Extracellular ATP and Some of its Analogs Induce Transient Rises in Cytosolic Free Calcium in Individual Canine Keratinocytes

Maja M. Suter, Flavio M. Crameri, James P. Slattery, Paul J. Millard, and Fernando A. Gonzalez

Departments of Pathology and Pharmacology, New York State College of Veterinary Medicine, Biotechnology Program, and Section of Biochemistry, Molecular and Cell Biology, Cornell University, Ithaca, New York, U.S.A.

Changes in intracellular free calcium ($[Ca^{++}]_i$) play an important role in a variety of biochemical reactions that lead to cellular responses such as proliferation and differentiation. The response of $[Ca^{++}]_i$ to extracellular nucleotides (ATP, UTP, ITP, and AMP-PNP) was determined in individual canine keratinocytes using the fluorescent probe fura-2 and digital video fluorescence imaging microscopy. In the presence of 1.8 mM extracellular Ca^{++}, 100 and 500 μM ATP caused a rapid (<9 sec) three- to twelvefold rise in $[Ca^{++}]_i$ above resting levels of 50–150 nM, followed by occasional fluctuations. Small responses were elicited with doses as low as 0.1 μM ATP. The response of cells stimulated with 500 μM ATP in Ca^{++}-free medium was characterized by 1.5 to 3 times rapid initial peak followed by a decrease of $[Ca^{++}]_i$ below resting levels. Loss of response occurred in the majority of keratinocytes preincubated for 30 min in Ca^{++}-free medium. UTP was as effective as ATP in stimulating rises in $[Ca^{++}]_i$ in keratinocytes. Smaller elevations in $[Ca^{++}]_i$ up to four- to fivefold resting levels were noted with 100 μM AMP-PNP or 500 μM ITP. Desensitization of cells was demonstrated when a second stimulation followed the primary ATP or UTP treatment.

These results are suggestive of the presence of purinergic receptors in the cytoplasmic membrane of canine keratinocytes. Experiments using the calcium channel blocker lanthanum suggest that ATP-induced initial rises and sustained levels of $[Ca^{++}]_i$ are dependent on the release of Ca^{++} from intracellular stores. These intracellular Ca^{++} stores appear to be rapidly depleted after removal of extracellular calcium ($[Ca^{++}]_e$), thereby abolishing ATP-induced $[Ca^{++}]_i$ increases.
J Invest Dermatol 97:223–229, 1991

Extracellular adenosine triphosphate (ATP) at low micromolar concentrations can influence many biologic processes including platelet aggregation, vascular tone, neurotransmission, cardiac function, neutrophil migration, and muscle contraction [1,2]. ATP interacts with P_2 purinergic receptors on the cytoplasmic membrane [1], and, in a variety of normal and neoplastic cells, extracellular ATP stimulates transient increases in $[Ca^{++}]_i$ [3–8]. In cultured human keratinocytes, increase in $[Ca^{++}]_i$ is an early event in terminal differentiation [9] and appears to be of primary importance in this process [10]. A rise in $[Ca^{++}]_i$ precedes morphologic changes of differentiation and growth inhibition [9]. Furthermore, the degree of proliferation and terminal differentiation of cultured keratinocytes is dependent on the concentration of extracellular calcium ($[Ca^{++}]_e$) [11–14], which in turn influences intracellular free calcium ($[Ca^{++}]_i$) levels directly [10].

The present study was performed to determine whether cultured canine keratinocytes respond to extracellular ATP and ATP analogs with increases in $[Ca^{++}]_i$. The results are indicative of the presence of P_2 purinergic receptors on the cytoplasmic membrane of canine keratinocytes that are subject to regulatory control by desensitization.

Manuscript received August 24, 1990; accepted for publication January 28, 1991.

Dr. Gonzalez's present address: Howard Hughes Medical Institute, Program in Molecular Medicine and Department of Biochemistry, University of Massachusetts Medical Center, Worcester, Massachusetts.

Supported by NIH/BRSG grant S7RR05462.

Presented in part at the Annual Meeting of the Society of Investigative Dermatology, Washington D.C., 1989 and 1990.

Reprint requests to: Dr. Maja M. Suter, Department of Pathology, New York State College of Veterinary Medicine, Cornell University, 325A VRT, Ithaca, NY 14853.

Abbreviations:

AMP-PNP: adenylyl-imidodiphosphate
ATP: adenosine-5′-triphosphate
Ca^{++}: calcium
$[Ca^{++}]_e$: extracellular calcium
$[Ca^{++}]_i$: intracellular free calcium
CT: cholera toxin
EGF: epidermal growth factor
EGTA: ethyleneglycol-bis-N,N,N′,N′-tetraacetic acid
FBS: fetal bovine serum
ITP: inosine-5′-triphosphate
La^{+++}: lanthanum
UTP: uridine-5′-triphosphate
WME: Williams' medium E

MATERIALS AND METHODS

Keratinocyte Cultures Long-term cultures of canine keratinocytes (between 4th and 8th passage) were seeded on 4.8-cm^2 glass coverslips at a concentration of $3–4 \times 10^4$ cells/cm^2, inserted into 6-well tissue culture plates, and grown in Williams' medium E (Gibco, Grand Island, NY) containing 10% fetal bovine serum (FBS), 10 ng/ml EGF (Collaborative Research, Lexington, MA), 10^{-10} M cholera toxin (CT; Schwarz-Mann, Orangeburg, NY), and antibiotics at 34°C as described earlier (supplemented WME) [15,16]. The cells were depleted of EGF, CT, and FBS 8–15 h prior to the experiments because cells maintained in medium supplemented with growth factors and serum did not respond. Between 36

0022-202X/91/$03.50

and 48 h after seeding, subconfluent monolayers were used for measurement of intracellular free calcium ($[Ca^{++}]_i$).

Loading and Cell Preparation for Imaging Subconfluent cultures of keratinocytes were incubated with 1 μM fura-2/AM (acetoxymethyl ester, Molecular Probes, Eugene, OR) in modified Tyrodes buffer (containing 135 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5.6 mM D-glucose, 10 mM Na-Hepes, 250 μM sulfinpyrazone (Sigma, St. Louis, MO), and 0.1% bovine serum albumin, pH 7.4) for 30 min at 34°C. After washing with modified Tyrodes buffer, the coverslips were mounted in a 1-ml chamber designed for flow-through operations. The flow chamber and all solutions that were flushed through the chamber were kept at 34° ± 0.5°C.

Calcium Measurement Levels of $[Ca^{++}]_i$ were determined by ratio imaging of cell-associated fura-2 fluorescence [3,17]. Experiments were performed either at 1.8 mM $[Ca^{++}]_e$ or in modified, calcium-free Tyrodes buffer supplemented with 100 μM ethyleneglycol-bis-N,N,N′,N′-tetraacetic acid (EGTA). In addition, selected experiments were conducted with 0.2 mM calcium in the extracellular solution. To determine the contribution of calcium influx to the rise in $[Ca^{++}]_i$ measured during ATP stimulation, keratinocytes were preincubated in 10 μM $LaCl_3$ (Fisher, Rochester, NY), a calcium channel blocker, for 5 min and then stimulated with 100 μM ATP in the presence of both lanthanum (La^{+++}) and Ca^{++}. In preliminary experiments, dose-response experiments with lanthanum were performed and 10 μM La^{+++} was found to be optimal. The data presented in this study represent a total of 105 experiments. Each treatment group consists of 4–5 experiments with an average of 10–15 cells/experiment.

The flow-through chamber was flushed with 4 times the chamber volume (4 ml) of nucleotide solution. Fura-2 fluorescence was imaged with a Nikon Diaphot inverted microscope using a Nikon 20x/NA 0.75 CF Fluor DL objective lens. Illumination at 340 nm and at 365 nm was accomplished by diverting light from a 150 W xenon source through one of two monochromators using a chopper mirror. Images were acquired with a microchannel plate image intensifier (Model KS-1380, Video Scope International) coupled to a silicon diode video camera (Model 70, Dage-MTI), digitized and averaged (eight frames at each wavelength) with a Tracor-Northern TN-8500 image processor and transferred to one of two 80-Mbyte hard disks. Thirty quantitative ratio images were taken with temporal resolution that was typically 1 ratio pair /9-sec interval over a period of approximately 4.5 min. Ratio images were generated after all image pairs had been collected and stored. Typically, five image pairs were taken of the cells in a resting stage before nucleotide stimulation. Then the nucleotide solution was flushed through the chamber in approximately 8 seconds. A background image from an area of the coverslip without cells was collected for both excitation wavelengths at the end of each experiment.

Background images were subtracted from the corresponding images of cell fluorescence, and the ratio (R) was generated in the image processor. The pixel values in the ratio images were measured and averaged on a frame-by-frame basis for each cell in the field of view to obtain the average ratio per cell as a function of time. Intracellular free calcium ($[Ca^{++}]_i$) was determined according to the equation:

$$[Ca^{++}]_i = K_d[(R - R_{min})/(R_{max} - R)]\beta,$$

where K_d = dissociation constant of the Ca^{++}/fura-2 complex (224 nM), $R = F_{340}/F_{365}$, i.e., the fluorescence at 340 nm excitation divided by the fluorescence at 365 nm excitation, R_{max} = R at saturated levels of Ca^{++} (10 mM $CaCl_2$), R_{min} = R at zero Ca^{++} (25 mM EGTA), and $\beta = F_{365}$(zero Ca^{++})/F_{365} (saturating Ca^{++}). At the end of each experiment day the fluorescence of a thin layer (130 μm) of 25 μM fura-2–free acid (Molecular Probes) in calcium-free, high-K^+ saline solution, supplemented with either 10 mM $CaCl_2$ (saturating Ca^{++}) or 25 mM EGTA (zero Ca^{++}), was measured to determine R_{max} and R_{min}. The value of β was determined for each pixel from the calibration images of fura-2. Average cell $[Ca^{++}]_i$ was determined using values for R_{max}, R_{min}, and β that were determined for the entire field.

The effect of stimulation with ATP or UTP on a second subsequent stimulation was determined by initial treatment of cells with ATP or UTP after frame 5, followed by the flushing of the chamber with buffer after frame 14, and second stimulation with either ATP or UTP after frame 18 or 24. For these desensitization experiments 40 quantitative ratio images were taken in 9-second intervals over a period of approximately 6 min. In these desensitization experiments ATP was used at a concentration of 100 μM. UTP was used at 100 or 500 μM as first and second stimulant. The higher UTP concentration was chosen because this nucleotide was less potent in heterologous desensitizations than ATP.

Negative Controls As negative control, chambers with fura-2–loaded cells were flushed with buffer twice during the experiment. In addition, the first flushing was performed with Ca^{++}-free buffer. In a third set of experiments cells were incubated with Ca^{++}-containing buffer, flushed with Ca^{++}-free buffer, and then flushed with Ca^{++}-containing buffer supplemented with 10 μM La^{+++}.

RESULTS

Resting Levels Resting levels of intracellular free calcium ($[Ca^{++}]_i$) in subconfluent canine keratinocytes range from 50–150 nM in the presence of 1.8 mM extracellular calcium. Flushing of the chamber with Ca^{++}-containing buffer did not have any effect on resting levels. In Ca^{++}-free medium, resting $[Ca^{++}]_i$ levels decreased in most cells to 10–50 nM within 36 seconds. After reintroduction of $[Ca^{++}]_e$, $[Ca^{++}]_i$ increased rapidly to normal resting levels in <27 seconds often associated with an initial slow transient elevation of twice these levels. When Ca^{++} was reintroduced in a buffer supplemented with 10 μM La^{+++}, the resting levels remained at 10–50 nM due to the Ca^{++}-channel blocking effect of La^{+++} (data not shown).

Stimulation with ATP in Ca^{++}-Containing Medium Keratinocytes were stimulated with adenosine-5′-triphosphate (ATP), uridine-5′-triphosphate (UTP), inosine-5′-triphosphate (ITP), or adenylyl-imidodiphosphate (AMP-PNP) at concentrations of 0.01–500 μM [3] in the presence of 1.8 mM $[Ca^{++}]_e$. Stimulation of keratinocytes with 100 μM ATP caused a rapid (within 9 sec) three- to twelvefold rise in $[Ca^{++}]_i$, followed by a slower and variable decrease to sustained values above resting levels (Fig 1, *cells A1–6*). One hundred micromolar ATP induced a maximal response and application of 500 μM ATP did not augment the magnitude of this response (Fig 1, *cells B1–6*). In many cells, sustained $[Ca^{++}]_i$ levels remained between 80 and 200 nM up to 270 sec after stimulation (end of experiment time) with a considerable increase in noise when compared with experiments conducted in Ca^{++}-free medium (compare *Fig 1* with *Fig 2*). This may represent fluctuations of a higher frequency than can be measured with 9-sec time resolution. Absolute $[Ca^{++}]_i$ levels varied sometimes between experiment days. However, the relation of maximal peaks to resting levels remained constant. Rare cells (<5%) responded with two slow fluctuations of higher magnitude (two- to fivefold; Fig 1, *cell B2*). Small and rapid initial responses up to 3 times resting levels were elicited with doses as low as 0.1 μM ATP (data not shown). No response was detected after incubation with 0.01 μM ATP. Canine keratinocytes maintained in supplemented WME up to the time of the experiment did not respond and depletion of cells of EGF, CT, and FBS of at least 6 h was necessary for detectable responses to occur. Although most cells reacted strongly to the stimulus, there were single cells (approximately 10%) in every experiment that did not respond regardless of the ATP concentration. Optimal responses similar to those described above occurred after stimulation of cells with 100 μM ATP in 0.2 mM $[Ca^{++}]_e$ (data not shown). Addition of La^{+++} to Ca^{++}-containing medium (1.8 mM) had no effect on ATP-induced initial

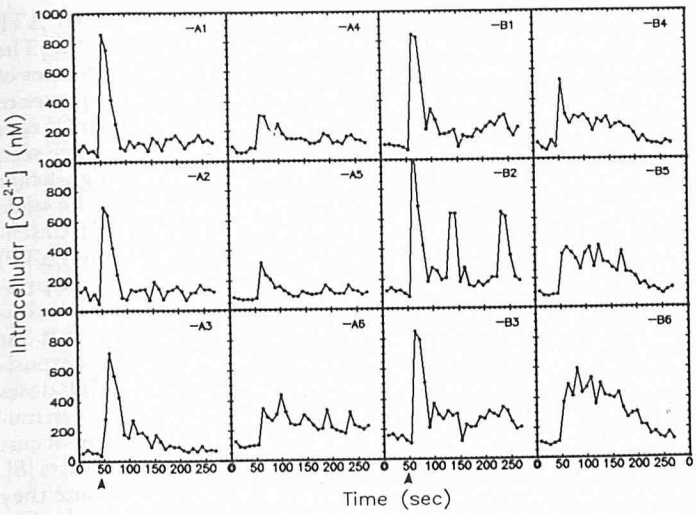

Figure 1. Changes in $[Ca^{++}]_i$ of 12 individual keratinocytes from four different experiments after ATP stimulation in Ca^{++}-containing medium. Three of 10–15 cells/experiment were chosen from each experiment to represent the variety of responses observed. One hundred μM ATP was used to stimulate cells *A1–6*, 500 μM ATP for cells *B1–6*. Stimulation *(arrowhead)* resulted in a rapid, synchronous, three- to twelvefold increase in $[Ca^{++}]_i$, followed by a slower and variable decrease to resting levels or sustained values above resting levels with increased noise. Rare cells (e.g., cell B2) responded with two slow fluctuations of higher magnitudes.

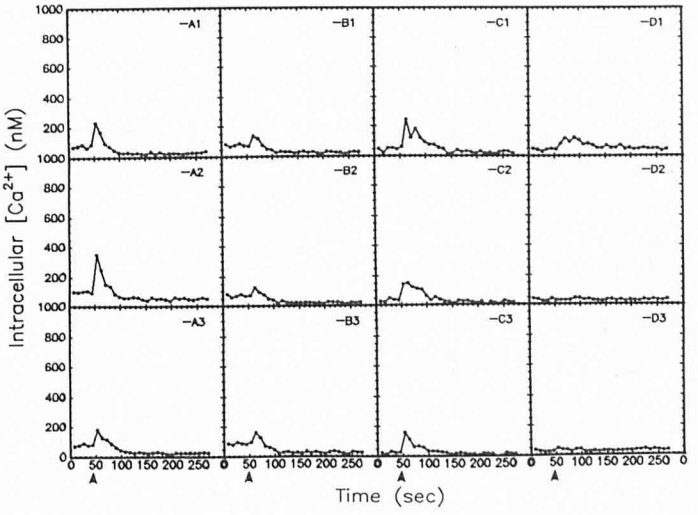

Figure 2. Changes in $[Ca^{++}]_i$ of 12 individual keratinocytes from four different experiments after ATP stimulation in Ca^{++}-free medium. From each experiment three representative cells were chosen. The Ca^{++}-containing buffer was replaced by a Ca^{++}-free solution supplemented with 100 μM EGTA and stimulation *(arrowhead)* with 500 μM ATP was carried out in Ca^{++}-free buffer. *A Cells,* incubation in Ca^{++}-containing buffer followed by Ca^{++}-free ATP stimulation; *B Cells,* change to Ca^{++}-free buffer 27 sec prior to stimulation; *C Cells,* 2 min of Ca^{++}-free incubation prior to stimulation; *D Cells,* Preincubation in Ca^{++}-free buffer for 30 min. Stimulation in Ca^{++}-free buffer resulted in a rapid, synchronous 1.5–4 times initial peak in most cells. This peak was followed by a rapid decrease of $[Ca^{++}]_i$ below resting levels in all cells. $[Ca^{++}]_i$ levels decreased below resting levels even in cells that did not respond to ATP stimulation *(cells D2, D3)*. Most keratinocytes preincubated in Ca^{++}-free medium for 30 min did not respond *(cells D1–D3)*.

and subsequent, transient elevations of $[Ca^{++}]_i$. The initial peak decreased over the same time course as seen in Ca^{++}-containing medium (72–90 seconds) and rare cells (<5%) showed an additional single large transient peak.

Stimulation with ATP in Ca^{++}-Free Medium Keratinocytes were stimulated with ATP in Ca^{++}-free buffer in four different experimental groups: (1) incubation in Ca^{++}-containing medium with subsequent stimulation in Ca^{++}-free medium (Fig 2, *A cells*); (2) change to Ca^{++}-free buffer 27 seconds prior to stimulation (Fig 2, *B cells*); (3) incubation in Ca^{++}-free buffer for 2 min prior to stimulation (Fig 2, *C cells*); and (4) 30 min incubation in Ca^{++}-free buffer prior to stimulation (Fig 2, *D cells*). Whereas groups (1) and (2) had normal resting levels at the time of stimulation, the prestimulation levels of most cells in group (3) and (4) were below normal resting levels, i.e., between 20 and 50 nM. The response of cells stimulated with 500 μM ATP in Ca^{++}-free medium was characterized by a 1.5 to 4 times rapid initial peak (<9 seconds) followed by a slower decrease (>45 seconds) to values below resting levels (<50 nM; Fig 2). Subsequent slow oscillations were observed only in <4% of cells and maximal levels of these oscillations did not exceed normal resting levels. Similar results were obtained when cells were preincubated for 27 seconds in Ca^{++}-free medium and then stimulated with ATP in the same medium. Loss of response occurred in the majority (88%) of keratinocytes preincubated for 30 min in Ca^{++}-free medium and the few cells that responded under these conditions showed only minimal increases in $[Ca^{++}]_i$ up to threefold, to 150–170 nM (Fig 2, *D cells*). The few cells that responded to ATP were those that maintained resting levels in the lower normal range (50–70 nM), whereas cells with resting levels below 50 nM $[Ca^{++}]_i$ did not respond.

Stimulation with UTP, ITP, and AMP-PNP One hundred μM UTP was as effective as 100 μM ATP in causing transient increases in $[Ca^{++}]_i$ in keratinocytes. When cells were stimulated with 100 μM AMP-PNP, initial peaks of $[Ca^{++}]_i$ were up to 4–5 times resting levels (Fig 3, *A cells*). The sustained levels of $[Ca^{++}]_i$ after the initial peak were similar to those seen after ATP stimulation. Five hundred μM ITP, the most effective dose tested, induced similar initial elevations in $[Ca^{++}]_i$ to maximally fourfold resting levels, followed by a slow decrease to resting levels without any subsequent transients (Fig 3, *B cells*).

Desensitization Experiments Possible desensitization of purinergic receptors was tested by a second stimulation with ATP or UTP. Treatment of cells with 100 μM ATP caused a partial desensitization of the response to a second 100 μM ATP stimulation 2 min later (Figs 4 and 5). Whereas most cells reacted to the first ATP treatment with six- to twelvefold increases in $[Ca^{++}]_i$, a second ATP incubation caused only three- to fivefold rises (Fig 5).

Heterologous desensitization was demonstrated when UTP treatment followed a first stimulation with ATP or vice versa. One hundred μM ATP prevented approximately half the keratinocytes from responding to a subsequent stimulation with 100 μM UTP whereas the other half responded, but with a second peak that was approximately half the magnitude of the first (Fig 6). A second stimulation using up to 500 μM UTP after the initial 100 μM ATP, however, resulted in $[Ca^{++}]_i$ increases of approximately the same magnitude (between 0.9× and 1.7× the initial peak). The reversed desensitization in which UTP was used for the initial stimulation followed by ATP showed the following results (Fig 6): UTP at a concentration of 100 μM was not able to prevent subsequent cellular responses in $[Ca^{++}]_i$ to 100 μM ATP. After a first stimulation of 500 μM UTP, 75% of the keratinocytes showed smaller peaks in response to 100 μM ATP (0.5× to 0.9× the initial UTP peak), whereas 25% of cells reacted with a $[Ca^{++}]_i$ increase equal to or higher than the initial rise. The cells that responded strongly to the first stimulation were most frequently the ones that were partially desensitized at the second stimulation.

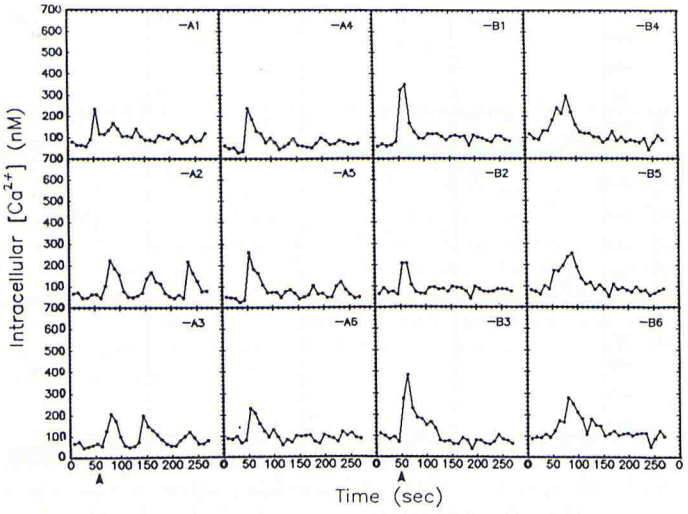

Figure 3. Response of keratinocytes to AMP-PNP and ITP. Three representative cells from four different experiments were chosen. *Cells A1–6,* Changes in $[Ca^{++}]_i$ of six individual keratinocytes after stimulation with 100 μM AMP-PNP *(arrowhead)* in Ca^{++}-containing medium. AMP-PNP caused four- to sixfold initial rises in $[Ca^{++}]_i$ and 25% cells responded with 1–4 equally large subsequent transients *(cells A2, A3). Cells B1–6,* Changes in $[Ca^{++}]_i$ of six individual keratinocytes after stimulation with 500 μM ITP *(arrowhead)* in Ca^{++}-containing medium. ITP stimulation induced initial elevations in $[Ca^{++}]_i$ up to fourfold resting levels, followed by a slow decrease to resting levels without subsequent fluctuations.

DISCUSSION

Extracellular ATP plays an important role in many biologic processes such as platelet aggregation, vascular tone, neurotransmission, cardiac function, and muscle contraction [1]. In many of these functions ATP is released from intracytoplasmic granules of activated cells [1]. Extracellularly, it binds to P_2 purinergic receptors and leads to activation of cells. Several different cell types including mouse A431 cells [3], mouse fibroblasts [7,8], aortic [6,18,19] and adrenal medullary [20] endothelial cells, human neutrophils [21], mouse macrophages [22,23], cardiac myocytes [4], rat hepatocytes [24,25], hepatocellular carcinoma cells [26], and Ehrlich ascites tumor cells [27] respond to micromolar doses of extracellular ATP by transient increases of intracellular free calcium $[Ca^{++}]_i$. The present study demonstrates that ATP induces transient increases of $[Ca^{++}]_i$ in canine keratinocytes, which is suggestive of the presence of P_2 purinergic receptors on the surface of these cells. Maximal rises in $[Ca^{++}]_i$ are induced by 100 μM ATP but small responses are seen with doses as low as 0.1 μM ATP. We have discovered significant heterogeneity among single keratinocytes as revealed by the wide range of the magnitude of the responses to ATP. The partial desensitization of keratinocytes by ATP to a second homologous (ATP) or heterologous (UTP) stimulation is further supportive of the presence of a receptor-mediated mechanism. The affinity of the keratinocyte P_2 purinergic receptor for UTP could be less than that for ATP, as higher doses of UTP are necessary to induce partial desensitization of cells to lower ATP concentrations and higher UTP doses are necessary to overcome desensitization of cells previously stimulated with lower doses of ATP. Similar rapid desensitization occurs after ATP stimulation in A431 cells [3,28] and mouse fibroblasts [8]. Performing these experiments on single cells is critical because they demonstrate directly that the same cell that is responding to the first stimulus is also responding to the second one, rather than having a subpopulation of cells responding to one stimulus and another subpopulation to another. In these studies, the unresponsiveness after a primary stimulation is not believed to be due to depletion of intracellular stores but is thought to be in fact a desensitization of receptors. This is supported by the fact that higher concentrations of UTP during the second stimulation overcome the desensitization of canine keratinocytes seen after stimulation with equal concentrations of ATP and UTP. Studies on Swiss 3T6 cells were suggestive of the presence of multiple ATP receptors [7,29].

Experiments to measure changes in $[Ca^{++}]_i$ were performed on subconfluent keratinocyte cultures. The majority of the cells is expected to be basal cells with a minority of cells in smaller groups showing a higher stage of differentiation. In the experiments presented no difference in reactivity to extracellular ATP was observed in small confluent cell clusters compared to single basal cells. Unfortunately, video imaging microscopy cannot be performed on highly differentiated cultures grown at the air-liquid interface due to technical incompatibilities. Selection of different keratinocyte clones may be necessary to address the question of keratinocyte heterogeneity in the reaction to extracellular ATP.

A rise in $[Ca^{++}]_i$ is an early event in terminal differentiation of keratinocytes [30] that precedes morphologic changes of differen-

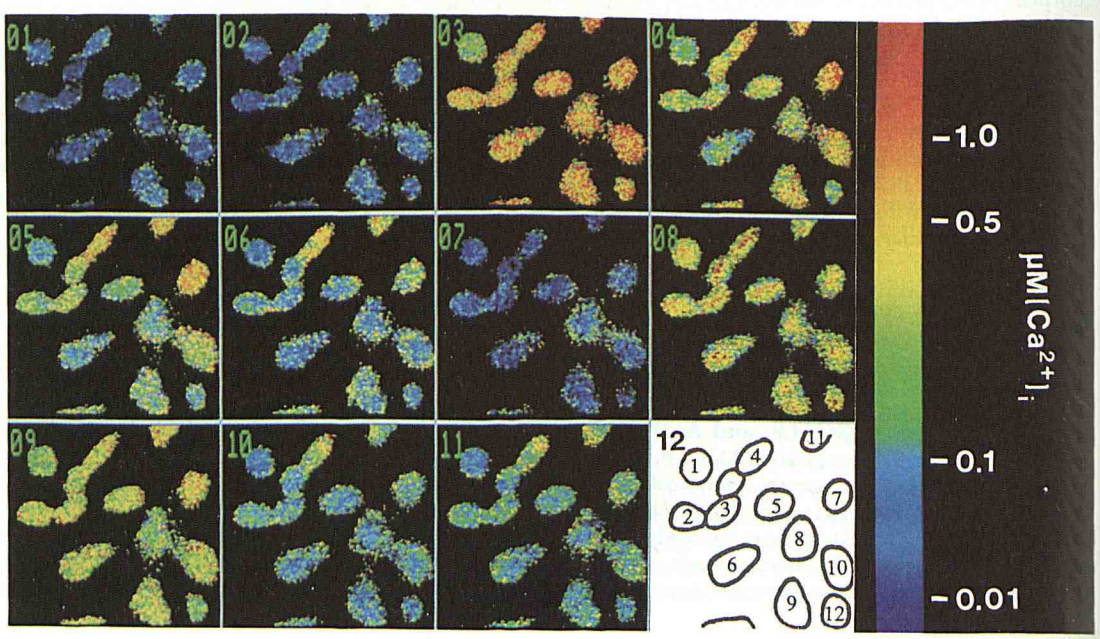

Figure 4. Homologous desensitization of ATP-induced rises in $[Ca^{++}]_i$ (same experiment as in *Fig 5*). Sequential ratio images of keratinocytes at different time points before *(1,2)* and after *(3–11)* stimulation with 100 μM extracellular ATP in the presence of 1.8 mM extracellular Ca^{++}. After washing of the chamber, cells were stimulated a second time with 100 μM ATP 126 sec after the first stimulation. *(1,2)* Images of $[Ca^{++}]_i$ resting levels taken 36 and 27 sec before addition of extracellular ATP; *(3–7)* 9, 18, 45, 81, and 117 sec after the first ATP addition, respectively, *(8–11)* 18, 27, 117, and 198 sec after second stimulation, respectively. *(12)* Cell map indicating position of cells from which data are shown in Fig 5.

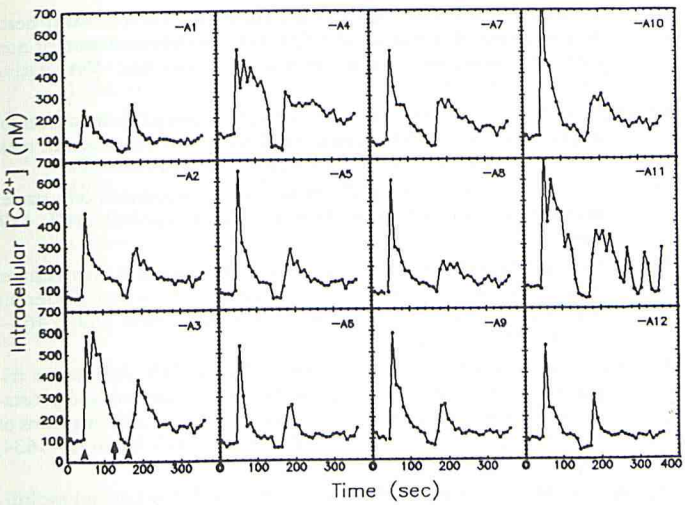

Figure 5. Homologous desensitization of ATP-induced rises in $[Ca^{++}]_i$ (same experiment as in *Fig 4*). The cell map in *Fig 4* corresponds to the cells shown in this figure. Changes in $[Ca^{++}]_i$ of 12 individual keratinocytes after treatment with 100 μM ATP in Ca^{++}-containing medium *(arrowhead)*, followed by washing with buffer after 81 sec *(arrow)*, and a second stimulation with 100 μM ATP *(arrowhead)*. Whereas the first stimulation induced six- to twelvefold initial increases in $[Ca^{++}]_i$, a second treatment with the same agonist resulted in peaks of only two- to fivefold resting levels.

tiation [9] and has been implicated as a crucial component to this process [10]. Extracellular ATP inhibits cornified envelope formation in human keratinocytes [31] and acts synergistically with other growth factors as a mitogen on A431 cells and mouse fibroblasts [32,33]. Extracellular ATP may also modulate keratinocyte growth by influencing other growth factors because it modulates EGF receptors on A431 epidermoid cells [28]. Exposure of A431 cells to low concentrations of ATP causes a loss of high-affinity EGF binding sites [28]. Extracellular ATP stimulates various early events in A431 cells that are reported to also occur as a result of treatment with conventional growth factors. These are increase in Ca^{++} efflux and influx, formation of inositol phosphate metabolites, and phosphorylation of the cytoskeletal protein ezrin [34]. In addition, ATP has recently been shown to increase levels of cellular diacylglycerol, stimulate the hydrolysis of phosphatidylcholine, and induce the release of arachidonic acid in these cells [35,36]. The ultimate biologic effect of extracellular ATP on keratinocytes in vivo is not known. The induction of $[Ca^{++}]_i$ increases by extracellular ATP is consistent with its playing a role in regulating keratinocyte growth and differentiation in vivo. A possible stimulatory function of extracellular ATP in wound repair has been proposed [32,33]. ATP and ADP are present in high concentration in platelets and may be released into the area of wounding along with polypeptide growth factors such as EGF, platelet-derived growth factor, and TGF α. In Balb/MK keratinocytes, extracellular ATP induces synergistic enhancement of DNA synthesis when combined with any of the polypeptides mentioned above [32]. ATP is also released into the extracellular space in the nervous system and at sensory nerve endings in the skin. These results are in contrast to findings reported by Harper et al [37], which suggested inhibition of keratinocyte growth by extracellular ATP. It should be noted that the culture medium of the experiments performed by Harper et al [37] contained 10% fetal calf serum, which is rich in ATPase, ADPase, and 5′-nucleotidase, and therefore it seems likely that this inhibitory effect of adenine nucleotides was in fact due to the formation of adenosine and not ATP. Such an inhibitory effect caused by formation of adenosine has been shown by Weisman et al [38]. In contrast, in the present paper, all experiments were performed in serum-free buffers that are free of ATPases. Furthermore, the response to extracellular ATP reported here is rapid and it seems unlikely that ATPase played a role in the process.

ATP mobilizes Ca^{++} from intracellular stores and also promotes entry of extracellular Ca^{++} in A431 cells [3,34]. The results obtained from experiments in which La^{+++} was used to block Ca^{++} influx in canine keratinocytes suggest that ATP-induced initial $[Ca^{++}]_i$ peaks are caused entirely by release of Ca^{++} from intracellular stores and are independent of influx of extracellular Ca^{++}. This has also been shown in human keratinocytes, in which transient rises in $[Ca^{++}]_i$ were identical in medium with or without calcium [31]. In canine keratinocytes, ATP induced smaller initial peaks and a subsequent decrease in $[Ca^{++}]_i$ below resting levels in experiments conducted in Ca^{++}-free medium. These results demonstrate an intracellular source of calcium for the ATP stimulus. Although the physiologic significance of a calcium-free environment may be questioned, such conditions offer important clues to the understanding of the mechanisms and dynamics of the calcium signal. The diminished response and the subsequent decrease in $[Ca^{++}]_i$ in the calcium-free medium may be due to rapid Ca^{++} efflux from the cells and to the rapid adaptation of the cells to the Ca^{++}-free environment. This drop of $[Ca^{++}]_i$ is most likely independent of ATP because it occurs in the absence of ATP if the $[Ca^{++}]_e$ is changed from a Ca^{++}-containing to a Ca^{++}-free medium. ATP accelerates this decrease of $[Ca^{++}]_i$ in response to lack of $[Ca^{++}]_e$. The ATP-induced release of $[Ca^{++}]_i$ from intracellular stores in the absence of a stimulated entry of Ca^{++} has been demonstrated in 3T6 cells [7]. This is in contrast to A431 cells in which extracellular ATP induces Ca^{++} efflux as well as greatly increases the rate of Ca^{++} entry within a few seconds [34]. The sensitivity of keratinocytes to $[Ca^{++}]_e$ changes is very high and response to extracellular ATP decreases dramatically in Ca^{++}-free medium to the point where it can be abolished. Keratinocytes appear to lose responsiveness to ATP if $[Ca^{++}]_i$ levels drop below 40 nM. This could represent a salvage signal when the concentration of $[Ca^{++}]_i$ is at an extremely low level that might endanger cell survival.

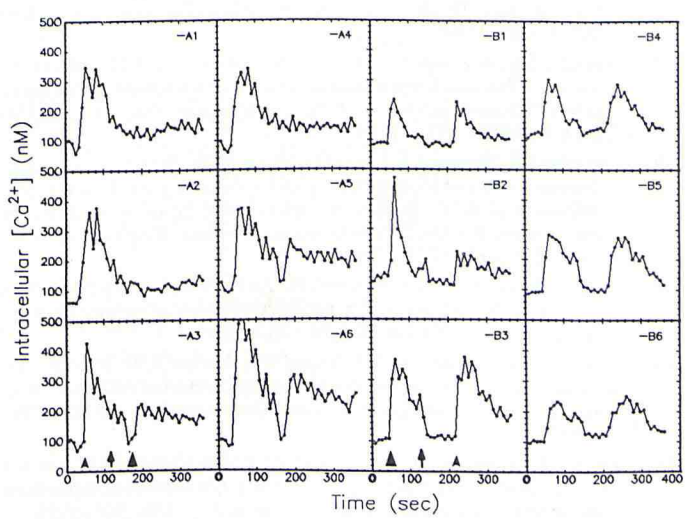

Figure 6. Heterologous desensitization of UTP-induced rises in $[Ca^{++}]_i$ with ATP *(cells A1–6)* and of ATP-induced rises in $[Ca^{++}]_i$ with UTP *(cells B1–6)*. *Cells A1–6,* Changes in $[Ca^{++}]_i$ of six individual keratinocytes after treatment with 100 μM ATP in Ca^{++}-containing medium *(arrowhead)*, followed by washing with buffer after 81 sec *(arrow)*, and a second stimulation with 100 μM UTP *(large arrowhead)*. In half the cells the second stimulation with UTP did not result in any $[Ca^{++}]_i$ rises *(cells A1, 2,* and *4)*, whereas the second half of the cells responded with a $[Ca^{++}]_i$ peak of up to half the height of the ATP stimulation *(cells A3, 5,* and *6)*. *Cells B1–6:* Reversed heterologous stimulation with a primary incubation with 100 μM UTP *(large arrowhead)* followed by a wash after 81 sec *(arrow)* and a subsequent stimulation with 100 μM ATP *(arrowhead)* after 171 sec did not inhibit a second equally high $[Ca^{++}]_i$ rise induced by ATP. Rare cells show a smaller second $[Ca^{++}]_i$ peak *(cell B2)*.

The present study demonstrates that ATP and some of its analogs induce transient rises in $[Ca^{++}]_i$ in canine keratinocytes grown in subconfluent cultures. This is suggestive of the presence of P_2 purinergic receptors in the cytoplasmic membrane of keratinocytes. The presence of a receptor-mediated mechanism is supported by the phenomenon of desensitization. In addition, the rapid response to AMP-PNP, a very poorly hydrolysable ATP analog, tends to rule out an ATPase or kinase mechanism. The fact that UTP was as effective as ATP is interesting. An ATP receptor acting best with ATP and UTP, and less well with ITP, has been described in other cell lines [21,22,27,34,39,40]. Extracellular ATP has a pronounced effect on $[Ca^{++}]_i$ in canine keratinocytes, which suggests that it may play an important role as a growth factor.

We thank Dr. Leon A. Heppel for his advice and critical review of the manuscript.

REFERENCES

1. Gordon JL: Extracellular ATP: effects, sources, and fate. Biochem J 233:309–319, 1986
2. Marks PW, Maxfield FR: Transient increases in cytosolic free calcium appear to be required for the migration of adherent human neutrophils. J Cell Biol 110:43–52, 1990
3. Gonzalez FA, Gross DJ, Heppel LA, Webb WW: Studies on the increase in cytosolic free calcium induced by epidermal growth factor, serum, and nucleotides in individual A431 cells. J Cell Physiol 135:269–276, 1988
4. De Young MB, Scarpa A: ATP receptor-induced Ca^{++} transients in cardiac myocytes: Sources of mobilized Ca^{++}. Am J Physiol 257:C750–C758, 1989
5. Nagelkerke JF, Dogterom P, De Bont HJGM, Mulder GJ: Prolonged high intracellular free calcium concentrations induced by ATP are not immediately cytotoxic in isolated rat hepatocytes. Changes in biochemical parameters implicated in cell toxicity. Biochem J 263:347–353, 1989
6. Hallam TJ, Pearson JD: Exogenous ATP raises cytoplasmic free calcium in fura-2loaded piglet aortic endothelial cells. FEBS Lett 207:95–99, 1986
7. Gonzalez FA, Rozengurt E, Heppel LA: Extracellular ATP induces the release of calcium from intracellular stores without the activation of protein kinase C in Swiss 3T6 mouse fibroblasts. Proc Natl Acad Sci USA 86:4530–4534, 1989
8. Gonzalez FA, Heppel LA, Gross DJ, Webb WW, Parries G: The rapid desensitization of receptors for platelet derived growth factor, bradykinin and ATP: Studies on individual cells using quantitative digital video fluorescence microscopy. Biochem Biophys Res Commun 151:1205–1212, 1988
9. Sharpe GR, Gillespie JI, Greenwell JR: An increase in intracellular free calcium is an early event during differentiation of cultured human keratinocytes. FEBS Lett 254:25–28, 1989
10. Hennings H, Kruszewski FH, Yuspa SH, Tucker RW: Intracellular calcium alterations in response to increased external calcium in normal and neoplastic keratinocytes. Carcinogenesis 10:777–780, 1989
11. Boyce ST, Ham RG: Calcium-regulated differentiation of normal human epidermal keratinocytes in chemically defined clonal culture and serum-free medium. J Invest Dermatol 81:33S–40S, 1983
12. Dale BA, Scofield JA, Hennings H, Stanley JR, Yuspa SH: Identification of fillagrin in cultured mouse keratinocytes and its regulation by calcium. J Invest Dermatol 81:90S–95S, 1983
13. Stanley JR, Yuspa SH: Modulation of the synthesis of pemphigoid and pemphigus antigens by extracellular calcium concentrations which regulate differentiation of epidermal cells. J Invest Dermatol 80:365, 1983
14. Al-Ani AM, Messenger AG, Lawry J, Bleehen SS, Macneil S: Calcium/calmodulin regulation of the proliferation of human epidermal keratinocytes, dermal fibroblasts and mouse B16 melanoma cells in culture. Br J Dermatol 119:295–306, 1988
15. Suter MM, Pantano DM, Augustin-Voss HG, Varvayanis M, Crameri FM, Wilkinson JE: Keratinocyte differentiation in the dog. Adv Vet Dermatol 1:252–264, 1990
16. Suter MM, Pantano DM, Flanders JA, Augustin-Voss HG, Dougherty E, Varvayanis M: Comparison of growth and differentiation of normal and neoplastic canine keratinocyte cultures. Vet Pathol 28:131–138, 1991
17. Millard PJ, Gross D, Webb WW, Fewtrell C: Imaging asynchronous changes in intracellular calcium in individual stimulated tumor mast cells. Proc Natl Acad Sci USA 85:1854–1858, 1988
18. Luckhoff A, Busse R: Increased free calcium in endothelial cells under stimulation with adenine nucleotides. J Cell Physiol 126:414–419, 1986
19. Pirotton S, Raspe E, Demolle D, Erneux C, Boeynaems J: Involvement of inositol 1,4,5-trisphosphate and calcium in the action of adenine nucleotides on aortic endothelial cells. J Biol Chem 262:17461–17466, 1987
20. Forsberg EJ, Feuerstein G, Shohami E, Pollard HB: Adenosine triphosphate stimulates inositol phospholipid metabolism and prostacyclin formation in adrenal medullary endothelial cells by means of P_2-purinergic receptors. Proc Natl Acad Sci USA 84:5630–5634, 1987
21. Kuroki M, Takeshige K, Minakami S: ATP-induced calcium mobilization in human neutrophils. Biochim Biophys Acta 1012:103–106, 1989
22. Greenberg S, DiVirgilio F, Steinberg TH, Silverstein SC: Extracellular nucleotides mediate Ca^{++} fluxes in J774 macrophages by two distinct mechanisms. J Biol Chem 263:10337–10343, 1988
23. Sung SSJ, Young JDE, Origilio AM, Heiple JM, Kaback HR, Silverstein SC: Extracellular ATP perturbs transmembrane ion fluxes, elevates cytosolic free $[Ca^{++}]$ and inhibits phagocytosis in mouse macrophages. J Biol Chem 260:13442–13449, 1985
24. Charest R, Blackmore PF, Exton JH: Characterization of responses of isolated rat hepatocytes to ATP and ADP. J Biol Chem 260:15789–15794, 1985
25. Sistare FD, Picking RA, Haynes RC Jr: Sensitivity of the response of cytosolic calcium in quin-2-loaded rat hepatocytes to glucagon, adenine nucleosides, and adenine nucleotides. J Biol Chem 260:12744–12747, 1985
26. Gilligan A, Prentki M, Glennon MC, Knowles BB: Epidermal growth factor-induced increases in inositol trisphosphate, inositol tetrakisphosphate, and cytosolic Ca^{++} in a human hepatocellular carcinoma-derived cell line. FEBS Lett 233:41–46, 1988
27. Dubyak GR, DeYoung MB: Intracellular Ca^{++} mobilization activated by extracellular ATP in Ehrlich ascites tumor cells. J Biol Chem 260:10653–10661, 1985
28. Hosoi K, Edidin M: Exogenous ATP and other nucleoside phosphates modulate epidermal growth factor receptors of A431 epidermoid carcinoma cells. Proc Natl Acad Sci USA 86:4510–4514, 1989
29. Gonzalez FA, Bonapace E, Belzer I, Friedberg I, Heppel LA: Two distinct receptors for ATP can be distinguished in Swiss 3T6 mouse fibroblasts by their desensitization. Biochem Biophys Res Commun 164:706–713, 1990
30. Yuspa SH, Hennings H, Tucker RW, Jaken S, Kilkenny AE, Roop DR: Signal transduction for proliferation and differentiation in keratinocytes. Ann NY Acad Sci 548:191–196, 1988
31. Pillai S, Bikle DD: Extracellular ATP increases intracellular calcium (Ca_i) and influences growth and differentiation of cultured human keratinocytes (abstr). J Invest Dermatol 94:566, 1990
32. Wang D, Huang N-N, Heppel LA: Extracellular ATP shows synergistic enhancement of DNA synthesis when combined with agents that are active in wound healing or as neurotransmitters. Biochem Biophys Res Commun 166:251–258, 1990
33. Huang N-N, Wang D, Heppel LA: Extracellular ATP is a mitogen for 3T3, 3T6, and A431 cells and acts synergistically with other growth factors. Proc Natl Acad Sci USA 86:7904–7908, 1989
34. Gonzalez FA, Alfonzo RG, Toro JR, Heppel LA: Receptor specific for certain nucleotides stimulate inositol phosphate metabolism and Ca^{++} fluxes in A431 cells. J Cell Physiol 141:606–617, 1990
35. Wang D, Huang N-N, Gonzalez FA, Heppel LA: Multiple signal transduction pathways lead to extracellular ATP-stimulated mitogenesis in mammalian cells. I. Involvement of protein kinase C-dependent and independent pathways in the mitogenic response of mammalian cells to extracellular ATP. J Cell Physiol (in press)
36. Huang N-N, Wang D, Gonzalez FA, Heppel LA: Multiple signal

transduction pathways lead to extracellular ATP-stimulated mitogenesis in mammalian cells. II. A pathway involving arachidonic acid release, prostaglandin synthesis, and cyclic AMP accumulation. J Cell Physiol (in press)

37. Harper RA, Flaxman BA, Chopra DP: Effect of pharmacological agents on human keratinocyte mitosis *in vitro*. I. Inhibition by adenosine nucleotides. Proc Soc Exp Biol Med 146:1032–1036, 1974
38. Weisman GA, Lustig KD, Lane E, Huang N, Belzer I, Friedberg I: Growth inhibition of transformed mouse fibroblasts by adenine nucleotides occurs via generation of extracellular adenosine. J Biol Chem 263:12367–12372, 1988
39. Davidson JS, Wakefield IK, Sohnius U, Van der Merwe PA, Millar RP: A novel extracellular nucleotide receptor coupled to phosphoinositidase-C in pituitary cells. Endocrinology 125:1989
40. Lustig KD, Sportiello MG, Erb L, Weisman GA: Consequences of P_{2Y} purinoceptor activation by ATP^{4-} in cultured endothelial cells (abstr). In: Biological Actions of Extracellular ATP. Meeting of the NY Academy of Science, Philadelphia, November 27–29, 1989

ANNOUNCEMENT

An international congress, "Dermatology 2000," will be held in Vienna, May 18–21, 1993, at the University of Vienna, Department of Dermatology.

Chairmen: Congress and Scientific Committee Chairman, Professor Klaus Wolff; Congress Vice Chairman, Professor Georg Stingl; Organising Committee Chairman, Professor Peter Fritsch.

Scientific Committee: Pavel Bartak (Prague), Martin Black (London), Ruggero Caputo (Milan), Enno Christophers (Kiel), Attila Dobozy (Szeged), Peter Dukor (Vienna), Louis Dubertret (Paris), Ervin Epstein (San Francisco), Malcolm Greaves (London), Yasumasa Ishibashi (Tokyo), Stephania Jablonska (Warsaw), Stephen Katz (Bethesda), José Mascaro (Barcelona), Constantin Orfanos (Berlin), Hans Rorsman (Lund), Jean-Hilaire Saurat (Geneva), Sam Shuster (Newcastle), John Stratigos (Athens), Hachiro Tagami (Sendai), Willem van Vloten (Utrecht).

The Scientific Programme will be designed to inform practising dermatologists about the potential impact of science on the future of their practice.

There will be 18 plenary lectures, 6 lunch-time sessions, 24 interactive Symposia, and 12 teaching symposia.

Topics of plenary lectures include somatic gene therapy for dermatology; new approaches to treatment of skin cancer; targeted drug delivery systems; dermatological surgery: a trend towards refinement; regulation of wound healing by biological response modifiers; modulation of keratinization; new micro-organisms — new diseases; targeted immunosuppression; mechanisms of skin allergy: an answer at last?; environmental dermatology: a slogan or reality?; neuropeptides/neurotransmitters/neurohormones: the neurocutaneous network — new mechanisms of skin disease; photosensitization — refinement for the future; cyclosporin, FK506, rapamycin and further down the road; plus other topics to be announced.

Areas covered by interactive and teaching symposia include psoriasis, melanoma, melanoma precursors, atopic dermatitis, contact dermatitis, acne, lymphoma, autoimmune disease, genodermatoses, fungal and parasitic infections, neonatal and paediatric dermatology, skin ageing, hair loss, drug eruptions, viral exanthems, intensive care dermatology, lasers, bullous diseases, STD, HIV infection, photoimmunology, herpes viruses, HPV, non-melanoma skin cancer, plus other topics to be announced.

Latest topics to be covered in "Controversies and Challenges" and "Hotline" sessions. Faculty includes over 100 internationally-recognized experts from all aspects of dermatology and cutaneous biology. Poster presentations will form an important and integral part of the overall programme — opportunities to present results in open forum.