formal saline for 24 h and paraffin-embedded. Skin sections (15 μm) were mounted onto gelatin-coated slides and dried overnight in a 50 °C oven before dewaxing with xylene and rehydrating through alcohols. Sections were then stained using the protocol outlined for cell surface staining of keratinocytes grown on covergrips. Primary antibodies were mouse monoclonal antibody raised to the β subunit of ATP synthase and rabbit polyclonal antibody raised to nm23-H1 and rabbit anti-mouse TRITC conjugate for visualization of ATP synthase. Ethical approval was obtained from the Liverpool Ethics Committee.

**Statistical Analysis**—Statistical analysis was carried out with analysis of variance, with the significant between groups determined by a Tukey post test.

**RESULTS**

**Increasing Cell Number Increases ATP in the Bathing Medium**—In previous studies (7), confluent cultures of normal human keratinocytes have been shown to release ATP in real-time experiments. However, this has not previously been shown in the human keratinocyte cell line, HaCaT, neither has release in keratinocytes been shown to be dependent on cell number. Therefore, HaCaT cells were seeded into 12-well plates at densities ranging from 100 to 1,000,000 cells/well and allowed to grow for 24 h. Complete medium was then removed, replaced with 0.5 ml of serum-free medium before 200-μl samples were collected and ATP concentrations measured using AMR.

**Fig. 1.** Cell number affects the ATP concentration of culture medium. HaCaT cells were seeded at varying densities from 100 to 1,000,000 cells/well and allowed to adhere and grow for 24 h. The ATP concentration of the medium was measured using AMR containing luciferin/luciferase. Experiments were performed in duplicate and are representative of two experiments. Data shown are mean ± S. E. (n = 2).

**Fig. 2.** Culture medium surrounding HaCaT cells contains more ATP than that surrounding SaOS-2 cells. HaCaT and the osteoblast-like cell line, SaOS-2, were seeded at 1 × 10^5 cells/well and allowed to adhere and grow for 24 h. Complete medium was removed and replaced with 0.5 ml of serum-free medium before 200-μl samples were collected and ATP concentrations measured using AMR. ATP, UDP, and 2-methylthio-5'-adenosine diphosphate had no effect on the concentration of ATP in the culture medium (after correction by subtraction of controls; i.e., nucleotides in the absence of cells), ADP, GTP, and UTP (10 μM) elevated ATP in the medium by ~250, 100, and 100 nM (5.2, 2.5-, and 2.5-fold), respectively (Fig. 3). This differs from the results found in SaOS-2 cells, where only ADP induced an increase in the concentration of ATP in the culture medium (43). The increase in ATP observed on addition of ADP to HaCaT cells was concentration-dependent (Fig. 4) and was also much greater than that reported in SaOS-2 cells (43). Furthermore, over a period of 90 min, although ATP is readily degraded by ecto-nucleotidases (Fig. 5A) when ATP (10 μM) is directly added to HaCaT cells, the ATP produced by interconversion of 10 μM ADP → ATP (Fig. 5B) appears to be relatively stable with time and is much lower at ~250 nM. Both the ATP-degrading and ATP-forming activities were confirmed as enzyme-mediated, because the results in the presence of cells (Fig. 5, solid squares and solid line) differed to those from identical experiments in the absence of cells (solid triangles and dotted line).

There are currently three known enzymes by which ADP can be converted to ATP at the cell surface, via adenylyl kinase (33–35), NDPK (33, 41, 42, 44), and F1F0 ATP synthase (36–39). Because the enzymes have been reported to be active in a number of different cell types, their role in the synthesis of extracellular ATP was investigated in the HaCaT cell line.

**Ecto-adenylate Kinase Converts ADP to ATP in the Culture Medium**—Adenylate kinase catalyzes the reaction, 2ADP ↔ ATP + AMP, and is inhibited by Ap5A (45). To investigate the effects of ecto-adenylate kinase on ADP conversion to ATP, increasing concentrations of Ap5A (10 μM to 300 μM) were added to confluent cultures of HaCaT cells, and the mixture was incubated for 10 min prior to addition of ADP (10 μM). The ATP concentrations of samples of culture medium were measured using AMR. For the increase in ATP, upon addition of ADP, to be due to adenylyl kinase activity, it would be expected that conversion would be reduced by Ap5A. However, untreated-Ap5A dose-dependently elevated the ATP concentration of culture medium when in the presence of 10 μM ADP (data not shown). When samples of untreated-Ap5A were analyzed high concentrations of contaminating ATP and ADP were observed (data not shown) in agreement with previous reports (46). Therefore, the experiment was repeated with apyrase type VII-treated Ap5A. Addition of ADP (10 μM) after a 10-min incubation with treated-Ap5A (10 μM to 300 μM), decreased the ATP concentration in the culture medium in a concentration-dependent manner (Fig. 4). Maximal inhibition of ADP to ATP conversion occurred with 10 μM ADP in the presence of 300 μM Ap5A (93% inhibition), suggesting that
almost all of the ADP to ATP conversion was due to the presence of ecto-adenylate kinase.

Presence of Ecto-F_{1}F_{0} ATP Synthase in HaCaT Cells—F_{1}F_{0} ATP synthase usually catalyzes the mitochondrial synthesis of ATP from ADP and orthophosphate using proton-motive force (47). However, there have also been recent reports showing a minor role in ADP → ATP conversion. Presence of ATP synthase at the cell surface was confirmed by confocal microscopy (Figs. 8 and 9). The cells were fixed in situ before the addition of antibodies to eliminate antibody-capping artifacts, and results are representative of parallel experiments. In unpermeabilized cells (Fig. 8), the β-subunit of ATP-synthase was shown to be cell surface-associated, because the pattern of staining differed to that in permeabilized cells (Fig. 9), where ATP synthase was more diffusely distributed particularly in the mitochondria. Optical sectioning through the z-axis also confirmed apical localization of antigen (data not shown). The β-subunit of ATP synthase was irregularly distributed on the surface of the cells, with punctate “hotspots” in cells at the periphery of colonies and negligible distribution in cells in the center of colonies. Cell surface localization of ATP synthase was also compared with that of EGF-receptor (EGFR, Fig. 8), which is an established cell surface marker in keratinocytes, particularly for flow cytometry. Fig. 8 shows EGFR and β-subunit of ATP synthase and the respective overlay of the two images. Collectively, this confirms the presence of ecto-F_{1}F_{0} ATP synthase in HaCaT cells.
Nucleoside Diphosphokinase Activity in HaCaT Cells—10 μM GTP and UTP was observed to increase the concentration of ATP in the culture medium (Fig. 3), suggesting the presence of ecto-NDPK in HaCaT cells. NDPK catalyzes the reaction, N1DP + N2TP ↔ N1TP + N2DP, via a covalent intermediate where the enzyme is phosphorylated at a histidine residue. Because ATP is released in relatively high concentrations in comparison with SaOS-2 cells, we postulated that there would also be a high degree of hydrolysis to ADP. The added GTP or UTP could then act as a phosphate donor in the NDPK reaction (GTP + ADP ↔ GDP + ATP or UTP + ADP ↔ UDP + ATP) thus elevating the concentration of ATP in the culture medium. NDPK activity is inhibited by high concentrations of 5′-diphosphates (49), which act as competitive inhibitors. Therefore, high concentrations of UDP (10 mM) were added in combination with much lower concentrations of GTP (20 μM) and ADP (10 μM) (Fig. 10). As in Fig. 3, UDP alone had no effect, whereas ADP or GTP alone resulted in low concentrations of ATP in the culture medium. In contrast, 10 μM ADP in the presence of 20 μM GTP resulted in ∼1.8 μM ATP in the culture medium. The ATP content of culture medium was also lower with UDP plus ADP than with ADP alone, but there was no effect on ATP content with UDP plus GTP in comparison with GTP alone. Addition of 10 mM UDP in the presence of ADP plus GTP reduced the ADP plus GTP response by 83% suggesting that NDPK has a major role in interconversion of di- and triphosphate nucleotides in HaCaT cells. However, because UDP did not completely abolish the ADP plus GTP response, another enzyme other than NDPK must be partly responsible. Presence of NDPK at the cell surface was confirmed by confocal microscopy (Fig. 11). The cells were again fixed in situ before the addition of antibodies to eliminate antibody-capping artifacts, and results are representative of parallel experiments. In unpermeabilized cells (Fig. 11A), NDPK was shown to be cell surface-associated: the pattern of staining differed to that in permeabilized cells (Fig. 11B). Optical sectioning through the z-axis also confirmed apical localization of antigen (data not shown). Similar to ATP synthase distribution, NDPK was also irregularly distributed, with punctate hotspots on the surface of some cells.

Collective Effects of Conversion Enzymes on ADP and GTP—To observe the contribution of each conversion enzyme,...
confluent HaCaT cells were incubated with the concentration of inhibitors/competitors that had been shown to provide maximal effects of ADP to ATP conversion in previous experiments. Inhibitors (300 μM apyrase-treated Ap5A or 48 μM oligomycin) were added alone or simultaneously 10 min prior to addition of nucleotides. ADP (10 μM), GTP (20 μM), or ADP plus GTP were then added in the presence or absence of 10 mM UDP, and the effects on ATP in the culture medium were analyzed (Fig. 12). As in Fig. 10, when nucleotides alone were added, the increase in ATP in the bathing medium was much greater after addition of ADP plus GTP (1530 ± 52 nM) than with either ADP (295 ± 40 nM) or GTP (140 ± 4 nM) alone and nor was the effect additive.

When adenylate kinase was inhibited by apyrase-treated Ap5A, the responses to ADP and GTP alone were reduced (Fig. 12). However, the concentration of ATP when ADP plus GTP were added simultaneously was much higher (500 ± 18 nM) than for the additive effect of each of the nucleotides when added alone (40 ± 2.5 nM total), suggesting the influence of another conversion enzyme, which we postulated to be NDPK. Furthermore, the effect with ADP plus GTP was greater than with ADP plus UTP (Fig. 13) suggesting that GTP is a better substrate than UTP, in agreement with previously published $K_{cat}$ and $K_m$ measurements for NDPK (49). In the presence of UDP to inhibit NDPK (Fig. 12), the response to ADP alone was reduced, whereas there was no effect with GTP alone. The further reduction with ADP plus GTP in the presence of UDP in comparison to with Ap5A corresponds with the amount of ATP produced by NDPK (249 ± 46 nM). To identify the role of ATP synthase in the formation of ATP from ADP plus GTP, oligomycin was used. However, as oligomycin is dissolved in Me2SO, the effects of the same concentration of Me2SO (2:125) were used as a control. Although Me2SO alone markedly reduced the amount of ADP → ATP conversion, it had no significant effect on GTP → ATP conversion. More interestingly, oligomycin had no effect on the response with GTP (Fig. 12), whereas it inhibited both the ADP response and the ADP plus GTP response by approximately equal amounts. Therefore,
of Adenylate Kinase—Each can use multiple phosphate donors (except for the forward reaction of adenylate kinase to generate further ADP, which can then be converted to ATP using the forward reaction once again.

ATP synthase is responsible for producing ~76 ± 32 nm ATP. However, the response with ADP plus GTP in the absence of inhibitors (~1500 ± 50 nm) cannot be fully accounted for by NDPK (249 ± 46 nm) and ATP synthase (76 ± 32 nm).

In the presence of all the inhibitors (Ap5A, oligomycin, and UDP) there was no ADP to ATP conversion; thus we postulate that the discrepancy between the ADP plus GTP response and the effects of each of the nucleotides alone is due to the reverse reaction of adenylate kinase, which can use a number of phosphate donors.

There are five isoforms of adenylate kinase numbered 1 through 5 (AK1–5), and when using AMP as the main substrate, each can use a number of phosphate donors (except for AK1 (50), which to date has only been tested with ATP). Out of the five isoforms, adenylate kinases 1–3 (51) and 5 (52) can all use GTP as a phosphate donor. Therefore, it appears that, in the presence of 20 μM GTP in combination with 10 μM ADP, both the forward and reverse reactions are taking place according to the reactions outlined in Fig. 14. This would also account for the decrease in ATP in the culture medium when GTP was added in the presence of the adenylate kinase inhibitor, Ap5A (Fig. 12).

Enzyme Localization in Normal Human Skin—To begin to identify the physiological role of nucleotide interconversion in vivo, the localization of F1F0 ATP synthase and NDPK (using the nm23-H1 antibody) was investigated in normal human skin sections. Both interconversion enzymes were differentially expressed, with high levels in the basal layer and diminishing levels in the differentiated layers with a high degree of colocalization (Fig. 15A). On closer inspection using optical sectioning through the z-axis, both enzymes were in the cytoplasmic domain in the basal layer (Fig. 15B) and thus are most likely to be mitochondrial. In the granular layer, nm23-H1 appeared to be around the periphery of the cell, whereas ATP synthase staining remained in the cytoplasmic domain (Fig. 15C). The tissue was fixed in situ before the addition of antibodies to eliminate antibody-capping artifacts, and results are representative of parallel experiments.
This study has identified three mechanisms for interconversion of nucleotides at the cell surface in HaCaT cells. The mechanisms identified include adenylate kinase, NDPK, and F1F0 ATP synthase with the conversion utilizing 5′-monophosphates, 5′-diphosphates, and 5′-triphosphates. This has major repercussions for P2 receptor studies where extracellular nucleotides are often added to cells before changes in cellular events are measured.

Previous studies have shown that normal human keratinocytes release ATP in real-time experiments (7), whereas UTP also has been shown to be constitutively released from 1321N1 cells (44). However, we now report that confluent cultures of HaCaT cells (a keratinocyte cell line) (11) also release ATP, because the ATP content of culture medium was dependent on cell number (Fig. 1). Furthermore, this release is much higher from HaCaT cells than from the same numbers of SaOS-2 cells (Fig. 2). There are currently several postulated mechanisms for ATP release, including cytolysis, ATP-binding cassette (ABC) proteins (15–20), and/or neighboring ion channels (53), or through vesicles during exocytosis (22–24). However, although the mechanism for ATP release by platelets (13) and neuroendocrine cells or neurons (14) is well documented, there remains very little consensus in non-neuronal cell types. In SaOS-2 cells, cytolysis has been discounted because sufficient lactate dehydrogenase could not be detected (43).

Because ADP → ATP conversion by NDPK has recently been observed in SaOS-2 cells (43), the changes in the concentration of ATP in the culture medium surrounding HaCaT cells were thus measured after addition of various nucleotides. In contrast to SaOS-2 cells, where only ADP had an effect (43), in HaCaT cells, ADP, GTP, and UTP significantly elevated the ATP content of the culture medium (Fig. 3). Furthermore, ATP was concentration-dependently elevated by ADP (Fig. 4). This first evidence for nucleotide conversion at the cell surface by this cell type. The conversion of ADP to ATP suggests either the presence of ecto-adenylate kinase, which converts 2ADP ↔ ATP plus AMP, or ecto-NDPK, which uses a phosphate donor in the reaction, N1TP + N2TP ↔ N1TP + N3DP, both of which have been reported to be at the cell surface in a number of various cell types (33, 41, 42, 44). Interestingly, because HaCaT cells release more ATP than SaOS-2 cells (Fig. 2), this may explain the differences in the nucleotides that can be converted by these two cell types. Although ATP is released, hydrolysis ecto-nucleotidases break it down to ADP, AMP, and adenosine reducing the potential for activation of P2 receptors (27). Therefore, it would be expected that higher concentrations of ADP would be present in the culture medium of HaCaT cells than SaOS-2 cells. This ADP could then be utilized by ecto-NDPK when GTP or UTP were added, and thus prolonging the concentration of ATP in the culture medium.

Because the measured ATP concentrations at any time point can be considered to be representative of the net effect of both ATP-degrading and ATP-generating enzymes, the changes in ATP concentration after addition of a bolus of ATP or ADP were measured over time. The effects were due to ecto-enzymes, because there was no conversion of ADP → ATP in the absence of cells (Fig. 5B), whereas relatively little hydrolysis was observed with ATP in the absence of cells (Fig. 5A). In the presence of cells, because the ATP concentration in the bathing medium was rapidly reduced with time (Fig. 5A), this is evidence that HaCaT cells express ecto-nucleotidases. However, there was comparatively little degradation of the ATP produced by interconversion enzymes when 10 μM ADP was added (Fig. 5B). It can, therefore, be assumed that the overall rate of production is equal to that of hydrolysis, and an equilibrium has been reached. Furthermore, the equilibrium appears to be reached almost immediately, and thus it was not necessary to analyze ATP measurements using the same ADP concentration at any other time point.

Interestingly, after 90 min, the background ATP concentration was similar to that generated by addition of ADP (~250 nm) suggesting that this concentration of ATP is able to persist in the extracellular environment. This level is just below that necessary to induce responses such as [Ca2+], elevation in this cell type (10), which in keratinocytes is linked to an increase in proliferation (8, 10). Because it is unlikely that all cells release ATP simultaneously, this also provides insights into how proliferation may be locally induced, because relatively few cells would be required to release ATP to elevate the extracellular concentration to that required for [Ca2+]i, elevation.

To identify whether the ADP → ATP conversion was due to ecto-adenylate kinase, the inhibitor Ap5A (45) was used. Confluent cultures were incubated with increasing concentrations (30–300 μM) of Ap5A, prior to addition of ADP. The ATP content of the culture medium was elevated in a concentration-dependent manner (data not shown). However, the purchased Ap5A was found to contain a significant amount of contaminating ADP and ATP (results not shown) in agreement with previous reports (46). Therefore, when the experiments were repeated in the presence of apyrase-treated Ap5A, where the contamination had been removed, the ATP content of the culture medium was concentration-dependently decreased by the apyrase-treated Ap5A (Fig. 6). At the maximal concentration (300 μM) of Ap5A, ADP → ATP conversion was inhibited by 93%, indicating that this is the predominant enzyme responsible for the conversion of ADP to ATP. The study using the contaminated Ap5A (data not shown), when in the presence of high concentrations of ATP and ADP, revealed that ATP synthesis via conversion could still take place. This suggests that another conversion enzyme, for example NDPK is present and active.

NDPK catalyzes the phosphorylation of 5′-diphosphates using 5′-triphosphates as phosphate donors and is inhibited by high concentrations of 5′-diphosphates. Therefore, the effects of UDP on ADP/GTP conversion to ATP were observed (Fig. 10). UDP not only inhibited the conversion of ADP to ATP, but also inhibited the conversion of ADP plus GTP to ATP, indicating that NDPK is present and active in HaCaT cells. However, there was no effect with GTP alone, suggesting that the response with GTP alone is not due to NDPK and ADP in the culture medium from hydrolysis as we at first postulated. Furthermore, the activity of this enzyme may not be at the cell surface. In a recent report, G protein-coupled receptor activation has been linked to the translocation of NDPK to the cell cortex, whereas it has also been suggested that NDPK has a role in vesicular trafficking (54). An antibody to nm23-H1 was used to observe localization in HaCaT cells (Fig. 11). Nm23-H1 is derived from a human gene, which has been cloned and shown to encode for a 17-kDa protein with NDPK activity (55). In our study, Nm23-H1 appears to be at the cell surface where expression is patchy with not all cells showing the same degree of expression (Fig. 11A) in agreement with previous studies (54). Therefore, it remains unclear as to whether the effects observed in our study due to NDPK are indeed extracellular events or whether conversion occurs on the inner surface of the membrane with subsequent ATP transportation out of the cell via vesicular release.

Because there have also been recent reports describing ADP → ATP conversion by a third ecto-enzyme, F1F0 ATP synthase (36–39), the role of this enzyme in the HaCaT cells was investigated. Presence of F1F0 ATP synthase localization was ob-
served on the cell surface of HaCaT cells using confocal microscopy (Fig. 8). Oligomycin, an inhibitor of the F_0 complex of ATP synthase inhibited ADP to ATP conversion in a concentration-dependent manner (Fig. 7). However, although maximal effects occurred on addition of 48 μM oligomycin, ADP to ATP conversion was not completely abolished, suggesting a minor role for this enzyme in the HaCaT cell line. Interestingly, NDPK expression at the cell surface is both inducible and patchy (54), and we have shown that F_Fo ATP synthase expression is also punctate (Fig. 8), suggesting that the ATP generated by interconversion is spatially limited. This has important consequences, because this may provide a mechanism whereby local rather than global concentrations of nucleotides are regulated at the cell surface to control the overall stimuli of proliferation and/or differentiation.

Because the effects of GTP alone could not be attributed to NDPK as we first thought, the combined role of all three enzymes was studied. Maximal concentrations of oligomycin, ApGpA, and UDP when added together almost entirely abolished the conversion of ADP or GTP to ATP (Fig. 12), suggesting that there are no further ecto-conversion enzymes present. However, the activity of NDPK, F_Fo ATP synthase, or adenylate kinase, when determined from the nucleotides added alone, could not fully account for the ATP content of the culture medium when the nucleotides were added simultaneously. Because conversion must be due to one of the enzymes already identified, we postulate that this discrepancy is likely to be due to the reverse reaction of adenylate kinase utilizing GTP. Out of the five adenylate kinase isoforms, adenylate kinases 1–3 (51) and 5 (52) can all use GTP as a phosphate donor in the reverse reaction when in the presence of AMP (Fig. 13). Similarly, this also accounts for the decrease in ATP when GTP was added in the presence of the adenylate kinase inhibitor, ApGpA (Fig. 12).

The discovery that the HaCaT cell line has three mechanisms for nucleotide interconversion has consequences not only in studies where the effects of nucleotides on cellular processes are observed, but also more importantly on the wider field of P2 receptor classification, where, unless these enzymes are accounted for, false interpretation will be likely. Normal human keratinocytes predominantly express P2Y_2 receptors and the HaCaT cell line expresses P2Y_4 receptors (10), which can be activated by both ATP and UTP. The conversion enzymes detected in HaCaT cells all convert ADP → ATP and thus channel the stimulus to the P2Y_2 and P2Y_4 receptor subtypes. Because activation of P2Y receptors is involved in proliferation, differentiation, and apoptosis in the epidermis (8, 10), localized release of nucleotides, hydrolysis, and subsequent interconversion may provide a system whereby these processes are regulated. Therefore, the localization of interconversion enzymes (NDPK and ATP synthase) was investigated in normal human epidermis.

In comparison with cultured HaCaT cells, distribution of ATP synthase and NDPK differed depending on the layer within the epidermis (Fig. 15). In the basal layer, expression of both enzymes was greater than in the differentiated layers, and the pattern of staining was in the cytoplasmic domain as would be expected for mitochondrial rather than cell surface expression. In the granular layer, the pattern of nm23-H1 staining was more pronounced at the cell surface and was almost entirely around the cell periphery, whereas ATP synthase remained in the cytoplasmic domain.

ATP is released by the mitochondria-rich keratinocytes in the basal layer (7). The released ATP activates P2Y_2 receptors, which are known to induce proliferation in normal keratinocytes and HaCaT cells (10), this is important, not only for normal epidermal homeostasis, but also for wound healing. It is notable that there is an apparent absence of cell surface interconversion enzymes in the basal layer where there is probably an adequate supply of extracellular ATP from release. If ecto-nucleotidases are also colocalized with ATP release sites, as has been shown for ecto-ATPases and ATP release sites in astrocytes (56), ATP will quickly break down to ADP, AMP, and adenosine (27) in the basal layer. These breakdown products, which are substrates for interconversion enzymes, could diffuse to the granular layer where cell surface interconversion enzymes are present. There is clearly the potential for generation of extracellular ATP in the granular layer. This may be important for ensuring that apoptosis can occur in the terminally differentiated cornified layer where P2X_receptors are localized (8), because elevated levels of ATP are necessary to activate this receptor subtype. Therefore, it appears that nucleotide interconversion is involved in the differentiation process in the epidermis and more importantly, it suggests that the generation of extracellular ATP may be spatially regulated.

Acknowledgments—We are grateful to Paul Rothwell and Christine McNamee for help with the immunofluorescence staining and Dr. Tomoko Kamishima and Professor Brian Beechey for advice, particularly on the use of oligomycin.

REFERENCES

1. Abbracchio, M. P., Boynaeams, J. M., Barnard, E. A., Boyer, J. L., Kennedy, C., Maier, B., Portugal, M. T., Rahn, W., Andrefsky, A. L., Steinman, G. A., and Burnstock, G. (2003) Trends Pharmacol. Sci. 24, 52–55
2. Khakh, B. S., Burnstock, G., Kennedy, C., King, B. F., North, R. A., Seguela, P., Voigt, M., and Humby, P. (2001) Pharmacol. Rev. 53, 107–118
3. Abbracchio, M. P., and Burnstock, G. (1994) Pharmacol. Ther. 64, 445–475
4. Palli, S., and Bille, D. D. (1992) J. Clin. Invest. 90, 42–51
5. Reimer, W. J., and Dixon, S. J. (1992) J. Am. Physiol. 263, C1040–C1048
6. Balevic, V., and Burnstock, G. (1998) Pharmacol. Rev. 50, 413–492
7. Dixon, C. J., Bowler, W. B., Littlewood-Evans, A., Dillon, J. P., Bille, G, Sharpe, G. R., and Gallagher, J. A. (1999) Br. J. Pharmacol. 127, 1680–1686
8. Greig, A. V., Linge, C., Terenghi, G., McGrouther, D. A., and Burnstock, G. (2003) J. Invest. Dermatol. 120, 1007–1115
9. Grischi-Stewart, U., Bardhim, M., Robson, T., and Burnstock, G. (1999) Cell Tissue Res. 296, 599–605
10. Burrell, H. E., Bowler, W. B., Gallagher, J. A., and Sharpe, G. R. (2003) J. Invest. Dermatol. 120, 440–447
11. Boukamp, P., Petrussevska, R. T., Breitkreutz, D., Hornung, J., Markham, A., Reisin, I., Gachet, C., Jacobson, K. A., Weisman, M., and Zimmermann, H. (1999) J. Cell Biol. 166, 761–771
12. Huang, N., Wang, D., and Heppel, L. A. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 7094–7098
13. Detwiler, T. C., and Feinmann, R. D. (1973) Biochemistry 12, 2462–2468
14. Unsworth, C. D., and Johnson, R. G. (1990) Ann. N. Y. Acad. Sci. 603, 353–363
15. Abraham, E. H., Prat, A. G., Gerweck, L., Seneveratne, T., Arceci, R. J., Kramer, R., Guidotti, G., and Cantiello, H. F. (1995) Proc. Natl. Acad. Sci. U. S. A. 90, 312–316
16. Reisin, I., Prat, A. G., Abraham, E. H., Amar, J. F., Gregory, R. J., Ausiello, D. A., and Cantiello, H. F. (1994) J. Biol. Chem. 269, 20584–20591
17. Schwiebert, R. M., Egner, M. E., Hwang, T, Fulmer, S. B., Allen, S. S., Cutting, G. R., and Gugino, W. B. (1995) Cell 101, 1063–1073
18. Prat, A. G., Reisin, I., Ausiello, D. A., and Cantiello, H. F. (1996) Am. J. Physiol. 270, C358–C354
19. Roman, R. M., Wang, Y., Lidsky, S. D., Feranchak, A. P., Lomri, N., Scharf, M., and Verderio, C. (2003) J. Biol. Chem. 278, 1354–1362
20. Homolya, L., Steinberg, T. H., and Boucher, R. C. (2000) J. Biol. Chem. 150, 1349–1359
21. Plesner, L. (1995) Int. Rev. Cytol. 158, 141–214
22. Christensen, H. (1990) Drug Disc. Res. 39, 337–352
23. Zimmermann, H. (1999) Trends Pharmacol. Sci. 20, 231–236
24. Goding, J. W., Terkelhau, R., Maurice, M., Deterre, P., Sali, A., and Belli, S. I. (1999) Immunol. Rev. 161, 11–26
25. Greiben, B., Anicuia, K., Remy, D., Stefan, C., Bollen, M., Emsen, E. L., and Slegers, H. (1999) J. Neurochem. 72, 826–834
26. Zimmermann, H. (1992) Biochem. J. 283, 345–365
27. Resta, R., Yamashita, Y., and Thompson, L. F. (1998) Immunol. Rev. 161, 95–109
28. Donaldson, S. H., Fisher, M., and Boucher, R. C. (2002) Am. J. Respir. Cell Mol. Biol. 26, 209–215
29. Dixon, C. J., Hall, J. F., and Boarder, M. R. (2003) Br. J. Pharmacol. 138, 272–278
30. Fisher, M., and Boucher, R. C. (2003) J. Biol. Chem. 278, 11256–11264
31. Moser, T. L., Stack, M. S., Asplin, I., Engblad, J. J., Hejrup, P., Evertt, L.,
Nucleotide Interconversion at the Keratinocyte Cell Surface

Hubachak, S., Schnaper, H. W., and Pizzo, S. V. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 2811–2816

Moser, T. L., Renan, D. J., Ashley, T. A., Roy, J. A., Goodman, M. D., Misra, U. K., Cheek, D. J., and Pizzo, S. V. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 6656–6661

Araaraki, N., Nagan, T., Niki, R., Toyofuku, A., Tanaka, H., Kuramoto, Y., Emoto, Y., Shibata, H., Magota, K., and Higuti, T. (2003) Mol. Cancer Res. 1, 931–939

Martinez, J. O., Jacquet, S., Esteve, J. P., Rolland, C., Cabezón, E., Champagne, E., Pineau, T., Georgeaud, V., Walker, J. E., Terce, F., Collet, X., Perret, B., and Barbaras, R. (2003) Nature 421, 75–79

Lazarowski, E. R., Boucher, R. C., and Harden, T. K. (2000) J. Biol. Chem. 275, 31061–31068

Buxton, I., Kaiser, R. A., Oxhorn, B. C., and Cheek, D. J. (2001) Am. J. Physiol. 281, H1657–H1666

Yegutkin, G. G., Henttinen, T., and Jalkanen, S. (2001) FASEB J. 15, 251–260

Buckley, K. A., Golding, S. L., Rice, J. M., Dillon, J. P., and Gallagher, J. A. (2003) FASEB J. 17, 1401–1410

Lazarowski, E. R., Homlya, L., Boucher, R. C., and Harden, T. K. (1997) J. Biol. Chem. 272, 24348–24354

Sinev, M. A., Sineva, E. V., Ittah, V., and Haas, E. (1996) Biochemistry 35, 6425–6437

Conant, A. R., Fisher, M. J., McLennan, A. G., and Simpson, A. W. (1998) Br. J. Pharmacol. 125, 357–364

Boyer, P. D. (1997) Annu. Rev. Biochem. 66, 717–749

Linnett, P. E., and Beechey, R. E. (1979) Methods Enzymol. 55, 472–518

Lasce, I., and Gonin, P. (2000) J. Bioenerg. Biomembr. 32, 237–246

Yoneda, T., Sato, M., Maeda, M., and Takagi, H. (1998) Mol. Brain Res. 62, 187–185

Wilson, D. E., Povey, S., and Harris, H. (1976) Ann. Hum. Genet. 39, 305–313

Van Rompay, A. R., Johansson, M., and Karlsson, A. (1999) Eur. J. Biochem. 261, 509–517

Cantiello, H. F. (1997) Biosci. Rep. 17, 147–171

Gallagher, B., Parrott, K. A., Szabo, G., and de S. Otero, A. (2003) J. Cell Sci. 116, 3239–3250

Kimura, N., Shimada, N., Nomura, K., and Watanabe, K. (1999) J. Biol. Chem. 265, 15744–15749

Joseph, S. M., Buchakjian, M. R., and Dubyak, G. R. (2003) J. Biol. Chem. 278, 23331–23342