

Ph. D. THESIS

**REGULATION OF INTRACELLULAR CALCIUM
HOMEOSTASIS IN HUMAN IMMORTALISED KERATINOCYTES**

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INTRODUCTION

The skin is one of the most important organs in our body in detecting stimuli coming from the environment and also in protecting against injurious substances and effects. It is composed of 3 distinct parts, the outer epidermis, the middle dermis and the inner subcutis, from which the epidermis is a cornified surface epithelium with 5 layers. Every layer of the epidermis is formed by specialised cells, called keratinocytes, that have distinct proliferation and differentiation properties in each and every strata.

In addition to the barrier function they perform as epidermal cells, keratinocytes are also immunologically active, they synthesise a number of cytokins (e.g. interleukins). Keratinocytes can present certain antigens (e.g. MHC-II) in their surface membrane, can migrate and phagocytose which is very important in the defence against infectious agents. These cells are essential in healing after wounds, but participate in hyperproliferative skin diseases (e.g. psoriasis) and.

A wide scale of signaling pathways are present in keratinocytes. Adenosine-trisphosphate (ATP) and bradykinin activate the inositol-triphosphate (IP_3) pathway, histamine exerts its effects through cyclic-adenosine-monophosphate (cAMP) while NO and arachidonic-acid increase the intracellular cyclic-guanosine-monophosphate (cGMP) concentration. There are receptors (EGF-receptor, $TGF\alpha$ -receptor or the receptors for cytokins) in the plasma membrane of keratinocytes that have tyrosine-kinase activity.

Purinoreceptors

ATP is known to be released as a cotransmitter in neuronal synapses (in the gastrointestinal tract, smooth muscle, endocrine and exocrine glands, sympathetic and parasympathetic neurons). In the epidermis the source of extracellular ATP is mainly the keratinocytes that die during injury. In keratinocytes ATP activates the phospholipase C (PLC) enzyme through specific P_{2Y} purinoreceptors and increases the concentration of IP_3 and diacyl glycerol. IP_3 initiates the release of calcium from the endoplasmic reticulum (ER) and the intracellular calcium concentration ($[Ca^{2+}]_i$) rises. The changes of $[Ca^{2+}]_i$ play an important regulatory role in cell function since transient increases in $[Ca^{2+}]_i$ are believed to induce growth and proliferation while prolonged elevations are thought to result in differentiation. Furthermore, the increased $[Ca^{2+}]_i$ will in turn

activate calcium-dependent enzymes (e.g. protein kinase C, PKC) and thus result in changes in the metabolic processes of the cells, in the transcription of genes and in the activity of certain membrane receptors and channels.

Properties and functions of the Protein Kinase C system in keratinocytes

Protein kinase C comprises a family of serine/threonine kinases. Up to date, at least 11 different isoenzymes have been identified, which can be classified into the groups of calcium- and phorbol ester-dependent 'classical' (PKC α , β I, β II, and γ ; cPKC), the calcium-independent 'novel' (PKC δ , ϵ , η , and θ ; nPKC), the calcium- and phorbol ester-independent 'atypical' (PKC ζ and $\lambda/1$; aPKC) isoforms, and the unique PKC μ . These isoforms possess a characteristic expression pattern in a given cell type, and isoenzyme-specifically regulate various cellular processes. Not only may some PKC isoforms be activated whereas others not for a given response, but different PKC isoenzymes may often have antagonistic effects on the same cellular events suggesting differential roles of specific PKC isoforms.

Emerging evidence suggest a pivotal role of PKC in the regulation of the proliferation and differentiation of keratinocytes. The PKC expression levels and the subcellular localization of certain isoforms (representing changes in their activity) are altered with calcium- and high cell density-induced differentiation. On the other hand, how PKC regulates the proliferation and differentiation, the function of cell membrane receptors and ionic channels, and the calcium homeostasis are not yet clear.

Store Operated Channels (SOC)

An increase in intracellular calcium concentration is associated with either the release of calcium from internal stores following the activation of specific receptors (e.g. P_{2Y} in keratinocytes), or the influx of calcium from the external medium through specific or non-specific ion channels. The latter channels could be ligand gated (e.g. P_{2X} purinergic receptor), voltage dependent, or sensitive to the changes of the intracellular environment. One of the most important calcium influx pathway on keratinocytes, the store operated channels (SOC), belong to this latter group, and are regulated through changes in the calcium content of the ER.

Recent work from our department has demonstrated SOC-s on keratinocytes. However, very little is known about the function and the possible regulation of these channels by PKC.

Mechanosensitive channels

The presence of mechanosensitive channels (MSC-s) in their surface membrane is a universal property of cells since mechanosensitivity plays an essential role in a number of physiological processes. MSC-s respond to membrane stress by changes in open probability, they may either be stretch-activated (SAC-s) or stretch-inactivated channels (SIC-s). The channels can be classified into 5 groups: SA cation channels, SA potassium channels (SAKs), SA anion channels, SA nonselective channels and Stretch-Inactivated channels.

The skin is almost continuously under mechanical stress. Previous reports have documented both cation- and anion-selective channels in keratinocytes with the latter being activated by osmotic stress. It is, therefore, of interest how changes in mechanical stress might influence the cellular processes in keratinocytes.

Oxidative agents, NO and peroxynitrite

Reactive oxidative compounds have recently emerged as an important mediators of various physiological functions and pathophysiological conditions. These factors play an essential role during growth, regulate the synthesis of different enzymes, transcription- and growth-factors and result in apoptosis in certain cases. Reactive oxygene is synthetised in the cell membrane, in the mitochondria, in the endoplasmic reticulum and in the peroxisome.

Peroxynitrite (ONOO^-), a potent oxidant, is formed in a near diffusion-limited reaction of superoxide (O_2^-) and nitric oxide (NO) and can directly oxidise some sulfate-groups of proteins. Although the biological half-life of peroxynitrite is short ($<0,1$ s), it has profound effects on keratinocytes by inducing apoptosis and necrosis. Nevertheless, the cellular effects of peroxynitrite (PN) on keratinocytes and on other skin-derived cell types is not well understood, but the activation of certain enzymes (poli(ADP-ribóz)-polymerase; PARP) and decreases in the concentration of cytoplasmic NAD^+ and ATP could be important in cytotoxic effects of PN.

OBJECTIVES

In our experiments we wanted to examine the properties of the purinergic signalling pathway in preconfluent –proliferating - and confluent –differentiating – HaCaT keratinocytes. In addition we also wished to study the regulatory role of protein kinase C in these processes. Since the release of calcium from the internal stores plays an important role in cell proliferation and differentiation, we first decided to describe the function of the store operated channels and their regulation through phosphorylation.

In a next set of experiments we wished to examine the mechanosensitive channels found in the surface membrane of keratinocytes. We were interested which MSC-s are present in keratinocytes, whether these cells are activated by hypotonic stress and can the PKC system alter their function and the transmembrane currents. It was also important to test if the intracellular calcium concentration was altered by the hypotonic solutions and would this altered $[Ca^{2+}]_i$ change the proliferation and differentiation of these cells.

Finally we examined the effects of oxidative compounds (PN and SIN-1) on the regulation of intracellular calcium homeostasis. We wished to test how would the different degree and duration of the $[Ca^{2+}]_i$ increase affect the apoptotic and necrotic effects of the oxidative compounds.

MATERIALS AND METHODS

Cell culture

HaCaT keratinocytes were continuously cultured in an endotoxin-free modified Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine and 10 μ l/ml antibiotic, antimycotic solution. Cells were plated on coverslips and incubated in DMEM at 37°C in 5% CO₂ before further study. To elucidate the effects of PKC keratinocytes were treated with 100 nM phorbol 12-myristate 13-acetate (PMA) for 16 h (overnight).

Some experiments were carried out on presheet cultures, while in another series of experiments cells were kept in culture until more than 70% confluence was reached. Under these conditions cells were still in a monolayer, but formed large "islands", consisting of usually more than 100 cells.

Intracellular calcium measurement

To introduce the calcium sensitive probe into the cytosol, cells were incubated with 5 μ M Fura-2 AM for 1 hour at 37°C. Before each measurement keratinocytes were kept at room temperature in normal Tyrodes' solution to allow constant distribution of the dye in the cytoplasm. The coverslips, containing the Fura-2 loaded cells, were then placed on the stage of a Nikon-Diaphot inverted fluorescent microscope. Excitation wavelength was altered between 340 and 380 nm by dual wavelength monochromator and an on-line connected microcomputer while the emission was monitored at 510 nm using a photomultiplier at an acquisition rate of 10 Hz per ratio. $[Ca^{2+}]_i$ levels were calculated from the ratio ($R = F_{340}/F_{380}$) of the fluorescence intensities measured with excitation wavelength being 340 (F_{340}) and 380 nm (F_{380}) as described in our earlier reports using *in vivo* calibration data. During the measurements the optical background fluorescence was determined on cell-free parts of the coverslips.

During the experiments we used a normal Tyrodes' solution and a calcium free Tyrodes' solution. Calcium free external solution had no added calcium and 1 mM EGTA. Hypotonic solutions (TY75 and TY50) had 25% or 50% lower NaCl as compared to the normal solution. The oxidative reagent peroxynitrite was dissolved in glucose- and Hepes- free Tyrodes' solution (pH 11), SIN-1 was applied in a similar vehicle (pH 7.5) immediately before measuring.

Membrane potential recordings with conventional microelectrodes

The membrane potential was recorded using conventional microelectrodes pulled from borosilicate glass capillaries and filled with 3 M KCl and had a resistance of 20-30 MΩ. To avoid changing the Cl⁻ concentration, the solution was exchanged to a modified Tyrodes' solution (TY) where half of the NaCl was exchanged to Na-glutamate before a hypotonic challenge. Hypotonic solutions had part of the Na-glutamate removed and had an osmolality approximately 75% (TY75) or 50% (TY50) of the original Tyrodes' solution. During the experiments cells were continuously superfused with either the modified Tyrodes' solution (TY) or with one of the hypotonic solutions (TY75 or TY50). Electrical signals were digitised at 1 kHz and recorded using Axoclamp 2B. Data were analysed using the pClamp 6.0.4. software.

Voltage- and current clamp measurements

Cells were voltage clamped in the whole-cell mode of the patch clamp technique using an Axopatch 200A amplifier. Pipettes were again pulled from borosilicate glass capillaries, but had resistances 2-3 MΩ when filled with an artificial internal solution, containing K-aspartate. The membrane potential changes were examined in current clamp mode. Passive electrical parameters of the cells were determined with 5 mV pulses of 40 ms duration. The holding potential was set to -40 mV. The linear capacitance of the cells was between 20 and 40 pF.

Western blot analysis

Cells after 70% confluence were washed with ice-cold phosphate-buffered saline (PBS), harvested in homogenisation buffer and disrupted by sonication on ice. Protein content of samples was measured by a modified BCA protein assay. Total cell lysates were mixed with SDS-PAGE sample buffer and boiled for 10 min at 100°C. The samples were subjected to SDS-PAGE according to Laemli (8% gels were loaded with 20-30 µg protein per lane) and transferred to nitrocellulose membranes. Membranes were then blocked with 5% dry milk in PBS and probed with the appropriate, monoclonal rabbit primary antibodies against different PKC isoenzymes (PKCα, ε, η, δ, β és ζ) and the keratinocyte differentiation marker involucrin. Peroxidase-conjugated goat anti-rabbit IgG antibodies were used as secondary antibodies, and the

immunoreactive bands were visualised by an ECL western blotting detection kit on light sensitive films.

Determination of the cellular proliferation

Cellular proliferation was determined by measuring the conversion of the tetrazolium salt MTT to formazan. Cells were plated in 96-well multititer plates (5000 cell/well density) in quadruplicates and were treated with different culturing media for the indicated times. Cells were then incubated with 0.5 mg/ml MTT for 3 hours, and the concentration of the formazan crystal (as the indicator of number of viable cells) was determined colorimetrically according to the manufacturer's protocol. When hypotonic stress was tested on cell proliferation/differentiation three different solution were used. Hypotonic culturing solution (LS50) was prepared by adding distilled water to NS to reach a final osmolarity as for TY50. This decreased the serum content of the medium. A low-serum culture medium (LS) was also used as a control. LS was prepared as LS50 but instead of distilled water Tyrodes' solution was used for supplementing NS. LS was thus isotonic and it had the same low serum content as LS50.

Immunofluorescent measurements

Cells were washed with ice-cold phosphate-buffered saline (PBS), fixed with ethanol 5 minutes and then the reaction was applied with a specific blocker solution containing 0,6% Triton X-100 and 1% BSA for 30 minutes. The fixed cells were treated with specific monoclonal rabbit primary antibodies against different PKC isoenzymes and purinoreceptors for 2 hours, and with secondary goat FITC-conjugated antibodies for 1 hour. Samples were then incubated with propidium-iodid for 1 hour to label the nuclei. The images of labelled keratinocytes were measured with fluorescence microscope.

RESULTS

Effect of protein kinase C on transmembrane calcium fluxes in HaCaT keratinocytes

In human keratinocytes the activation of the phosphatidyl-inositol (PI) pathway by ATP or the emptying of the intracellular calcium stores by cyclopiazonic acid (CPA) have been shown to activate calcium influx through SOC. It is, however, not clear if the changes in PKC activity or the proliferation and differentiation properties of these cells are able to modify the capacitative calcium influx.

Effect of PMA pretreatment in calcium influx

Repeated activation of the PI pathway with 30s long applications of ATP resulted in transient changes in $[Ca^{2+}]_i$ that showed only slight decrease in amplitude. The resting calcium concentration ($[Ca^{2+}]_{rest}$) returned to essentially the same value after the end of the ATP-evoked calcium transients as revealed by the negligible change in $[Ca^{2+}]_{rest}$ ($\Delta[Ca^{2+}]_{rest}$). Removing calcium from the extracellular medium suppressed but did not abolish the ATP-evoked change in $[Ca^{2+}]_i$. This indicated that both a release from the intracellular stores (endoplasmic reticulum, ER) and an influx from the external environment contributed to the formation of the calcium transients. Overnight pretreatment of the cells with PMA neither altered $[Ca^{2+}]_{rest}$ (79 ± 4 vs. 80 ± 4 nM, in control and in PMA pretreated cells, respectively; $n=20$ for both groups) nor did it influence the overall kinetics of the calcium transients. These observations suggested that the changes of the PKC activity do not play a direct regulatory role in $[Ca^{2+}]_i$ and the PI pathway.

The average elevation in $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$) was, however, significantly greater – except for the first transient- in treated cells. This was accompanied by a substantial increase (>20 nM on average) in the resting $[Ca^{2+}]_i$ following every calcium transient. In the absence of external calcium $\Delta[Ca^{2+}]_{rest}$ was a substantially reduced, the calcium concentration returned to the original level after each transient as it did in control cells. These observations suggested that the continuous increase in resting $[Ca^{2+}]_i$ seen on PMA pretreated cells under normal external conditions was predominantly due to an influx of calcium from the extracellular space.

CPA-induced calcium influx in control and PMA-treated keratinocytes

To test the function of store operated channels the intracellular stores were emptied using the calcium ATP-ase blocker cyclopiazonic acid in the absence of external calcium. The addition of CPA (10 μ M) caused a transient elevation in $[Ca^{2+}]_i$ both in control and in PMA pretreated cells. Both the average increase in $[Ca^{2+}]_i$ and the maximal rate of rise of the CPA-evoked calcium transients were essentially the same in the two populations. These observations further strengthened that PMA did not influence the amount of calcium stored in the ER. $[Ca^{2+}]_i$ returned close to a resting level in the continuous presence of CPA indicating the complete emptying of the ER. The re-administration of calcium into the external solution caused an elevation in $[Ca^{2+}]_i$ both in control and in PMA pretreated cells as expected from the SOC-s being open. The rate of increase was, however, greater by 47% in PMA pretreated cells indicating that the SOC-s had been activated by the PMA treatment.

Effect of PMA treatment to the PKC isoenzyme pattern in preconfluent cells

To elucidate which PKC isoenzyme(s) might be responsible for the above described effects of PMA, Western-blot analysis of control and PMA pretreated keratinocytes was carried out. As expected from previous studies, HaCaT keratinocytes expressed 6 types PKC isoforms: the classical PKC α and β , the novel δ , ϵ and η , and the atypical ζ . In preconfluent cells an overnight incubation with PMA resulted in the selective and significant downregulation of PKC α , whereas the overall expression of the other isoforms was not affected by the phorbol ester treatment.

Confluent cells have altered calcium entry through store-operated channels

The experiments described above were repeated on HaCaT cells forming a monolayer on the coverslips. Repeated application of ATP onto control keratinocytes caused calcium transients with gradually declining amplitude as it did on isolated cells. The rapid rise seen in isolated cells was preceded by a slow phase. $[Ca^{2+}]_{rest}$ was not different in preconfluent as compared with confluent cells. The amplitude of ATP-evoked calcium transients were greater in confluent cells (155 $\pm$ 19 nM) than in proliferating keratinocytes (116 $\pm$ 8 nM). An overnight treatment of the cells with PMA, again, neither altered $[Ca^{2+}]_{rest}$ nor affected the kinetics of the ATP-evoked calcium

transients, it even left the initial slow phase intact, and the $\Delta[\text{Ca}^{2+}]_{\text{rest}}$ was completely absent.

The CPA-induced $[\text{Ca}^{2+}]_i$ elevations and their characteristic parameters in control and PMA pretreated confluent cells were similar to those measured on preconfluent cells indicating that reaching confluence did not, dramatically, alter these stores. On the other hand, examining the calcium influx through SOC-s in confluent cells revealed a characteristic alteration in their kinetics. In contrast to the continuous rise observed on preconfluent cells, $[\text{Ca}^{2+}]_i$ on confluent cells reached an early peak and then it began to decline. Calcium influx must have ceased or was drastically reduced, indicating either a desensitisation or a strong inactivation of the influx pathway. It should also be noted that the initial rate of rise was somewhat larger in confluent as compared with non-confluent cells. Calcium influx with similar kinetics and amplitude were observed in PMA pretreated cells further strengthening the idea that PMA has little effect on SOC-s in confluent cells.

Altered effect of PMA on PKC isoenzyme pattern in confluent keratinocytes

Overnight treatment of the confluent keratinocytes with PMA had a characteristically different effect on the isoenzyme pattern than it did on preconfluent cells. In confluent cells both PKC β and PKC ϵ were downregulated. These observations suggest a direct role of PKC α in the regulation of available SOC in HaCaT keratinocytes. They also imply a possible role for other isoemzymes (β and ϵ) in the modulation of the kinetics of these channels.

Stretch-activated channels influence the membrane potential and alter the proliferation of HaCaT keratinocytes in vitro

Effect of hydrostatic pressure on electrophysiological properties of the keratinocytes

During the course of the experiments a correlation was found between the actual membrane potential (MP) of the cells and the amount of solution in the bath. To quantify this observation we changed the level of the solution in the recording chamber, from a starting value of 3 mm to 3 cm, while continuously monitoring the membrane potential of the cells under current clamp. Together with the increase in solution level the cell became hyperpolarised, from a starting value of $-20 \pm 6,5$ mV to $-55 \pm 12,4$ mV.

Upon the removal of solution opposite changes were obtained, the membrane potential of the cells became depolarised. Due to these observations all experiments were carried out with solution levels at or below 5 mm.

Hypotonic solutions and membrane potential

Under our standard conditions the MP of HaCaT cells was found to be -29.4 ± 3.8 mV as measured with the conventional microelectrode in modified Tyrode's solution (n=10). Upon the addition of a hypotonic solution (TY75) the membrane potential increased and reached a new, more negative, stable value. Further increasing the stress, by applying a more hypotonic solution (TY50), caused a further hyperpolarisation, which again reached a new stable value. The TY75 solution caused a 9.2 ± 1.3 mV while the TY50 solution a 27.2 ± 5.2 mV shift towards more negative membrane potentials.

Similar observations were made using the whole-cell mode of the patch clamp technique. The resting MP measured under these condition in modified TY solution, was -19.7 ± 2.3 mV, not significantly different from that reported above. Upon changing the solution to TY50 the cells became hyperpolarised, however, the actual potential change was somewhat smaller (-11.4 ± 0.5 mV).

Protein kinase C isoenzymes play a crucial role in the regulation of keratinocyte proliferation and differentiation. We tested if the alteration of PKC might influence the response of HaCaT cells to hypotonic stress. Although the resting membrane potential of the PMA treated cells (-20.4 ± 2.8 mV) was not different statistically from that of the control cells, the application of the hypotonic solution, TY50, brought about a larger hyperpolarisation, to -38.7 ± 3.4 mV.

Membrane currents are altered by hypotonic stress

To describe the currents activated by a hypotonic challenge, membrane currents were measured using the whole-cell mode of the patch clamp technique. Cells were voltage clamped to -40 mV and subjected to a pulse protocol consisting of a brief hyperpolarisation to -80 mV and then a 600 ms long depolarisation to various membrane potentials in TY and TY50 solutions. The current measurements were carried out on the same cells on which the membrane potential measurements. The total membrane current (I_{tot}) in hypotonic solution, which is calculated as the difference

between the values at the end of the depolarising step and at the end of the prepulse to -80 mV, was greater than in TY solution. To describe this current the capacitive and ohmic components were subtracted from the current traces and presented as depolarisation evoked responses ($I(V_m)$). Control and PMA pretreated cells had essentially identical currents in Tyrode's solution. Following the hypotonic challenge $I(V_m)$ became larger with the effect being more pronounced for the PMA pretreated cells. Cell capacitance was monitored throughout the experiment and were found to be essentially identical for the control (27.2 ± 2 pF, $n=31$) and the PMA pretreated cells (27.9 ± 2.6 pF, $n=16$). Following the hypotonic challenge the cell capacitance increased (28.6 ± 2.6 pF) indicating a slight, but not significant increase in cell volume.

The removal of chloride slightly but not significantly shifted the resting potential of keratinocytes to depolarised values (-14.2 ± 2.8 mV, $n=6$). The addition of chloride free TY50 caused a hyperpolarisation to -19.4 ± 3.9 mV, which was significantly smaller than the change in chloride containing solutions. The removal of chloride essentially eliminated the increase in $I(V_m)$ following the hypotonic challenge at all membrane potential tested. These observations suggested an important role for chloride ions in hypotonic solution-evoked responses.

Effect of hypotonic stress in intracellular calcium concentration

Since the activation of MSC-s is often accompanied by a rise in $[Ca^{2+}]_i$ we tested if hypotonic solutions would induce an alteration in $[Ca^{2+}]_i$. We measured the hypotonic solution- and the phospho-inositide agonist, ATP-induced calcium transients.

The measured changes in $[Ca^{2+}]_i$ could be classified into two groups. Upon the application of the hypotonic solution $[Ca^{2+}]_i$ became elevated, and this elevation was either transient, it reached a definite peak and then declined in the continuous presence of the hypotonic solution (termed "transient"), or it was prolonged, lasting as long as the hypotonic solution was applied (termed "slow"). Transient responses were readily detectable for both TY75 and TY50 solutions with the larger change in tonicity causing a larger elevation in $[Ca^{2+}]_i$. The slow responses were only seen when the TY50 solution was applied, that is, the TY75 solution failed to induce a slow change in $[Ca^{2+}]_i$ on normal HaCaT cells. The amplitude of the calcium transient was, on average, larger on PMA pretreated than on control cells in line with the data obtained in the electrophysiological studies. Moreover, on PMA pretreated cells already the TY75

solution was capable of initiating a detectable slow type response. Both on control and on PMA pretreated cells the elevation in $[Ca^{2+}]_i$ induced by the hypotonic stress critically depended on the presence of external calcium. The removal of calcium from the external medium essentially prevented the formation of a calcium transient during the hypotonic challenge. The observed changes in $[Ca^{2+}]_i$ suggest that certain calcium-activated channels might also play some role in the hypotonic medium induced answers. These data clearly establish that a hypotonic challenge is followed by an elevation of $[Ca^{2+}]_i$, the amplitude but not the time course of which depends on the pretreatment with PMA.

Hypotonic stress alters the proliferation and differentiation of keratinocytes

HaCaT cells if continuously cultured in hypotonic solution (LS50) did not proliferate at all. This was not due to the partial serum removal since cell proliferation in isotonic solution with low serum content (LS) was continuous, although reduced compared to that of the normal culture medium (NS). In parallel with the reduced proliferation rate, cell differentiation, as assessed by measuring the amount of the terminal differentiation marker involucrin at day 10, was increased. A sudden hypotonic stress (when LS was exchanged to LS50) was accompanied by an increase in proliferation rate. These observations suggest that the cells under continuous hypotonic conditions stop at a certain phase of the cell-cycle (as indicated by the failure to proliferate or differentiate), but a sudden hypotonic stress induces cell proliferation and suppresses differentiation.

Intracellular calcium dependent signalling regulate oxidative stress sensitivity of HaCaT keratinocytes

Effects of peroxynitrite on the calcium influx in keratinocytes

The application of 10 μ M peroxynitrite caused a dramatic increase in $[Ca^{2+}]_i$ that was usually not reversible, or only slightly so, after its removal. The findings point to a membrane perturbing effect of peroxynitrite causing sustained influx of calcium. The application of the vehicle solution or decomposed PN did not cause any measurable elevation in $[Ca^{2+}]_i$.

Effects of SIN-1 in the intracellular calcium homeostasis of HaCaT keratinocytes

Other oxidative compounds (SIN-1, NO) also affected $[Ca^{2+}]_i$. SIN-1 was applied in concentrations 1, 2.5 and 5 mM, in the presence and absence of extracellular calcium. To monitor the loading of the internal stores the well-known PI activator ATP (180 μ M, 20 s) was administered before and after the 300 s long application of SIN-1. In the presence of external calcium SIN-1 caused a concentration-dependent, marked increase in $[Ca^{2+}]_i$. The amplitude of the ATP-evoked calcium signal was essentially the same in each SIN-1 concentration. When calcium was removed from the extracellular solution SIN-1 was still capable of causing an elevation in $[Ca^{2+}]_i$. This increase was, however, much less pronounced than in normal external calcium. In the absence of external calcium the ATP-evoked calcium transient following SIN-1 application was markedly reduced in amplitude as compared to that obtained before the application. Higher concentrations of SIN-1 caused greater reduction in the amplitude of the ATP-induced calcium signal. These data clearly indicate that SIN-1 was capable of both mobilising calcium from the internal stores and to induce an influx of calcium through the surface membrane.

DISCUSSION

The present study investigated the regulation of calcium homeostasis, transmembrane calcium currents, proliferation, differentiation in HaCaT keratinocytes.

These cells, just as a number of other cells that employ the PI pathway for increasing cytosolic $[Ca^{2+}]_i$, exhibit SOC activity. The overnight treatment with the general PKC activator PMA caused a specific alteration in the isoenzyme pattern by markedly downregulating PKC α and hardly affecting other isoenzymes in preconfluent cells. PMA pretreatment resulted in an elevated calcium influx. These observations imply that the phosphorylation state of the channel could be linked to its function. The results of the experiments with cyclopiazonic acid in which the rate of calcium influx was 47% greater in PMA-treated cells, confirmed this idea. Examining the calcium influx through SOC-s in confluent cells revealed a characteristic alteration in its kinetics, indicating either a desensitisation or a strong inactivation of the influx pathway. A prolonged PMA treatment downregulated the PKC β and PKC ϵ isoenzymes in confluent cells, but did not modify the kinetics or the amplitude of the calcium influx. These results suggested that PMA has little effect on SOC-s in confluent cells.

Mechanical forces are believed to induce proliferation and/or differentiation of keratinocytes. HaCaT cells have mechanosensitive channels in their surface membrane. Our results suggest that mechanical stress, through the activation of MSC-s cause marked and reversible alteration in the membrane potential (hyperpolarisation) and in the intracellular calcium concentration on keratinocytes. Changes in the hydrostatic pressure induced similar alterations in the membrane potential. The above changes were due, mainly, to the activation of chloride channels. The hypotonic solution mediated $[Ca^{2+}]_i$ increase depended on the presence of external calcium. These observations suggest that certain calcium-activated channels also play some role in the response to mechanical stress. The activation of PKC with PMA resulted in an increased responsiveness to hypotonic stress, as demonstrated by the augmented chloride current. Continuous culturing of the cells in a hypotonic solution was found to suppress cell proliferation. On the other hand, a sudden hypotonic stress was accompanied by an increase in proliferation rate and a reduction of the production of involucrin. These observations suggest that the changes in rather than sustained mechanical forces play a crucial role in the regulation of keratinocyte proliferation.

Production of cytotoxic compounds during inflammatory processes induce apoptosis and necrosis through changing the calcium homeostasis of the cells. Peroxynitrite and SIN-1 were found to cause a marked increase in intracellular calcium concentration in HaCaT keratinocytes. The effects of SIN-1 were reversible. Both the release from the internal stores and the influx from the extracellular space were parts of the response to SIN-1. PN, on the other hand, caused prolonged and irreversible changes, triggering the continuous influx of $[Ca^{2+}]_i$ by decreasing the integrity of the cell membrane.

***In extenso* articles that provide the basis of the thesis:**

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* Edina Bakondi és Mónika Gönczi contributed equally to the work.

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H. Papp, G. Czifra, J. Lázár, J. Boczán, **M. Gönczi**, L. Csernoch, L. Kovács, T. Bíró: Protein kinase C isoenzymes regulate proliferation and high cell density-mediated differentiation in HaCaT keratinocytes. *Exp. Dermatol.* 2003: 12:1-14. **IF: 2,234**

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