Toxicology in Vitro 27 (2013) 402–408

Contents lists available at SciVerse ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit

Chemicals inducing acute irritant contact dermatitis mobilize intracellular calcium in human keratinocytes

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ARTICLE INFO

Article history:
Received 4 July 2011
Accepted 2 August 2012
Available online 10 August 2012

Keywords:
Chemical irritant
In vitro irritation model
Irritant contact dermatitis
Skin
Keratinocyte
Calcium signaling

ABSTRACT

Intracellular Ca^{2+} increase is a common feature of multiple cellular pathways associated with receptor and channel activation, mediator secretion and gene regulation. We investigated the possibility of using this Ca^{2+} signal as a biomarker for a reaction to chemical irritants of normal human keratinocytes (NHK) in submerged primary cell culture. We tested 14 referenced chemical compounds classified as strong (seven), weak (four) or non- (three) irritants in acute irritant contact dermatitis. We found that the strong irritant compounds tested at 20–40 mM induced an intracellular Ca^{2+} increase measurable by spectrofluorimetry in an automated test. Weak and non-irritant compounds however did not increase intracellular Ca^{2+} concentration. We further investigated the mechanisms by which the amine heptylamine, classified as a R34 corrosive compound, increases intracellular Ca^{2+}. Heptylamine (20 mM) induced an ATP release that persisted in the absence of intra- and extra-cellular Ca^{2+}. In addition, we found that this ATP activates NHK purinergic receptors that subsequently cause the increase in intracellular Ca^{2+} from sarcoplasmic reticular stores. We conclude that measuring the intracellular Ca^{2+} concentration in NHK is a suitable and easy way of determining any potential reaction to soluble chemical compounds.

1. Introduction

The epidermis, being the topmost living layer of the skin, is constantly exposed to external injury from such as mechanical stimulation, chemical irritants and noxious temperatures. Contact dermatitis is one of the most frequent skin diseases. It comprises a major portion of occupational dermatoses in industrialized societies, resulting in considerable social and economic implications. It can be divided into irritant contact dermatitis and allergic contact dermatitis depending on the production or not of specific antibodies. Irritant contact dermatitis is defined as a localized inflammation of the skin caused by contact with toxic compounds such as metals, cleaning solutions, detergents, cosmetics, industrial chemicals and latex rubber. Depending on the time course of the skin reaction, irritation may be classified as acute, delayed or chronic i.e. developing slowly after exposure (Chew and Maibach, 2003). Historically, the irritation index during acute irritant dermatitis due to a single exposure to a chemical has been determined *in vivo* using the Draize skin irritation test on rabbits (Draize et al., 1944). As a measure of acute irritancy potential, each chemical is given a score based on erythema and edema grade (Bagley et al., 1996).

Despite the universal acceptance of this assay, the correlation between animal and human irritancy has come under question since, for some cases, chemicals have been misclassified using *in vivo* rabbit data (York et al., 1996). In view of this as well as ethical concerns, the 7th amendment to the European Cosmetics Directive stimulated the development of alternative tests for the assessment of the potential toxicological effects of substances.

Abbreviations: ATP, adenosine triphosphate; $[Ca^{2+}]_i$, intracellular calcium concentration; DMEM, Dulbecco's Modified Eagle's Medium; DMSO, dimethyl sulfoxide; EGF, epidermal growth factor; EGTA, ethyleneglycol-bis(β-aminoethyl)-*N*,*N*,*N*′,*N*′-tetraacetoxymethyl ester; F/F0, normalized ratio of the Fluo-4 AM fluorescence (F) to the basal fluorescence (F0); HBSS, Hanks' Balanced Salt Solution; IL1α, interleukine 1α; IL8, interleukine 8; NHK, normal human keratinocytes; NRR, neutral red release; PGE2, prostaglandin 2; PLC, phospholipase C; TNFα, tumor necrosis factor α; DMIPA, dimethylisopropylamine; PPADS, pyridoxal-phosphate6-azophenyl-2′-4′-disulfonate; SLS, sodium lauryl sulfate; SEM, standard error of the mean.

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http://dx.doi.org/10.1016/j.tiv.2012.08.010

Recently, the scientific advisory committee of the European Centre for the Validation of Alternative Methods (ECVAM) began accepting the use of *in vitro* tests on reconstructed epidermis to distinguish between skin irritating and non-irritating chemicals (Gerberick et al., 2008). Cellular end points measured in alternative skin irritation assays concern keratinocyte viability and cytokine (IL1α, TNFα) release (see review in Macfarlane et al., 2009; Gibbs, 2009). The viability is determined by measuring cell cytotoxicity (Gay et al., 1992; Triglia et al., 1991), loss of membrane integrity (Faller and Bracher, 2002; Osborne and Perkins, 1994) and mitochondrial metabolism (Chan et al., 2005), and the cytokine release is measured by ELISA. Irritant and corrosive compounds may also induce the secretion by keratinocytes of other inflammatory mediators such as PGE2 (Corsini and Galli, 1998; Coquette et al., 2003) and ATP (Mizumoto et al., 2003). However, none of these assays considers the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) as a putative biomarker of acute irritancy potential.

The first advantage is that the intracellular Ca^{2+} signal is ubiquitous and corresponds well with the diversity of the cellular response expected using a large spectrum of chemicals. Another advantage is that the $[Ca^{2+}]_i$ is easy to measure using a fluorescent probe and experiments can be miniaturized in 96 well dishes using an automated spectrofluorimeter. With this in mind, we firstly measured the variations in $[Ca^{2+}]_i$ of NHK in response to 14 chemical compounds chosen according to their skin irritancy potential and structural diversity, and found a link between Ca^{2+} increase and irritancy potential. We then analyzed the cellular mechanisms by which the skin corrosive compound heptylamine induces intracellular Ca^{2+} increase. Here we demonstrate how chemical stimulations by heptylamine, at concentrations that do not alter cell viability (10–20 mM), cause a Ca^{2+}-independent release of ATP from NHK which then activates metabotropic purinergic receptors, in turn bringing about the increase in $[Ca^{2+}]_i$. We conclude that this Ca^{2+} mobilization in NHK in response to chemical stimuli may be a relevant biomarker to detect potential keratinocyte reactions *in vitro* and test compound toxicity in acute irritant contact dermatitis.

2. Materials and methods

2.1. Normal human keratinocyte (NHK) primary cultures

Normal human keratinocyte (NHK) primary cultures were prepared from healthy human foreskin biopsies. Samples were obtained as surgical waste tissues. This study has been performed according to the Declaration of Helsinki. Informed consent of the patients or their legal representative was in agreement with the article L1245-2 of the French Public Health Code relating to the use of surgical waste tissues. Skin explants were conserved at 4 °C for up to 12 h in Hanks' balanced salt solution (HBSS; Invitrogen, Carlsbad, CA) supplemented with penicillin–streptomycin (200 U/ml; Invitrogen). Underlying mesenchyme was removed and the explants then decontaminated by washing in HBSS supplemented with ethanol (30%, v/v) and in HBSS supplemented with penicillin–streptomycin (200 U/ml). The explants were then cut into ~0.5 cm^2 pieces and incubated for 16 to 20 h at 4 °C with dispase grade II in PBS (24 U/ml; Roche, Indianapolis, IN) supplemented with penicillin–streptomycin (100 U/ml). Epidermal layers were lifted from the dermis and mechanically dissociated before being incubated in HBSS supplemented with trypsin–EDTA (0.5 mg/ml; Invitrogen) for 20 min at 37 °C. The resulting epidermal cell suspension was filtered through a cellular strainer (pore size 70 μm) and centrifuged at 1200 r.p.m. (200 g) for 6 min. Epidermal cells were re-suspended and cultivated in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen) supplemented with Ham's F-12 (30%; Invitrogen), fetal calf serum (FCS, 10%; Invitrogen), L-glutamine (2 mM; Invitrogen), penicillin–streptomycin (100 U/ml; Sigma–Aldrich, St Louis, M), insulin (5 μg/ml; Sigma), hydrocortisone (0.5 μg/ml; Sigma) and EGF (10 ng/ml; Sigma). To obtain keratinocyte proliferation, cells were plated at 80.10^3 - cells/cm^2 and co-cultured with feeder mitomycin C-treated 3T3 fibroblasts (30.10^3 cells/cm^2) in Falcon flasks (37 °C, 95% O_2–5% CO_2). Keratinocytes were allowed to proliferate and the culture medium was renewed every 2–3 days.

Having reached confluence after 8 to 15 days *in vitro*, the NHK were isolated using a two-step trypsination procedure: co-cultures were treated with trypsin–EDTA in HBSS (0.5 mg/ml) for ~1 min to dislodge inactivated 3T3 fibroblasts and then NHK were dislodged by a longer trypsin–EDTA treatment (~3 min). The NHK were then centrifuged at 1200 r.p.m. (200 g) for 6 min. Samples were either re-suspended in DMEM supplemented with FCS (20%) and DMSO (10%) for cryopreservation (3.10^6 cells/ml in cryovials) or were used for experiments. For experiments, the NHK were re-suspended in the complete culture medium described above and cultivated at 8.10^3 cells/cm^2 in black 96-well dishes for 5–7 days without 3T3 cells. Experiments were performed on NHK having reached confluence.

2.2. Intracellular Ca^{2+} measurements

All experiments were performed at room temperature (22–25 °C) in an isotonic (296 ± 5 mOsM) Krebs solution consisting of (in mM): 130 NaCl, 3 KCl, 1 $MgCl_2$, 10 Hepes, 2.5 $CaCl_2$ and 10 glucose. Variations in the $[Ca^{2+}]_i$ were measured with the Tecan infinite F500 spectrofluorimeter-luminometer (Tecan, Research Triangle Park, NC) on NHK isolated using the two-step trypsination procedure and cultured without 3T3 fibroblasts in black 96-well Greiner (Dutscher, France) dishes with clear bottoms. Cells were loaded with Fluo-4 AM (5 μM; Invitrogen) for 30 min (37 °C, 95% O_2–5% CO_2) and washed twice with Krebs solution. Fluo-4 fluorescence (F) was recorded by exciting the probe at 485 nm. Variation in the calcium concentration was normalized to the basal fluorescence (F0) as the ratio F/F0.

2.3. Extracellular ATP measurements

The luciferin-luciferase detection of ATP was performed in Krebs solution using the Tecan infinite F500 spectrofluorimeter-luminometer. NHK were isolated using the two-step trypsination procedure and cultured without 3T3 fibroblasts for 2 to 5 days at 4.10^3 cells/cm^2 in white 96-well Greiner dishes with clear bottoms. Dishes were placed in Krebs solution 10 min before the experiment in the presence of luciferin-luciferase (FLAAM, Sigma-Aldrich) at a final concentration of 0.04%. To determine the amount of ATP released from the cells, a calibration curve was also constructed using known concentrations of ATP in solution (10, 100, 1000, 10000 pM) according to product information (Sigma technical bulletin n° BAAB-1). Controls were performed with each drug solution to eliminate any drug effect on luciferase activity and to check for ATP contamination in stock solutions.

2.4. Chemicals and solutions

Experiments were performed in isotonic Krebs solution. To maintain iso-osmolarity, some solutions were prepared with an appropriate reduction in NaCl, which was replaced by D-mannitol in the corresponding control solutions. A Ca^{2+}-free external solution was prepared by substituting Ca^{2+} with Na^+ in the presence of EGTA (400 μM). All chemicals were purchased from Sigma-Aldrich. The classification of chemicals given by Bagley et al.