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ORIGINAL PAPER

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Calcium entry into keratinocytes induces exocytosis of lysosomes

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Abstract During the final steps of epidermal differentiation, extracellular calcium ions enter keratinocytes and induce transglutaminase activity and cornified envelope formation. In other cell types, entry of calcium mediated by ionophores has been reported to induce exocytosis of lysosomes. In this study, we investigated whether lysosomes of keratinocytes might exhibit a similar behaviour. Ionomycin treatment induced cornified envelope formation in keratinocytes, but also morphological changes including plasma membrane blebbing, although no immediate alteration in cell viability could be detected. The activity of the soluble lysosomal enzymes cathepsin C and β-galactosidase in the culture medium was increased upon ionomycin treatment. Cell leakage did not seem to be responsible for this phenomenon, as suggested by measurements of the cytosolic enzymes adenylate kinase and dipeptidylpeptidase III in the culture medium. Metabolic labelling followed by immunoprecipitation showed that ionomycin induced release of cathepsin D into the culture medium. Simultaneously, lysosome-associated membrane proteins (Lamps) 1 and 2 were detected at the cell surface of ionomycin-treated keratinocytes by biochemical and morphological approaches. These results suggest that upon ionomycin treatment, calcium entry stimulates exocytosis of lysosomes in keratinocytes.

Keywords Keratinocyte · Calcium · Ionomycin · Lysosome · Exocytosis

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Abbreviations *AK* Adenylate kinase · *BSA* Bovine serum albumin · *DMSO* Dimethylsulphoxide · *DPPIII* Dipeptidylpeptidase III · *DTT* Dithiothreitol · *EDTA* Ethylene diaminotetraacetic acid · *EGTA* Ethylene glycol-*O*,*O*′-bis(2-aminoethyl)-*N*,*N*,*N*′,*N*′-tetraacetic acid · *LDH* Lactate dehydrogenase · *PBS* Phosphate-buffered saline · *SDS* Sodium dodecylsulphate

Introduction

Lysosomes are highly flexible specialized organelles of eukaryotic cells. They form an acidic compartment in which various components captured by phagocytosis, pinocytosis or isolated from the cytoplasm by autophagy are degraded. Aside from this degradative function of lysosomes, considered for a long time to be the final stage of endocytic and autophagic pathways, it has been recognized that these organelles can undergo exocytosis [1]. Such lysosomal exocytosis is regulated and has been demonstrated as the cellular emergency response to repair a ruptured plasma membrane [2]. In epithelial cell types such as type II pneumocytes or granular epidermal keratinocytes, the content of lamellar bodies, which exhibit lysosomal properties, is secreted by exocytosis into the extracellular space. This lamellar body content contributes to elaborate the lung surfactant or to seal keratinocytes of the cornified barrier [3, 4]. Similarly, melanosomes which are lysosome-related organelles elaborated in pigment-producing melanocytes, are also exocytosed and eventually re-endocytosed by neighbouring keratinocytes, which transfer the pigments into their own lysosomal compartment [5].

The properties of lysosomes have been poorly studied in keratinocytes. One specific event in keratinocyte differentiation which could influence the properties of their lysosomes is the sudden increase in cytosolic calcium that occurs when keratinocytes reach the upper granular layer, just before entering the stratum corneum [6]. During this event, calcium ions activate epidermal transglutaminase I and consequently initiate the conjugation of components of the cornified envelope [7]. Simultaneously, this cytoso-

lic calcium increase might influence the behaviour of the lysosomal compartment and could possibly trigger the exocytosis of peripheral lysosomes as shown in fibroblasts and kidney epithelial cells [8]. If verified, this phenomenon could explain the finding that the lysosomal enzymes cathepsin B and cathepsin D are secreted by keratinocytes undergoing terminal epidermal differentiation at the air-liquid interface [9].

In the present study, we investigated the effects of treatment with the ionophore ionomycin to induce an abrupt increase in cytosolic calcium [10] on the exocytosis of lysosomes in cultured keratinocytes.

Materials and methods

Chemicals and antibodies

NHS-SS-biotin and streptavidin-agarose were obtained from Pierce (Rockford, Ill.). Triton X-100, glycine, EDTA and formaldehyde were obtained from Merck (Darmstadt, Germany). Ionomycin, ethidium bromide, acridine orange, Pepstatin A, leupeptin, aprotinin, sodium deoxycholate, Tween-20 and BSA were purchased from Sigma-Aldrich (Bornem, Belgium). NaOH was purchased from Carlo Erba Reagenti (Rodano, Italy). Protein A- and G-agarose were obtained from Roche (Basel, Switzerland). SDS and DTT were obtained from Acros (Geel, Belgium). Rabbit polyclonal antibody against human cathepsin D was obtained from DAKO (Glostrup, Denmark). Mouse monoclonal antibody against CD9 (Syb-1) was a kind gift from Dr. E. Rubinstein (INSERM U268, Hôpital Paul Brousse, Villejuif, France). Mouse monoclonal antibody against human Lamp-1 (clone H4A3) and Lamp-2 (clone H4B4) developed by J.T. August and J.E.K. Hildreth was obtained from the Developmental Studies Hybridoma Bank (DSHB) developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242. Mouse monoclonal antibodies against human kinesin were obtained from Exbio (Praha, Czech Republic)

Cell culture and treatments

Normal adult skin samples were obtained during plastic surgery (Dr. B. Bienfait, Clinique St. Luc, Namur-Bouge) and keratinocytes were isolated by the trypsin float technique [11]. Primary cultures were propagated in complete keratinocyte growth medium (KGM-2; Clonetics, Verviers). Proliferating primary cultures were trypsinized and keratinocytes were plated into secondary cultures at 6×10^3 cells/cm^2. When the cells covered approximately 50% of the culture substratum, keratinocytes were cultured in autocrine growth medium by excluding bovine pituitary extract, insulin, transferrin, epinephrine and epidermal growth factor from the KGM-2 medium. Keratinocytes proliferate autonomously in subconfluent autocrine cultures [12]. Confluence of the cultures induces growth arrest and terminal differentiation of the keratinocytes, characterized by the induction of early epidermal differentiation markers such as suprabasal keratins 1 and 10 [13]. In postconfluent autocrine keratinocyte cultures, the expression of keratin 10 is downregulated, whereas involucrin, a late marker of differentiation, is upregulated [14]. Treatments were performed on confluent keratinocyte cultures using 0.15 m*M* calcium-containing autocrine growth medium, unless otherwise specified. Stock solutions of ionomycin were prepared in DMSO. Vehicle-treated control treatments were performed using medium containing 0.3% DMSO, which is the equivalent of a treatment using 30 μ*M* ionomycin.

Cornified envelope formation

Formation of insoluble cornified envelopes was evaluated according to the method described by Leigh and Watt [15]. Postconfluent keratinocyte cultures which express cornified envelope precursors involucrin [14] and loricrin (our unpublished results) were either control-treated or treated with 10 μ*M* ionomycin for 4 h and the cells were lysed in an aqueous solution containing 2% SDS and 20 m*M* DTT. After 20 min at room temperature, the cell interiors had dissolved and cornified envelopes could be visualized on an inverted phase-contrast microscope. Pictures were taken using an Olympus digital camera.

Cytotoxicity test

Treatment-induced cytotoxicity was evaluated using the ethidium bromide-acridine orange staining assay. Following treatment, cells were stained using a 100-fold diluted solution of 1 mg/ml ethidium bromide and 0.3 mg/ml acridine orange and immediately observed using a microscope equipped for epifluorescence with FITC-specific filters (Olympus AX70). Green fluorescent, acridine orange-stained viable cells and orange fluorescent, ethidium bromide-stained dead cells were counted in three separate visual fields per treatment condition. The ratio of viable to total cells was then calculated in order to evaluate treatment-induced cytotoxicity. Means of triplicate control vs treated experiments were statistically compared using the unpaired Student's *t*-test.

Enzyme activity assays

Keratinocytes were grown in six-well plates (Becton-Dickinson, Le Claix de Pont, France). Following treatment, medium was collected and detached cells and cellular debris were spun down in a microfuge at 1000 *g* for 5 min (Thermo Savant, Holbrook, N.Y.). Cells were lysed in PBS containing 0.5% (v/v) Triton X-100 and lysates were cleared by centrifugation at 13,000 *g* for 5 min. Cathepsin C activity was assayed at pH 5.5 in medium and cell lysates according to the method described by Santilman et al. [16] using Gly-Arg-7-amino-4-methylcoumarin (Gly-Arg-AMC) as a fluorogenic substrate (Bachem, Basel, Switzerland). Release of the fluorescent moiety (AMC) was measured using an SPF Aminco spectrofluorometer (SLM Instruments, Urbana, Ill.) (excitation 365 nm, emission 450 nm). Measurements were corrected for dilutions and results are expressed as the ratio of enzyme activity detected in medium to the total enzyme activity detected in medium and cell lysates. Means of triplicate results were compared using the unpaired Student's *t*-test.

The activity of β-galactosidase was assayed in medium and lysates according to the method described by Wattiaux et al. [17] using methylumbelliferyl-β-D-galactoside as fluorogenic substrate (Sigma). Release of the fluorescent moiety (MUB) was measured using an SPF Aminco spectrofluorometer (excitation 365 nm, emission 450 nm). Results are expressed as the ratio of enzyme activity detected in medium to the total enzyme activity detected in medium and cell lysates. Means of triplicate results were compared using the unpaired Student's *t*-test.

LDH activity was assayed in medium and lysates using the CytoTox assay (Promega, Leiden, The Netherlands) according to the manufacturer's instructions. DPPIII activity was assayed in medium and lysates using Arg-Arg-naphthylamide as substrate (Bachem; 0.25 m*M* final concentration in 125 m*M* Tris buffer at pH 8). Release of the fluorescent moiety (naphthylamide) was measured using an SPF Aminco spectrofluorometer (excitation 335 nm, emission 415 nm). Measurements were corrected for dilutions and incubation time. Results are expressed as the ratio of enzyme activity detected in medium to the total enzyme activity detected in medium and cell lysates. Means of triplicate results were compared using the unpaired Student's *t*-test. Adenylate kinase (AK) activity was assayed in medium and lysates using the Toxilight assay (Cambrex, Verviers, Belgium) according to the manufacturer's instructions.

Metabolic labelling

Keratinocytes grown in 6-cm Petri dishes (TPP, Trasadingen, Switzerland) were starved in methionine/cysteine-free basal medium (KBLM-2, Clonetics) for 1 h and then pulse-labelled with 150 µCi per dish Tran^{35}S-label (ICN, Asse-Relegem, Belgium) for 30 min (cathepsin D) or 1 h (CD9, Lamp-1). Cells were chased in autocrine growth medium for the indicated times before analysis of cathepsin D, CD9 and Lamp-1.

Cathepsin D immunoprecipitation, SDS-PAGE and phosphorimaging

After the required incubation times, the cells were washed, then scraped and lysed in 1 ml lysis buffer (PBS containing 1% Triton X-100 and the protease inhibitors pepstatin A at 5 µg/ml, leupeptin at 1 µg/ml and aprotinin 0.02 mg/ml). The lysates were centrifuged for 15 min at 13,000 *g* at 4°C and the resulting supernatants were collected, preabsorbed with protein A-agarose for 1 h and used for immunoprecipitation. Lysates were incubated with 2 µl rabbit polyclonal antibody against cathepsin D for 1 h at 4°C on a test tube rotator. Immune complexes were captured by incubation with protein A-agarose for 1 h at 4°C. The agarose beads were then washed five times with RIPA buffer (PBS, 0.5% sodium deoxycholate, 1% Triton-X100, 0.1% SDS) and the immune complexes were released from the beads by boiling the samples for 5 min in two times concentrated Laemmli sample buffer [18]. Immunoprecipitates were separated on 10% SDS-polyacrylamide (SDS-PAGE) gels according to the method of Laemmli [18]. After electrophoresis, gels were dried and ^{35}S-labelled proteins were visualized using a Cyclone apparatus with StoragePhosphor screens (Packard Bioscience, Meridien, Ct.).

Cell surface biotinylation, CD9, Lamp-1 and Lamp-2 immunoprecipitation

The cell surface appearance of CD9, Lamp-1 and Lamp-2 was detected using the procedure described by Rohrer et al. [19] with slight modification depicted in Fig. 4B. After the required incubation times with Tran^{35}S-label and chase periods, the cells were chilled on ice and washed five times with ice-cold PBS. NHS-SS-biotin was added at a concentration of 2 mg/ml in PBS for 45 min on ice, with occasional rocking. Biotinylation was stopped by washing four times with 50 m*M* glycine in PBS. The cells were scraped and lysed in 1 ml lysis buffer as described above for cathepsin D, and the lysates were centrifuged for 15 min at 13,000 *g* at 4°C. The resulting supernatants were collected, preabsorbed with protein G-agarose for 1 h and used for immunoprecipitation. Lysates were incubated with 5 µl mouse monoclonal antibody against CD9, Lamp-1 or Lamp-2 for 1 h at 4°C on a test-tube rotator. Immune complexes were captured by incubation with protein G-agarose for 1 h at 4°C. The agarose beads were then washed five times with RIPA buffer and the immune complexes were released from the beads by boiling the samples for 10 min in 100 µl 0.5% SDS. The samples were vortexed and the beads were pelleted. The supernatants containing the immunoprecipitated proteins were added to 900 µl lysis buffer and incubated with streptavidin-agarose overnight at 4°C. The beads were then pelleted and half of the supernatant was precipitated using a simple methanol/chloroform method. Briefly, the supernatant was mixed with 500 µl methanol and vortexed, then 150 µl chloroform was added and the mixture was vortexed again. After centrifugation for 1 min at 13,000 *g*, supernatants were carefully removed, 400 µl methanol was added followed by vortexing and centrifugation for 1 min at 13,000 *g*. Supernatants were removed, the pellets were dried and suspended in Laemmli sample buffer. Meanwhile, the streptavidin-agarose beads linked to the biotinylated proteins were washed three times with RIPA buffer. Biotinylated CD9, Lamp-1 or Lamp-2 were eluted from the beads by boiling the samples for 5 min in two times concentrated Laemmli sample buffer containing 0.2 *M* DTT. Precipitated supernatants and biotinylated proteins were analysed by SDS-PAGE as described above using 15% acrylamide gels for analysis of CD9 and 7.5% acrylamide gels for analysis of Lamp-1 and Lamp-2.

Indirect immunofluorescent labelling

Procedures for indirect immunofluorescent labelling of cell surface proteins were as described by Amos and Lotan [20]. Following treatments, keratinocytes cultured on glass coverslips were washed several times with chilled PBS and incubated for 30 min on ice with H4A3 mAbs against Lamp-1 or KN-01 mAbs against kinesin (1/20 dilution in PBS/1% BSA), washed, incubated for 30 min on ice with Alexa 488-conjugated anti-mouse antibodies diluted in PBS/BSA (Molecular Probes, Eugene, Ore.), washed, fixed with 4% formaldehyde for 30 min, washed and mounted with Mowiol mounting medium (Molecular Probes). For intracellular labelling of proteins, cells were fixed with 4% formaldehyde for 30 min, washed, permeabilized using 0.1% Triton X-100 in PBS/1% BSA for 30 min and incubated for 30 min with H4A3 mAbs against Lamp-1 or KN-01 mAbs against kinesin (1/20 dilution in PBS/1% BSA/0.1% Triton X-100), washed, incubated for 30 min on ice with Alexa 488-conjugated anti-mouse antibodies diluted in PBS/1% BSA/0.1% Triton X-100, washed and mounted with Mowiol mounting medium. The immunofluorescent labellings were visualized on an Olympus AX70 epifluorescence microscope using FITC-specific filters. Images were acquired using a Zeiss AxioCam in conjunction with Zeiss AxioVision computer software (Carl Zeiss, Jena, Germany).

Results

Treatment with ionomycin induces membrane blebbing and cornified envelope formation

Confluent keratinocyte cultures exhibited a typical polyedric morphology (Fig. 1Aa). Cells were control-treated with 0.3% DMSO which was used as a diluent for ionomycin. Treatment with 30 µ*M* ionomycin resulted in the formation of plasma membrane blebs after 15 min (Fig. 1Ac, arrows). Blebs were still observed after 30 min with ionomycin (Fig. 1Ad, arrows), but after 60 min of treatment, cells had become rounded and blebs were no longer visible (Fig. 1Ae). These alterations in cell morphology were apparently calcium-dependent since they did not occur when 5 m*M* EGTA was included in the culture medium during treatment with 30 µ*M* ionomycin for 60 min (Fig. 1Ab). The cytotoxicity of ionomycin was determined using the ethidium bromide/acridine orange assay [21]. Cell viability in control cultures corresponded to 99.8±0.4% (n=3). Treatment with 30 µ*M* ionomycin for 60 min resulted in 99.3±0.7% (n=3) of viable cells, while chelation of calcium ions with 5 m*M* EGTA parallel to incubation with ionomycin for 60 min gave 99.8±0.3% (n=3) cell viability. These results suggest that the blebbing observed after short-term 30 µ*M* ionomycin treatment was not linked to immediate cell death induced by the treatment.

Ionomycin has been reported to induce activation of transglutaminase and formation of cornified envelope in epidermal keratinocytes [22]. As shown in Fig. 1Ba, insoluble cornified envelopes were not detected in control keratinocyte cultures. Treatment with 10 µ*M* ionomycin for 5 h induced the formation of insoluble cornified envelopes (Fig. 1Bb). This event was apparently calcium-

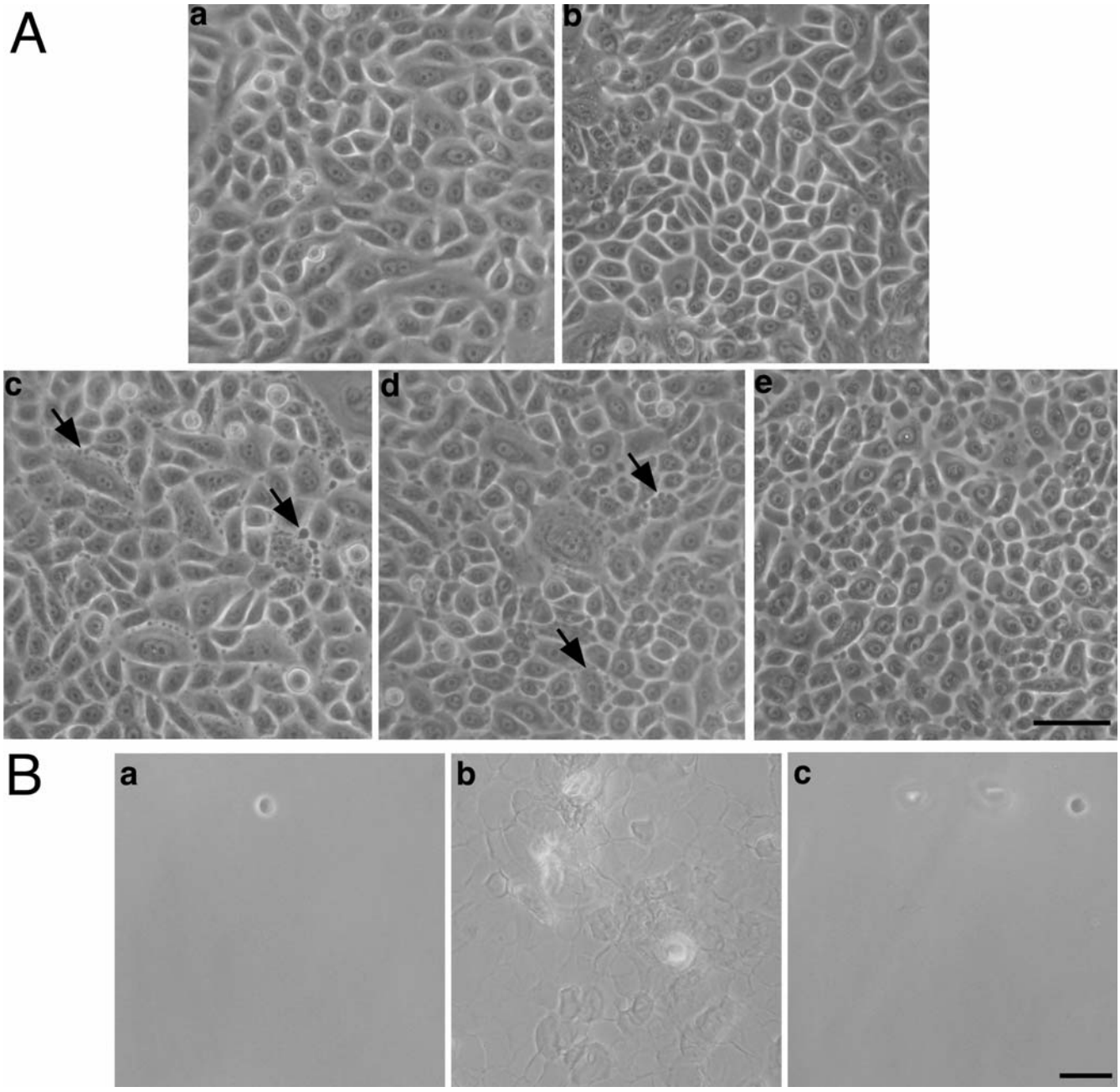


Fig. 1A, B Ionomycin treatment induces calcium-dependent membrane blebbing, cell rounding and cornified envelope formation in keratinocytes. **A** Keratinocyte cultures were treated with DMSO-containing medium for 60 min (*a*), medium containing 30 μ*M* ionomycin and 5 m*M* EGTA (*b*), or treated for 15 (*c*), 30 (*d*) and 60 min (*e*) with medium containing ionomycin. After the indicated times, the cultures were photographed using an inverted phase-contrast microscope (*arrows* membrane blebs appearing upon ionomycin treatment, *bar* 100 μm). **B** Keratinocyte cultures were treated with DMSO-containing medium (*a*) or medium containing 10 μ*M* ionomycin (*b*), then cornified envelope formation was assessed as described in Materials and methods (*bar* 100 μm)

dependent since cornified envelopes were not observed when the experiment was performed in the presence of 5 m*M* EGTA (Fig. 1Bc).

Treatment of keratinocytes with ionomycin triggers the release of soluble lysosomal proteins

Calcium ionophores have been shown to induce the secretion of lysosomal proteins in several cell types [23]. We sought to determine whether ionomycin treatment could also initiate the same response in keratinocytes. Cathepsin C (EC 3.4.14.1) is a soluble lysosomal exopeptidase [24]. As shown in Fig. 2A, cathepsin C activity detected in the culture medium of untreated keratinocytes was very low, suggesting that the constitutive exocytosis of lysosomes was weak in keratinocytes. Treatment of keratinocytes with 10 μ*M* ionomycin did not induce any significant increase in the extracellular activity of cathepsin C after 15, 30 or 60 min. This result is intriguing since incubation

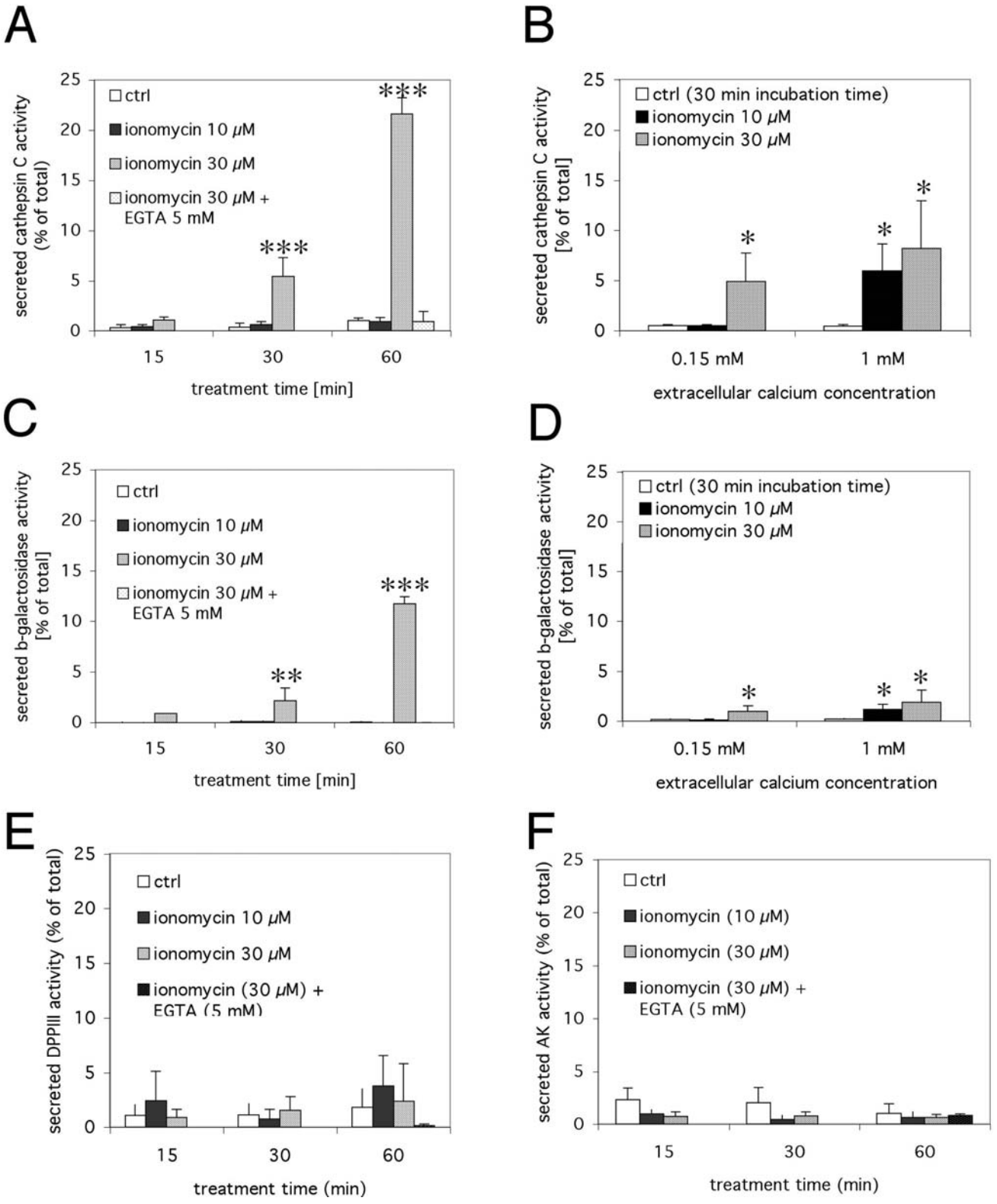


Fig. 2A–F Treatment of keratinocytes with ionomycin induces release of cathepsin C and β-galactosidase. Keratinocyte cultures were treated for 15, 30 or 60 min with vehicle-containing medium or medium containing ionomycin (10 or 30 μ*M*; 0.15 m*M* calcium) (**A**, **C**, **E**, **F**). Other cultures were treated for 30 min with medium containing 0.15 m*M* or 1 m*M* calcium or medium containing 0.15 m*M* or 1 m*M* calcium and 10 μ*M* or 30 μ*M* ionomycin (**B**, **D**). Following treatment, the enzymatic activities of cathepsin C (**A**, **B**), β-galactosidase (**C**, **D**), DPPIII (**E**) and adenylate kinase (**F**) were assayed in the culture medium and in the corresponding cell lysates. To verify if the effects of ionomycin treatment on enzyme release were calcium-dependent, keratinocyte cultures were treated with medium containing 30 μ*M* ionomycin and 5 m*M* EGTA for 60 min. Data (control vs treated) were compared using the unpaired Student's *t*-test (n=3 for all treatments except control, 10 μ*M* and 30 μ*M* ionomycin treatments for 30 min at 0.15 m*M* calcium, and 30 μ*M* ionomycin treatments for 60 min at 0.15 m*M* calcium where n=6, *P<0.05, ***P<0.001)

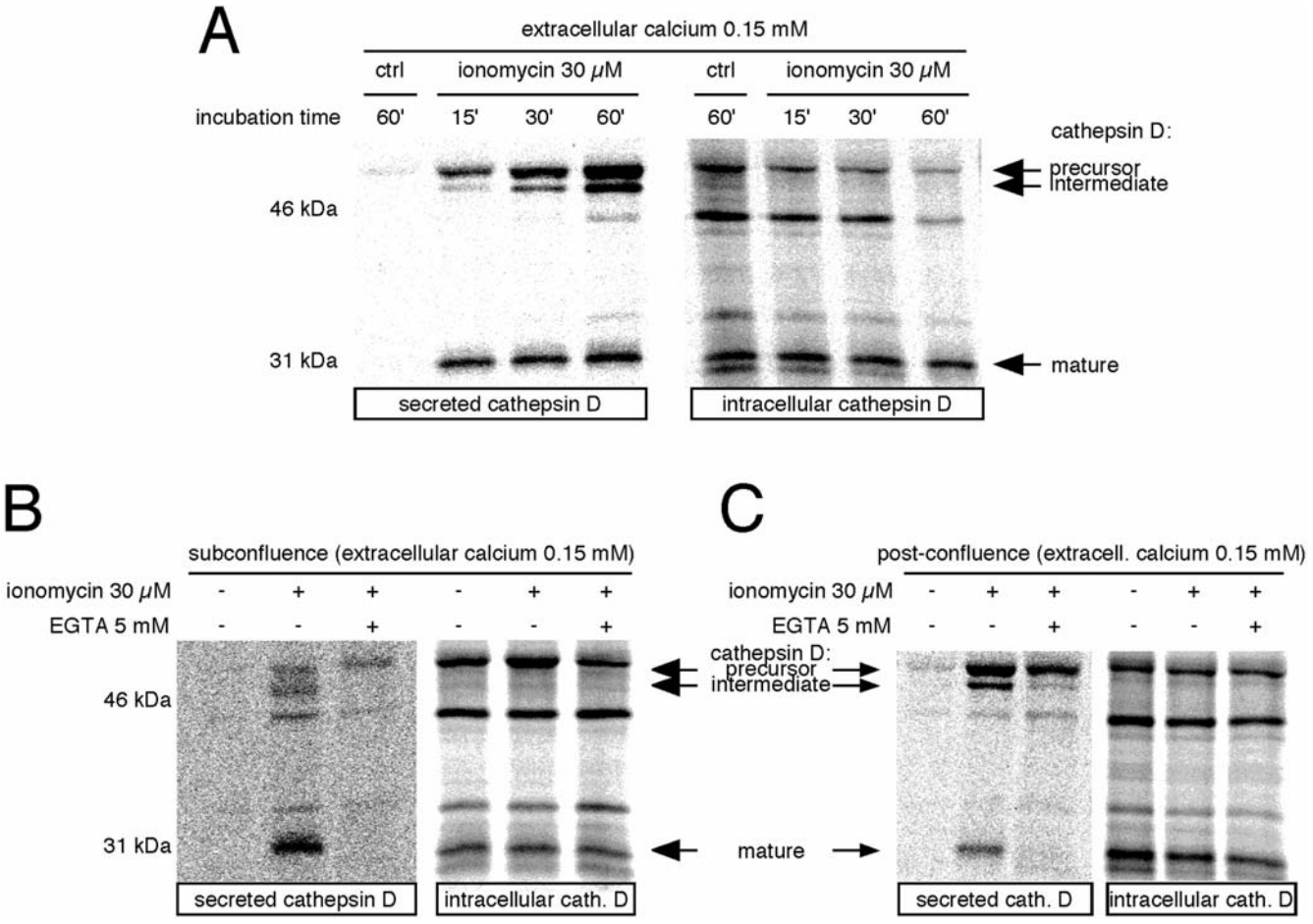


Fig. 3A–C Treatment of keratinocytes with ionomycin induces release of cathepsin D. **A** Following pulse-chase (30 min/18 h) metabolic labelling, the time-dependent effects of ionomycin treatment on cathepsin D secretion were investigated in confluent keratinocyte cultures either treated for 60 min with control medium or medium containing 30 μ*M* ionomycin for 15, 30 or 60 min. Following treatments, cathepsin D was analysed by immunoprecipitation from the culture medium and the corresponding lysates followed by SDS-PAGE and autoradiography. To determine whether the effect of ionomycin treatment on cathepsin D release was linked to cell density of the cultures, cathepsin D secretion was investigated in subconfluent (**B**) and postconfluent (**C**) keratinocyte cultures, which were treated for 60 min with either control medium or medium containing 30 μ*M* ionomycin following pulse-chase (30 min/18 h) metabolic labelling. To determine whether the effect of ionomycin treatment on cathepsin D release was calcium-dependent, cells were incubated for 60 min with 5 m*M* EGTA and 30 μ*M* ionomycin. Analysis of cathepsin D was then performed as described

with 10 μ*M* ionomycin has been reported to trigger exocytosis of lysosomes in fibroblasts and some epithelial cell types [23]. We hypothesized that an ionomycin concentration of 10 μ*M* might not be sufficient to induce exocytosis of lysosomes in keratinocytes, possibly because the extracellular calcium concentration is lower in the KBM-2 culture medium used for keratinocytes (0.15 m*M*) compared to its concentration in the culture medium used in studies of other cell types (1 m*M*). Thus, we tested an ionomycin concentration of 30 μ*M*. While treatment with 30 μ*M* ionomycin for 15 or 30 min triggered a small but significant release of cathepsin C activity, treatment with 30 μ*M* ionomycin for 60 min induced the release of about 20% of cathepsin C activity. The observed dose- and time-dependent release of cathepsin C activity by ionomycin-treated keratinocytes was apparently calcium-dependent, since chelation of calcium ions with 5 m*M* EGTA prevented cathepsin C secretion upon treatment with 30 μ*M* ionomycin for 60 min. To investigate the hypothetical involvement of the extracellular calcium concentration in the exocytosis of lysosomes upon ionomycin treatment, we analysed the effect of incubation of keratinocytes for 30 min with 1 m*M* calcium and 10 μ*M* ionomycin and observed a significant release of cathepsin C activity (Fig. 2B).

β-Galactosidase (EC 3.2.1.23.) is a soluble lysosomal glycosidase [17]. As shown in Fig. 2C, treatment with 30 μ*M* ionomycin for 30 or 60 min induced the release of a significant β-galactosidase activity into the culture medium. Incubation with 5 m*M* EGTA prevented the release of β-galactosidase activity upon treatment with 30 μ*M* ionomycin for 60 min. As shown in Fig. 2D, incubation of keratinocytes for 30 min with 1 m*M* calcium and 10 μ*M* ionomycin induced a significant release of β-galactosidase activity.

We tested whether the integrity of the plasma membrane barrier was affected by treatments with ionomycin.

We assayed the activity of the cytosolic enzyme LDH (EC 1.1.1.27) in the culture medium and lysates of keratinocytes treated as described above. Unexpectedly, LDH release by keratinocytes was variable and independent of the treatment (data not shown), which prompted us to investigate the release of other cytosolic enzymes. The activity of DPPIII (EC 3.4.14.4) [25, 26] was assayed in the cell lysates and the culture medium of keratinocytes treated as described in Fig. 2. As shown in Fig. 2E, DPPIII activity recovered in culture did not exceed 5% of the total activity irrespective of the treatment. We assayed the activity of AK, another cytosolic enzyme (EC 2.7.4.3) [27], in the cell lysates and the culture medium of keratinocytes treated as above. Secreted AK activity was detectable in all analysed culture media, but remained low (<5% of total activity) and no significant difference was detected between the different treatments (Fig. 2F).

Cathepsin D is a lysosomal aspartic endoprotease. The biosynthesis of the enzyme, which undergoes a characteristic proteolytic maturation, has been well studied [28]. Cathepsin D is synthesized in the endoplasmic reticulum as a 52 kDa precursor, then matures into a 48 kDa intermediate form which is finally cleaved into a 31 kDa mature form associated with lysosomes [29]. We employed pulse-chase analysis of metabolically labelled keratinocytes in order to investigate the effects of ionomycin on secretion of both inactive and active forms of cathepsin D. For appropriate detection of the mature form, we employed a long chase time lapse (18–24 h). Following treatments, cathepsin D was immunoprecipitated from the conditioned medium and the radioactive protein was detected by Phosphorimaging after SDS-PAGE. As shown in Fig. 3A, untreated keratinocytes give rise to secretion of a very low amount of cathepsin D precursor but no intermediate or mature forms. Upon treatment for 15, 30 and 60 min with 30 μ*M* ionomycin, mature cathepsin D was released into the culture medium, suggesting that ionomycin triggers exocytosis of lysosomes. Unexpectedly, ionomycin treatment also triggered release of some precursor and intermediate forms of cathepsin D.

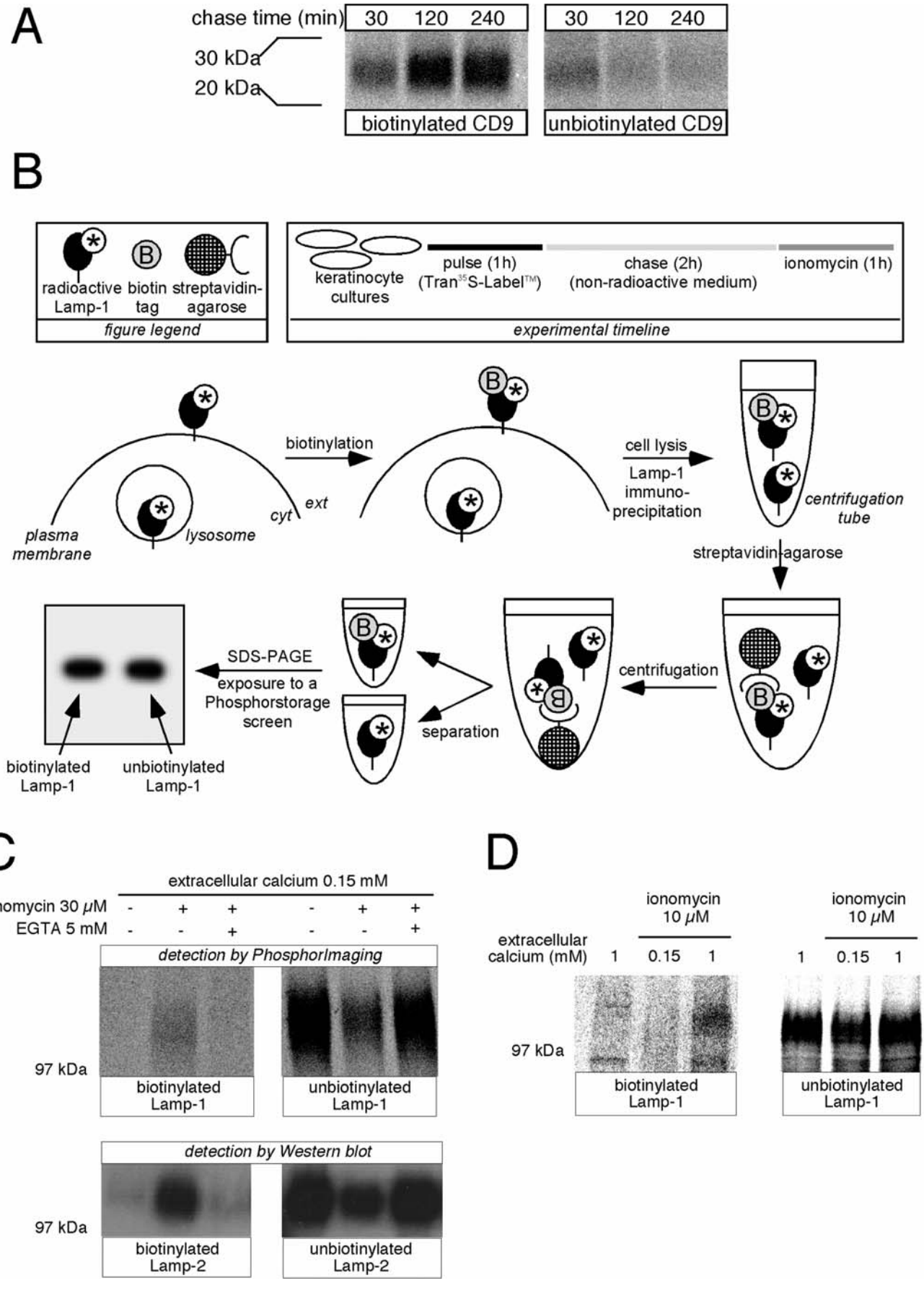
This result prompted us to analyse cathepsin D release upon ionomycin treatment also in subconfluent and postconfluent cultures in order to examine whether release of cathepsin D upon ionomycin treatment might be influenced by the cell density of the cultures. In subconfluent cultures (Fig. 3B) and postconfluent cultures (Fig. 3C), ionomycin treatment again induced release of mature, intermediate and precursor forms of cathepsin D. When calcium ions were chelated using EGTA during treatment with ionomycin, a strong reduction in the amount of released mature or intermediate cathepsin D was seen, while release of the precursor did not seem to be prevented to the same extent by EGTA. Ionomycin treatment of keratinocytes seems thus to induce a calcium-dependent secretion of mature and intermediate forms of cathepsin D, which have been associated with lysosomes in fibroblasts [29]. Ionomycin also seems to induce calcium-independent secretion of cathepsin D precursor. These results are reminiscent of the observations of Gardella et al. [30], who demonstrated that dendritic cells secrete mature and precursor forms of cathepsin D upon stimulation with ionomycin. These authors also noticed that secretion of the mature form is prevented upon parallel incubation with EGTA, but not secretion of cathepsin D precursor.

Ionomycin treatment induces the appearance of Lamp-1 and Lamp-2 at the cell surface of keratinocytes

We investigated the fate of Lamp-1 and Lamp-2 upon ionomycin treatment. Lamp-1 and Lamp-2 are lysosomal membrane proteins [31]. They are heavily glycosylated and carry many specific polylactosamine residues, leading to apparent molecular weights around 120 kDa. We used two different approaches to detect the putative appearance of Lamp-1 and Lamp-2 at the surface of cells treated with ionomycin: biotinylation of exposed surface proteins, and immunofluorescent surface labelling. As a control, we used the plasma membrane marker CD9, which is a transmembrane protein of the tetraspanin family that is constitutively expressed on the cell surface of keratinocytes [32]. As shown in Fig. 4A, some biotinylated radioactive CD9 was detected as early as 30 min after the metabolic labelling pulse. Detection of CD9 on the cell surface had increased by 120 min, apparently reaching a maximum. Simultaneously, the amount of unbiotinylated intracellular CD9 was low and had decreased after 120 min of chase time.

When the same analysis was performed on unbiotinylated cells, no CD9 was bound to streptavidin-agarose (data not shown). We applied this technique to analyse the

Fig. 4A–D Ionomycin treatment induces Lamp-1 and Lamp-2 at the cell surface. **A** The appearance of CD9 was detected at the cell surface of keratinocytes using the biotinylation-based technique described by Rohrer et al. [19]. Keratinocyte cultures were subjected to pulse-chase (1 h/30 min, 2 h, 4 h) metabolic labelling. After the required incubation times, cell surface proteins were biotinylated as described in Materials and methods. Cells were then lysed, CD9 was immunoprecipitated and biotinylated CD9 was analysed by precipitation using streptavidin-agarose, SDS-PAGE and autoradiography, next to unbiotinylated intracellular CD9. **B** Diagram outlining the experimental timeline and the biotinylation-based technique for analysing the appearance of Lamp-1 at the plasma membrane of keratinocytes (*cyt* cytoplasm, *ext* extracellular space). **C** Keratinocyte cultures were subjected to pulse-chase (1 h/2 h) metabolic labelling and treated for 60 min with control medium, medium containing 30 μ*M* ionomycin, and medium containing ionomycin and 5 m*M* EGTA. After the required incubation times, cell surface proteins were biotinylated as described. Cells were then lysed, Lamp-1 was immunoprecipitated and biotinylated Lamp-1 was analysed by precipitation using streptavidin-agarose, SDS-PAGE and autoradiography, next to unbiotinylated intracellular Lamp-1. Biotinylated and unbiotinylated Lamp-2 were similarly analysed and detected by immunoblotting. A representative of duplicate results is shown. **D** Keratinocyte cultures were subjected to pulse-chase (1 h/2 h) metabolic labelling and treated for 30 min with medium containing 1 m*M* calcium, medium containing 0.15 m*M* calcium and 10 μ*M* ionomycin or medium containing 1 m*M* calcium and 10 μ*M* ionomycin. After the required incubation times, cell surface and intracellular Lamp-1 was detected as described for **C**


A
chase time (min)
30 120 240
30 120 240
30 kDa
20 kDa
biotinylated CD9
unbiotinylated CD9
B
radioactive Lamp-1
biotin tag
streptavidin-agarose
figure legend
keratinocyte cultures
pulse (1h) (Tran35S-Label™)
chase (2h) (non-radioactive medium)
ionomycin (1h)
experimental timeline
plasma membrane
lysosome
cyt
ext
biotinylation
cell lysis
Lamp-1 immuno-precipitation
centrifugation tube
streptavidin-agarose
centrifugation
separation
SDS-PAGE
exposure to a Phosphorstorage screen
biotinylated Lamp-1
unbiotinylated Lamp-1
C
extracellular calcium 0.15 mM
ionomycin 30 μM - + + - + +
EGTA 5 mM - - + - - +
detection by PhosphorImaging
97 kDa
biotinylated Lamp-1
unbiotinylated Lamp-1
detection by Western blot
97 kDa
biotinylated Lamp-2
unbiotinylated Lamp-2
D
ionomycin 10 μM
ionomycin 10 μM
extracellular calcium (mM) 1 0.15 1
1 0.15 1
97 kDa
biotinylated Lamp-1
unbiotinylated Lamp-1

appearance of Lamp-1 at the surface of keratinocytes. Neosynthesized Lamp-1 has been shown in other cell types to localize in lysosomes by 120 min [33, 34]. After this incubation time, keratinocytes were kept in control medium, treated with ionomycin, or treated with ionomycin and EGTA. After 60 min, cell surface proteins were biotinylated, and biotinylated and unbiotinylated Lamp-1 were detected according to the procedure depicted in Fig. 4B. In control cells, Lamp-1 did not localize at the cell surface (Fig. 4C), but was mainly intracellular. Ionomycin treatment induced a weak but detectable biotinylation of Lamp-1, suggesting that some of the protein was then located at the cell surface. Furthermore, this appearance of Lamp-1 at the surface of cells was not observed in the presence of EGTA. Lamp-1 and Lamp-2 have a similar molecular weight. To be able to detect Lamp-1 and Lamp-2 separately from the same initial cell lysates, biotinylated and intracellular Lamp-2 were detected by Western blotting. As shown in Fig. 4C, ionomycin treatment resulted in the biotinylation of some Lamp-2 in a calcium-dependent manner. In a similar manner to the release of soluble lysosomal enzymes, we found that Lamp-1 could be biotinylated at the surface of keratinocytes treated with 10 μ*M* ionomycin only when the extracellular calcium concentration was raised to 1 m*M*, confirming that at this calcium concentration, ionomycin is able to trigger the exocytosis of lysosomes at a concentration of 10 μ*M* (Fig. 4D).

The second approach used to detect cell surface localization of Lamp-1 was indirect immunofluorescent labelling of nonpermeabilized cells with an antibody directed to the luminal domain of Lamp-1 [20]. As shown in Fig. 5, unpermeabilized keratinocytes did not exhibit any Lamp-1 immunofluorescence on the cell surface (Fig. 5a) in contrast with cells treated with ionomycin (Fig. 5b) which exhibited a punctate immunofluorescence pattern reminiscent of the observations of Rodriguez et al. [23], who performed similar experiments using rat kidney cells. Permeabilized keratinocytes exhibited typical perinuclear Lamp-1 immunolabelling (Fig. 5c) [35]. We used an antibody to kinesin as a control, since kinesin is an intracellular protein associated with microtubules [23]. Unpermeabilized keratinocytes did not exhibit kinesin immunolabelling when they were left untreated (Fig. 5d) or treated with ionomycin (Fig. 5e), suggesting that no antibody can reach intracellular antigens. As shown in Fig. 5f, kinesin immunolabelling of permeabilized keratinocytes is reminiscent of centrosome labelling observed by Neighbors et al. in epithelial PtK1 cells [36].

Taken together, our results suggest that ionomycin treatment induces a calcium-dependent exocytosis of some lysosomes, leading to the incorporation of lysosomal membranes into the plasma membrane.

Discussion

In this study, treatment of epidermal keratinocytes with ionomycin induced the formation of temporary cellular blebs. Although cellular viability did not seem to be affected by this treatment, the increase in cytosolic calcium led to the appearance of numerous cornified envelopes, presumably as a result of transglutaminase-1 activity induction. In addition to this stimulation of late epidermal differentiation, the rise in calcium concentration in the cytosol of keratinocytes would induce exocytosis of lysosomes, as suggested by the release of some soluble lysosomal proteins into the culture medium, and the cell surface appearance of two lysosomal integral membrane proteins.

Several roles have been attributed to lysosomes in keratinocytes [37, 38, 39]. Further functions could be suspected if, as shown in other cell types [23], keratinocytes have the ability to respond to a rise in the cytosolic calcium concentration by exocytosis of some of their lysosomes. At an extracellular calcium concentration of 0.15 m*M*, we observed no release of the activity of soluble lysosomal enzymes during incubation of keratinocytes with 10 μ*M* ionomycin, but treatment with 30 μ*M* ionomycin resulted in a small percentage of the total lysosomal enzyme activities (10 to 20%) being released into the extracellular medium. Lysosome exocytosis, as indicated by the release of the activities of soluble lysosomal enzymes and the cell surface localization of Lamp-1, could be induced in keratinocytes incubated with 10 μ*M* ionomycin at an extracellular calcium concentration of 1 m*M*. These results suggest that a limited fraction of the lysosome population, possibly the membrane proximal organelles [8], undergo exocytosis. Such a result is similar to the limited secretion of hexosaminidase activity reported by Rodriguez et al. in ionomycin-treated fibroblasts, in which lysosome exocytosis can be induced by treatment with 10 μ*M* ionomycin [23].

The calcium-dependent release of the intermediate and mature cathepsin D peptides supports the hypothesis of an ionomycin-induced exocytosis of lysosomes. We also analysed the presence of integral lysosomal membrane proteins at the cell surface in order to investigate if the release of soluble enzymes was the result of effective exocytosis, as demonstrated in fibroblasts [23]. Lamp-1 and Lamp-2 were chosen as these typical lysosomal membrane proteins are not transported to the cell plasma membrane except in some particular cell types [31] or under particular conditions [40]. Using surface biotinylation and immunofluorescent labelling without cell permeation, we found that in untreated keratinocytes, Lamp-1 was not expressed on the cell surface. However, treatment of keratinocytes with ionomycin induced some biotinylation of Lamp-1, suggesting that a proportion of this protein was then relocated to the plasma membrane. Recently, another marker of lysosomal membranes, the tetraspanin CD63, has also been shown to be exposed at the cell surface of rat kidney cells in response to an enhanced cytosolic calcium concentration [8].

Incubation with ionomycin has been shown to affect the intracellular pH in several cell types [41, 42, 43, 45]. Increasing the pH of acidic vesicles by treatment with ammonium chloride inhibits the endosomal dissociation of the lysosomal enzymes from the mannose 6-phosphate receptors [46, 47], and therefore induces the secretion of the

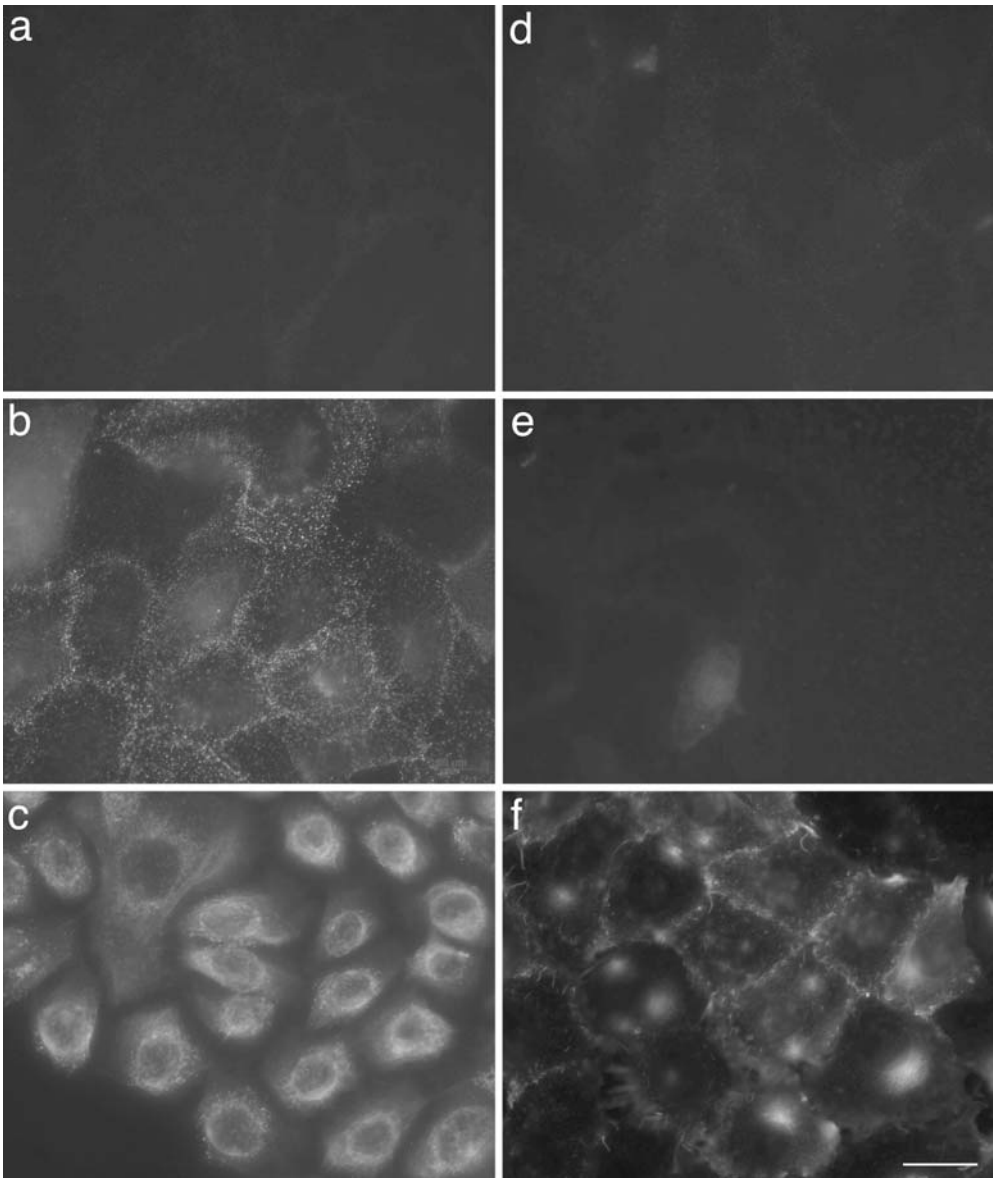


Fig. 5a–f Ionomycin treatment induces Lamp-1 immunolabelling at the cell surface. Keratinocyte cultures seeded on glass coverslips were treated for 60 min with vehicle-containing medium (**a**, **d**) or medium containing 30 μ*M* ionomycin (**b**, **e**) and subjected to Lamp-1 (**a**, **b**) or kinesin (**d**, **e**) immunofluorescent labelling. Other keratinocyte cultures were fixed, permeabilized and subjected to immunofluorescent labelling for Lamp-1 (**c**) or kinesin (**f**). Immunolabellings were visualized as described in Materials and methods. Photographs were taken using the same exposure time for all samples. A representative of duplicate experiments is shown (*bar* 20 μm)

precursor form of cathepsin D in several cell types [48, 49]. We cannot exclude the possibility that incubation with ionomycin could induce an alkalinization of the endosomes and thereby trigger the observed release of cathepsin D precursors.

Interestingly, calcium-regulated exocytosis of lysosomes could be a means by which wounded cells, i.e. cells which exhibit torn plasma membranes, acquire new material for plasma membrane repair [2]. This process could be of primary importance for cells which are very often mechanically stressed, such as epidermal keratinocytes exposed at the surface of the body, especially keratinocytes of the basal layer which guarantee epidermal anchorage on the connective dermal tissue through the epidermodermal junction [50]. Such a repairing role for lysosomes, particularly membrane proximal lysosomes [8], might explain why the lysosomal compartment is well developed in basal keratinocytes [51].

Calcium-dependent exocytosis of keratinocyte lysosomes could also be implicated in epidermal differentiation. Indeed, calcium has been identified as one major regulator of epidermal differentiation [7]. Several reports have already suggested links between epidermal differentiation and release of lysosomal enzymes by these cells [9, 52, 53]. These secreted hydrolases are thought to be involved in epidermal desquamation, the process by which the epidermis naturally loses keratinocytes from the surface of the cornified layer. A massive entry of calcium into the cytosol of differentiating keratinocytes present in the upper granular layers has been identified as concomitant to late differentiation and cornification of keratinocytes. Aside from the activation transglutaminase-1, which then conjugates components of the cornified envelope [7], this entry of calcium might trigger the exocytosis of lysosomes in granular keratinocytes and induce the release of lysosomal enzymes into the extracellular space.

Granular keratinocytes contain lamellar bodies which contain a mixture of lipid lamellae and lysosomal enzymes and undergo exocytosis at the transition between the granular and cornified layers [54, 55]. The exocytosis of lamellar bodies has been reported to be stimulated by a decreased cellular calcium concentration [4, 56, 57] and to be inhibited by an increased calcium concentration [58]. The calcium-dependent exocytosis of lysosomes which we report seems thus to be regulated differently from lamellar body exocytosis.

In conclusion, our results suggest that keratinocytes can respond to an enhanced cytosolic calcium concentration by exocytosis of some of their lysosomes. Whether this lysosomal exocytosis does really happen in vivo in answer to calcium during epidermal differentiation awaits specific demonstration.

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