

Down-regulation of protein kinase C isoforms in irritant contact dermatitis

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Protein kinase C (PKC) isoforms are important in cell signal transduction associated with regulation of cell proliferation and differentiation. In this study, alterations of PKC isoform levels in irritant patch test reactions were detected by Western immunoblots. 4 chemically and structurally different irritants, 4% and 8% hydrochloric acid (HCl); 1% and 2% sodium hydroxide (NaOH); 20% and 40% nonanoic acid (NON) in 1-propanol; and 5% and 10% sodium dodecyl sulfate (SDS) were applied on the backs of mice in Finn Chambers and fixed with surgical dressings. The patches were kept on the skin for 24 h and removed. 24 h after removal, mild erythema with thickening of skin without vesicles was observed in all irritated skin, except that 4% HCl and 1% NaOH treated skin showed unremarkable skin reaction. No visible skin reaction was detected in vehicle-treated skin. PKC isoform α , β , γ , and δ levels in irritated skin revealed a 10% to 65% decrease compared to vehicle treated skin. These results indicate that in HCl, NaOH, NON and SDS-induced irritation, activation of the PKC related cell signal transduction cascade may be involved, and that PKC mediated events may be a common phenomenon in irritant contact dermatitis.

Key words: protein kinase C; irritant contact dermatitis; sodium dodecyl sulfate; sodium hydroxide; hydrochloric acid; nonanoic acid; immunoblot © Munksgaard, 1998.

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Irritant contact dermatitis (ICD) is believed to be a non-immunological skin reaction (1). The spectrum of ICD is much wider than allergic contact dermatitis (ACD) and ranges from slight scaling of the stratum corneum, through redness, whealing, and deep caustic burns, to an eczematous condition indistinguishable from ACD (1). Although ICD is much more common than ACD, the pathological mechanism of ICD is not well-defined. It is not at all clear how irritants applied to the skin cause skin inflammation. Studies have revealed distinct histopathological changes induced by different irritants, indicating the multitude of ways by which irritants may interact with components of the skin (2, 3). However, the biochemical process of ICD and the sequence of events which ultimately cause inflammatory reaction are not well understood.

In recent years, research in the field of cell signal transduction has made enormous progress. Cell response to extracellular signals, such as hormones, antigens, cytokines and growth factors, is mediated through a specific set of mechanisms, the signal transduction cascade, that regulates cell func-

tion (4, 5). In this process a system of different cellular components is assembled to guarantee a defined and successful process to keep the numerous events in cell growth, differentiation, cytokine production and adhesion molecule expression in order (4, 5). The protein kinase C (PKC) family, which comprises at least 12 isoenzymes, has been demonstrated to play an important role in the signal transduction pathway that utilizes secondary messengers generated from the turnover of membrane phospholipids (Fig. 1) (4–6). These enzymes can be divided into 3 groups: (i) conventional PKC (cPKC): (α , β 1, β 2, γ) which require charged phospholipids, diacylglycerol or phorbol ester and calcium for optimal activity; (ii) novel PKC (nPKC): (δ , θ , η /L, μ) which require charged phospholipids, diacylglycerol or phorbol esters, but no calcium for activity; (iii) atypical PKC (aPKC): (λ / τ , ς) whose activity depends only on negatively charged phospholipids (4–6).

Different PKC isoforms have shown distinct tissue distribution and specific functions (4–6). Most PKC isoforms are present in skin and investigations have revealed that PKC plays an im-

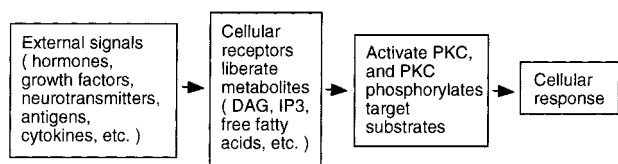


Fig. 1. Schematic view of PKC in cell signal transduction. Extracellular signals, such as hormones, growth factors, cytokines, neurotransmitters, and antigens bind to their cellular receptors, and through activation of various phospholipases, a variety of lipid metabolites are generated. Many of these, such as diacylglycerol (DAG), inositol-triphosphate (IP₃), lysophospholipids, free fatty acids, phosphatidate, as well as cardiolipin and arachidonic acid have been shown to be activators (second messengers) of PKC. Ca⁺⁺ which is required for the activation of conventional PKC, is released from the endoplasmic reticulum by the action of IP₃. Activated PKC can phosphorylate a variety of target substrates resulting in a cascade reaction leading to appropriate cell response (4–7).

portant role in proliferation and differentiation of keratinocytes, Langerhans' cells (LC) and melanocytes (7). Recently, a rôle for PKC in the migration of LC from epidermis and also in the induction of contact hypersensitivity has been demonstrated (8, 9). In a preliminary study in our laboratory, a decrease (down-regulation) of mouse skin PKC α , β , γ and δ protein levels in sodium dodecyl sulfate (SDS) induced ICD was detected. Whether this down-regulation is specific for SDS-ICD or is a general phenomenon in ICD is unknown. The aim of this study was to examine PKC isoform α , β , γ and δ levels in mouse skin patch tested with chemically and structurally different irritants: nonanoic acid (NON), hydrochloric acid (HCl), sodium hydroxide (NaOH), as well as SDS, in an attempt to elucidate the role of PKC in the pathogenesis of ICD.

Materials and Methods

Animals

10 to 12 week-old female BALB/c mice were purchased from Charles River Laboratories, Wilmington, MA.

Preparation of solutions

NaOH, HCl (Sigma Co., St. Louis, MO, USA), and SDS (Life Technologies, Inc. Gaithersburg, MD, USA) were diluted in double distilled water. NON (Sigma) was diluted in 1-propanol (Sigma). The concentrations (w/v) of irritants were: 1% and 2% for NaOH; 4% and 8% for HCl; 5% and 10% for SDS; and 20% and 40% for NON. Test concentrations were chosen according to literature reports and our own experience to obtain a mild to moderate skin reaction (2, 3, 10, 11).

Patch test and harvesting of skin samples

The backs of mice were shaved and patch test filter paper disks (Allerderm, CA), wet with test solutions (40 μ l each), were applied with Finn Chambers (Allerderm, CA) and fixed in place with surgical dressings. Patches containing vehicles (water or 1-propanol) served as controls. Each mouse was treated with 1 irritant. A total of 3 patches were applied on each mouse with 2 different concentration of irritants on one side and vehicle on the corresponding opposite side. The patches were kept on the skin for 24 h and removed. 24 h post-removal, skin biopsies (0.2 $\times$ 0.2 cm²) were obtained from the test and control sites. Skin reaction was evaluated by visual examination. The biopsies were placed epidermis side down on an ice-cold Petri dish and subcutaneous tissue was quickly removed with a razor blade. The tissues were then snap frozen in liquid N₂ until used.

Preparation of mouse skin extracts and SDS-polyacrylamide gel electrophoresis (PAGE)

Mouse skin specimens were pulverized under liquid nitrogen with a stainless steel pulverizer. The powder was then homogenized in extraction buffer containing 25 mM tris HCl (Sigma), 5 mM ethylenediaminetetraacetic acid (EDTA, Fisher Scientific Company, NJ, USA), 20 mM mercaptoethanol (Sigma), 1 mM phenylmethylsulfonyl fluoride (PMSF, Sigma), 5% glycerol, and 0.5% Triton X-100 (Sigma), as well as 10 μ g/ml pepstatin, leupeptin and aprotinin (Sigma). The homogenate was centrifuged at 13,000 $\times$ g for 60 min at 4°C. The supernatant was collected and protein concentration was measured by the method of Bradford (Bio-Rad, Hercules, CA). The proteins were separated by SDS-PAGE. 20 μ g protein was used per slot of minigel (Bio-Rad). The supernatant was mixed with 4 volumes of sample buffer [125 mM tris-HCl, pH 6.8; 10% (v/v) glycerol; 2% (w/v) SDS; 5% (v/v) β -mercaptoethanol and 0.025% bromophenol blue (Sigma)] and boiled for 4 min before loading onto the minigel. After electrophoresis, the gel was placed on a polyvinylidene difluoride (PVDF) membrane (Amersham Life Science, England) and the proteins were transferred in a wet transfer system. After transfer, the membrane was removed and rinsed in buffer containing 10 mM tris, pH 7.5, 100 mM NaCl, and 0.1% tween 20 (TBST, Sigma), air dried and stored in a desiccator at 4°C until immunodetection.

Immunoblots

The membrane blots were placed in blocking buffer containing 5% bovine serum albumin (BSA,

Sigma) in TBST for 1 h at room temperature. After washing with TBST, the blots were incubated with primary antibodies for 1 h at room temperature with agitation. The following monoclonal antibodies obtained from Transduction Laboratories, KY, USA were used for PKC isoform detection: monoclonal mouse anti PKC α (raised against an 18 kDa fragment of rat brain PKC α corresponding to amino acid positions 270–427); mouse anti PKC β (raised against a 23 kDa protein fragment corresponding to amino acids 126–324 of human PKC β which recognizes both PKC β_1 and β_2); mouse anti PKC γ (raised against 499–697 of rat PKC γ) and mouse anti PKC δ (raised against a 20.3 kDa protein fragment corresponding to amino acids 114–289 of rat PKC δ). The concentrations of anti PKC α , β , γ and δ were 0.25 $\mu\text{g/ml}$; 1 $\mu\text{g/ml}$; 0.1 $\mu\text{g/ml}$ and 0.5 $\mu\text{g/ml}$ respectively. After incubation, the immunoblots were further washed and incubated with a secondary antibody of horseradish peroxidase (HRP)-conjugated sheep anti-mouse IgG (Amersham Life Science, England) 1:5000, at room temperature for 20 min. PKC isoforms were detected by using Hyperfilm-ECL (Amersham Life Science) after reaction with ECL reagents (Amersham Life Science, England). PKC signals were quantified using a Scan Analyzer densitometer with Biosoft (Cambridge, UK) Software. The results were expressed as arbitrary densitometer units.

The immunodetection of PKC was monitored by using rat brain PKC as positive control. PKC from rat brain was purified by a procedure similar to that described by us previously (12). In brief, whole brain from a rat (Sprague-Dawley or Long-Evans) was homogenized in 7 volumes of 20 mM tris-HCl, pH 7.4 containing 2 mM EDTA, 0.5 mM ethylene glycol bis-(β -aminoethyl ether) N,N,N',N' – tetra acetic acid (EGTA), 1 mM PMSF and 10 mM mercaptoethanol. The homogenate was centrifuged at 20,000 g for 20 min and PKC was purified by sequential ion-exchange (DEAE-cellulose), molecular sieve (Sephadex G-200) and affinity (phenyl Sepharose) chromatography. All operations involving enzyme isolation were carried out at 4°C. The final preparation was stored at –80°C in small aliquots. This preparation represents a mixture of PKC isozymes present in adult rat brain and served as positive control for immunoblots.

The specificity of α , β and γ isoforms of PKC detected was verified by competitive Western blot as per Signal Transduction Laboratory (13). Skin PKC was identified by the same immunoblotting method, with the exception that the monoclonal antibodies of PKC were incubated with varying concentrations of the purified corresponding rat

brain PKC isoforms α , β and γ for 12 h at room temperature prior to the assay. For this purpose, rat brain PKC isoforms α , β and γ were isolated by further chromatography of rat brain PKC (prepared as above) on hydroxyapatite which resolved the proteins into 3 calcium-, phosphatidylserine-, and diolein-stimulated peaks: PKC γ (eluting at KPO_4 concentration of 0.14 M), PKC β (0.17 M KPO_4), and PKC α (0.19 M KPO_4) (14). To prevent any overlap between the individual isozymes, only the peak activity tubes were used for further studies. PKC activity was assayed as per published procedures (14). PKC isoforms detected by immunoblots could be completely inhibited by 100 $\mu\text{g/ml}$ of the corresponding rat brain PKC isoform isolated by us (data not shown).

Results

Visual examination of patch test reactions

Patch test of vehicles used showed no visible changes compared to normal uninvolved skin. Well-defined erythema with slight thickness of skin was seen in all irritated sites, except that 4% HCl and 1% NaOH treated skin showed unremarkable reaction. The intensity of the reactions increased with increasing concentration of irritants. No vesicles, bullae, abrasion, exudation and eschar formation were detected.

PKC α , β , γ and δ isoform expression in normal mouse skin

The immunoblots of PKC isoform α , β , γ and δ in normal mouse skin are shown in Fig. 2. The pep-

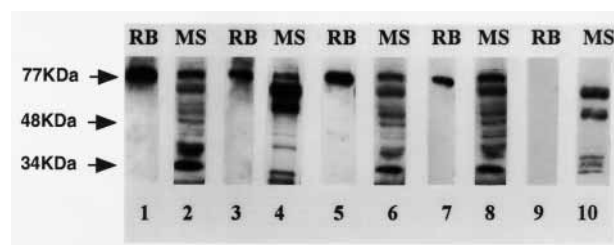


Fig. 2. Expression of PKC- α , - β , - γ and - δ in normal mouse skin. Extracts from rat brain (1.5 μg per lane) and normal mouse skin (20 μg per lane) were subjected to SDS-PAGE and analyzed by immunoblotting with mouse monoclonal antibodies against PKC α (lanes 1, 2); β (lanes 3, 4); γ (lanes 5, 6) and δ (lanes 7, 8). RB: rat brain, MS: mouse skin. Lanes 9 and 10 are negative controls which show the extracts of rat brain and mouse skin respectively, reacted with second antibody only, i.e. incubation of primary anti PKC antibody was not performed. No band is seen in extract of rat brain (lane 9). However, with mouse skin several bands at regions lower than 77 kDa are visible (Lane 10), but none around 77–80 kDa is detected indicating that the 77 kDa bands in lanes 1–8 are PKC.

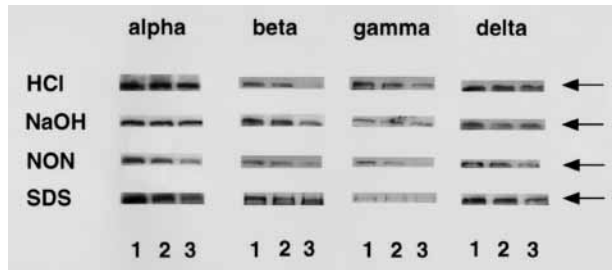


Fig. 3. Immunoblots of isoform- α (alpha); - β (beta); - γ (gamma); and - δ (delta) in HCl, NaOH, NON and SDS irritated skin. Lane 1, control; Lane 2, 4% HCl, 1% NaOH, 20% NON and 5% SDS treated skin; Lane 3, 8% HCl, 2% NaOH, 40% NON and 10% SDS treated skin. The bands in the region of 77–80 kDa molecular weight of native PKC isoform are shown. Similar data were obtained in three different mice but only the results from 1 experiment are presented.

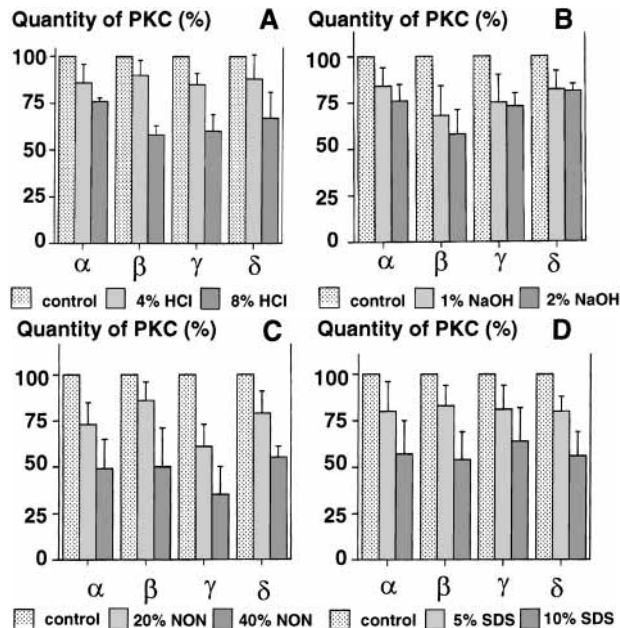


Fig. 4. Densitometric quantification of PKC isoform α , β , γ and δ in HCl (A), NaOH (B), NON (C) and SDS (D) irritated skin. Mean $\pm$ SEM (bars) of three independent experiments are shown. PKC level of 100% (arbitrary densitometer units) represents that from control skin. A 10% to 65% decrease of PKC isoform level is observed in irritated skin.

tide band in the region of 77–80 kDa molecular weight was regarded as PKC.

PKC α , β , γ and δ levels in irritated skin

The immunoblots of PKC isoforms α , β , γ and δ in irritated and control mouse skin are shown in Fig. 3. Densitometric quantification of these data, shown in Fig. 4, reveals that PKC levels in the irritated skin decreased with increasing concentration of irritants.

Discussion

This study shows that: (i) mouse skin PKC isoform α , β , γ and δ were down-regulated in HCl, NaOH, NON as well as in SDS induced irritant patch test reactions; (ii) down-regulation of mouse skin PKC, at least with regard to isoforms α , β , γ and δ , may be a general event in mouse ICD.

The PKC family is a group of serine/threonine protein kinases which plays a crucial role in cell signal transduction that regulates cell function (Fig. 1). Upon stimulation of the cell, cytosolic (soluble) PKC is translocated to the membrane (particulate fraction). Activated PKC may then be degraded by proteolytic cleavage resulting in diminished PKC activity in the cells (so called down-regulation) (4–5). A clear picture of the distribution, translocation and degradation of PKC isoenzymes within distinct signaling pathways in different cells is far from established. In some cell types, certain PKC isoenzymes are constitutively present in the particulate fraction, and depending upon the cell type and external stimuli, down-regulation, translocation without down-regulation or over expression of PKC isoenzymes could be observed (4–7). However, translocation of PKC and down regulation are generally accepted as processes involved in the activation of PKC (4–7).

Recently, Renneche et al. (15) reported differential immunological recognition of phosphorylated forms of PKC μ by different PKC μ antibodies. When keratinocytes or mouse skin in vivo was treated with the phorbol ester 12-O-tetradecanoyl-phorbol-13-acetate (PMA, a PKC activator), one of the antibodies, SC-639, could not recognize phosphorylated PKC μ , which mimicked PMA induced down-regulation. However, PKC μ level did not seem to be altered when antibody from another source, P26720 was used for detection. Since phosphorylation of PKC is a concomitant event of PKC activation (4–5), a decrease in PKC level detected by antibodies SC-639 in this case, although it may not be down-regulation, still indicates PKC activation.

In the present study of irritant patch test reactions, PKC isoforms α , β , γ and δ were found to be decreased, suggesting that in the process of ICD, activation of PKC dependent signal transduction pathway may be involved. Considering the rich content of lipids and phospholipases in skin (16), it is conceivable that when skin is exposed to irritants, phospholipid metabolites may be generated through an unknown process causing PKC activation. Studies by Elias et al. (16) have demonstrated that even in the stratum corneum, small amounts of phospholipids and abundant phospholipase are present. Furthermore, the involve-

ment of a variety of cytokines in ICD has been identified, and studies have shown that many cytokines could induce phospholipid degradation and PKC activation (4–5). Activation of PKC has also been demonstrated to be essential in cytokine expression. Lisby et al. (17) investigated the modulatory effects of irritants, dimethyl sulfoxide (DMSO) and SDS on the expression of epidermal tumor necrosis factor (TNF α), an important component in ICD. It was found that both DMSO and SDS as well as PMA were able to up-regulate TNF α mRNA and that this up-regulation could be abolished by inhibition of PKC (17). These results indicate that the expression of TNF α mRNA is PKC-dependent. Therefore, we may visualize a complicated network of PKC interaction in ICD.

Our results are consistent with the earlier observations of PMA treated mouse skin. Following topical application of PMA to mouse skin, down-regulation of PKC α , β and δ as well as skin inflammation were observed (18, 19). Reynolds et al. (20) also noted that PKC isoform β is down-regulated in SDS-induced ICD as well as in urushiol-induced ACD and atopic dermatitis.

Activation of PKC seems to be a general phenomenon in ICD. In this study, 4 different irritants at 2 different concentrations were studied: 2 acids (1 organic, NON; 1 inorganic, HCl); 1 base (NaOH) and 1 surfactant (SDS). Pathological studies have clearly indicated different but distinct changes in HCl, NaOH, NON and SDS irritated skin (2, 3, 21). Reaction to acid irritation usually includes fixation of epidermal cells and preservation of cell outlines, whereas dissolution of lipids and partial destruction of squamous cells through a breakdown of the keratin cross-links are evident in alkali irritated skin (21). Surfactants can soften squamous cells, sometimes inducing erosion with removal of lipid (21). The chemical properties of the irritants will certainly affect the mode of skin barrier penetration and the interactions with skin components and thus influence the metabolites generated. However, although the histological changes and clinical processes are dependent upon the irritant inducing ICD, similar changes in cytokine profiles and in cell-adhesion molecules have been demonstrated for ICD elicited by different irritants, indicating a common pathway in ICD (22–24). In vivo studies on skin enzyme release have shown that liberation of enzymes, such as acid phosphatase, is a common phenomenon in skin irritated with HCl, NaOH, SDS as well as acetic acid (25, 26). Therefore, it is reasonable to suggest a general involvement of phospholipid turnover and activation of PKC in ICD. Direct analysis of skin phospholipid metabolism is needed to resolve this issue.

With some irritants, down-regulation of PKC isoforms is significant only at higher concentrations (Fig. 4). Since PKC plays an important part in cell proliferation, one may suggest that down-regulation of PKC may be associated with hyperplasia during ICD. Remarkable hyperplasia observed at 24 h after removal of the irritant in 10% and 20% SDS irritated mouse skin (unpublished data) seems to support this suggestion. However, on treatment of mouse skin with 20% SDS for only 6 h, where necrosis of skin without any sign of hyperplasia was detected, PKC α , β , γ and δ were also down-regulated (unpublished data). These results suggest that decrease of PKC may not be confined to the hyperplastic skin and therefore its significance need to be investigated further.

Since PKC level in skin is regulated both by protein synthesis (mRNA controlled) and degradation, the decreased level of PKC in irritated skin may be a result of reduced expression of PKC mRNA. Human T84 cells treated with PMA induced an increase in mRNA expression of PKC δ and enzyme translocation without a change in PKC δ level (27). On the other hand, a decrease in both mRNA expression and protein level of PKC α was also noticed in these studies (27). The diversity of PKC isoforms as well as their distribution and regulation in different types of cells in response to a variety of stimuli is well known (4–7). Therefore, an examination of the levels of mRNA expression of various PKC isoforms in ICD, in progress in our laboratory, will help elucidate the role of PKC in ICD. Results presented here, using methods of immunodetection, clearly show that in ICD, (whether induced by acids, bases or surfactants), levels of PKC α , β , γ and δ are lower than in controls. Down-regulation (post activation ?) of PKC may, therefore, be a common event in ICD.

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