Recovery of gap junctional intercellular communication after phorbol ester treatment requires proteasomal degradation of protein kinase C

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Reversible down-regulation of gap junctional intercellular communication (GJIC) is proposed to be an important cellular mechanism in tumor promotion. Gap junction function is modified by a variety of tumor promoters, including the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA). Treatment of cells with TPA results in the activation and subsequent depletion of the TPA-responsive protein kinase C (PKC) isoforms. PKC-induced degradation of the PKC isoforms α, δ and ε was recently shown to occur via the ubiquitin–proteasome pathway. In the present study we investigated the role of the proteasome in the TPA-induced modification of GJIC in IAR20 rat liver epithelial cells. TPA exposure of IAR20 cells induced hyperphosphorylation of gap junction protein connexin43 and inhibition of GJIC. Prolonged TPA treatment induced down-regulation of PKCα, δ and ε and a reduction in the total PKC activity, which was associated with recovery of GJIC. Co-treatment of IAR20 cells with TPA and the proteasomal inhibitor MG132 suppressed down-regulation of PKCα, δ and ε and caused prolonged PKC activity. Under these conditions, the recovery of GJIC was blocked. The general PKC inhibitor GF109203X reversed the effect of MG132, indicating that the prolonged TPA-induced inhibition of GJIC caused by MG132 was due to the prolonged PKC activity. These results indicate that proteasomal degradation of PKC is one mechanism by which the recovery of GJIC after TPA treatment is regulated.

Introduction

Gap junctions are specialized membrane domains enriched in intercellular channels between adjacent cells (1). Each channel is formed when six integral membrane proteins, termed connexins, in each of the opposing plasma membranes become aligned. These channels are found in most animal tissues and provide for the direct transfer of ions, amino acids, nucleotides and second messengers between neighboring cells. Gap junctions have been shown to play important roles in embryonic development, cellular differentiation, and growth control. Most tumor cells have reduced or abolished gap junctional intercellular communication (GJIC), and this has led to the hypothesis that inhibition of GJIC is a critical step in multistage carcinogenesis (2–4). Supporting this hypothesis are several studies in which transformed cell lines with deficient GJIC become communication-efficient and normalize their cell growth through transfection of connexin cDNA (5–7).

A variety of tumor-promoting chemicals and oncogenes inhibit GJIC (8–13). The phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) is a highly potent tumor promoter and is extensively used for the study of mechanisms underlying tumor promotion. TPA increases connexin43 (Cx43) phosphorylation and concomitantly causes inhibition of GJIC in a number of cell types (14,15). The TPA-induced decrease in GJIC is thought to be due to a change in the permeability of the single gap junction channels (16,17). TPA has also been reported to decrease GJIC by inhibiting the assembly of Cx43 into gap junctions (18).

TPA is a protein kinase C (PKC) activator and the TPA-induced inhibition of GJIC is proposed to be due to Cx43 phosphorylation via PKC (17). PKC is a superfamily of phospholipid-dependent serine/threonine kinases, involved in a multitude of cellular processes (19). The various PKC isoforms are divided into three groups, according to the structures of their regulatory domains. These include the classical PKCs, α, βI, βII and γ, the novel PKCs, δ, ε, η and θ, and the atypical PKCs, ζ, τ, ν and μ. The various PKC isoforms show distinct cofactor requirements, biochemical regulation and tissue distributions. Moreover, the biological function and activity of members of the PKC family are closely regulated by their intracellular localization and degradation. When epithelial cells are exposed to TPA, PKC isoforms become phosphorylated and rapidly translocate from the cytosol to membranes (20–22). Prolonged stimulation leads to PKC dephosphorylation and proteolytic degradation (23). The combined effects of activation and down-regulation of PKC isoforms may play important roles in TPA-induced tumor promotion.

The ubiquitin–proteasome system plays an essential role in the selective and programmed degradation of cell cycle regulatory proteins, tumor suppressors, proto-oncogenes and components of signal transduction pathways. Dysfunction in several ubiquitin-mediated processes causes pathological conditions, including malignant transformation (24). In the ubiquitin–proteasome system, proteins are targeted for degradation by covalent ligation to polyubiquitin chains. The protein is then recognized by the 26S proteasome, a 2000 kDa ATP-dependent proteolytic complex. This barrel-shaped structure comprises the central 20S proteasome, which provides for degradation, and the 19S proteasome, which recognizes polyubiquitin-tagged proteins (25). It was recently shown that prolonged stimulation with TPA induces ubiquitination of PKCα, δ and ε (26). Similarly, activated PKCζ is ubiquitinated in HeLa cells (27) and activated PKCη is ubiquitinated in baby

Abbreviations: Cx43, connexin43; DMSO, dimethyl sulfoxide; DMEM, Dulbecco’s modified Eagle’s medium; ERK, extracellular signal-regulated kinase; GJIC, gap junctional intercellular communication; PBS, phosphate-buffered saline; PKC, protein kinase C; TPA, 12-O-tetradecanoylphorbol-13-acetate.

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hamster kidney cells (28). The ubiquitin–proteasome system has been proposed as the major route of degradation for PKC isoenzymes (29).

Phorbol esters, and other classes of tumor-promoting chemicals, usually down-regulate GJIC in a reversible fashion. It is thought that this reversible down-regulation of functional communication is an important cellular process during tumor promotion (30,31). However, the molecular mechanisms underlying the reversible inhibition of GJIC are still poorly understood. The aim of the present study was to investigate whether dysfunction in the proteasomal degradation of PKC affects the TPA-induced transient inhibition of GJIC. We use the rat liver epithelial cell line IAR20 and show that when the proteasomal degradation of the PKC isoenzymes α, δ and ε is inhibited, recovery of GJIC after TPA stimulation is blocked. We conclude that the proteasomal degradation of PKC plays an essential role in the recovery of GJIC after prolonged TPA treatment.

Materials and methods

Cells, reagents and antibodies

The rat liver epithelial cell line IAR20 was obtained from the International Agency for Research on Cancer (Lyon, France). The cells were originally isolated from normal inbred BD-Iv rats, and express Cx43, but not connexin26 or connexin32 (32,33). The cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (Gibco BRL Life Technologies, Inchinnan, UK). TPA, GF109203X, G and leupeptin were obtained from Sigma (St Louis, MO), and dissolved in dimethyl sulfoxide (DMSO). The anti-Cx43 antiserum was made in rabbits injected with a synthetic peptide consisting of the 20 C-terminal amino acids of Cx43 (34). Monoclonal antibodies for PKC α, β, δ, ε and t were obtained from Transduction Laboratories (Lexington, KY). Goat anti-rabbit and goat antimouse secondary antibodies conjugated to horseradish peroxidase were from Bio-Rad (Hercules, CA).

Determination of GJIC by scrape loading

Quantitative scrape loading was performed as previously described (35). IAR20 cells (10 × 10⁴) were plated onto 60 mm Petri dishes (Costar, Cambridge, MA) 48 h prior to experiments. To reduce the risk of cell detachment from the dish during the scrape loading procedure the growth medium was replaced with DMEM with 1% fetal bovine serum after 24 h. Before scrape loading the confluent cell layer was washed twice with phosphate-buffered saline (PBS). Two milliliters of 0.05% (w/v) Lucifer Yellow (Sigma) dissolved in PBS without Ca²⁺ and Mg²⁺ was added to the cell monolayer, which was cut 5–6 times with a surgical scalpel. After 3.5 min the Lucifer Yellow solution was removed, the dish rinsed four times with PBS, fixed in 4% formaldehyde in PBS and mounted with a glass coverslip prior to acquiring digital monochrome images by means of a COHU 4912 CCD camera (COHU Inc., San Diego, CA) and a Scion LG-3 frame grabber card (Scion Corp., Frederick, MD). Analysis was done using the public domain program NIH Image (developed at the US National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/) and Microsoft Excel (35). The levels of GJIC were determined as distance of diffusion of the dye from the scalpel cut. Exposing the cells to 30 μM chlordane for 1 h was shown to result in a complete block of GJIC. Thus, the fluorescent cells following such exposure have obtained the dye directly through the scrape process and were used to define zero GJIC. The data are presented as means ± SE relative to control. Statistically significant differences between samples in a given experiment were identified using one-way analysis of variance (ANOVA) with Tukey–Kramer multiple comparisons test.

Western blotting

Cells were seeded and treated as for the scrape loading experiments. Following exposure as indicated, the dishes were washed with PBS, the cells scraped into 500 μl of SDS electrophoresis sample buffer (10 mM Tris, pH 6.8, 15% w/v glycerol, 3% w/v SDS, 0.01% w/v bromophenol blue and 5% v/v 2-mercaptoethanol) and sonicated. The extract was heated for 5 min at 95°C, separated by 8% SDS–PAGE and transferred to nitrocellulose membranes as described (36). The blotting membranes were developed with 4-chloro-1-naphthol (Bio-Rad) for the anti-Cx43 antiserum and with chemiluminescence reagent (Pierce, Rockford, IL) for the PKC isoenzyme antibodies.

Measurement of PKC activity

PKC activity was measured using the Protein Kinase C Assay Kit RPN77 from Amersham (Little Chalfont, UK), according to the manufacturer’s instructions. Briefly, the cells were homogenized by sonication and phospholipids (phosphatidylserine), TPA, a peptide that is specifically phosphorylated by PKC and [³²P]ATP were added and incubated for 20 min at 37°C. The mixture was then applied to peptide binding paper discs and the discs were washed to remove unbound [³²P]ATP. Incorporation of [³²P] was determined by scintillation counting. It was confirmed that the assay was linear with regard to time and amount of cell extract used.

Results

Proteasomal inhibitors prevent recovery of GJIC after TPA treatment

TPA has been shown to induce inhibition of GJIC in a number of cell types (15,18). We investigated the effect of TPA on GJIC in confluent IAR20 cells by scrape loading. As shown in Figure 1A, 100 ng/ml TPA induced complete inhibition of GJIC after 30 min incubation. Figure 1B shows that TPA reduced GJIC in a dose-dependent manner. TPA-induced inhibition of GJIC has been shown to occur concomitantly with...
hyperphosphorylation of Cx43 (37). Western blots of Cx43 in untreated IAR20 cells showed three bands. These bands are referred to as NP, P1 and P2. NP has previously been shown to be the non-phosphorylated form of Cx43 and P1 and P2 two different phosphorylated forms (38). As shown in Figure 1C, TPA exposure for 15 min resulted in a shift to the P2 band, while the intensity of the NP and P1 bands decreased.

The TPA-induced inhibition of GJIC was a reversible process, in agreement with previous studies in other cell types (Figure 2A and B). After the initial rapid decline at 30 min, GJIC increased to ~40% compared with unexposed cells after 2 h of TPA treatment. After 4 h of TPA treatment, GJIC had increased to ~70%. Complete recovery of GJIC to control values in the continuous presence of TPA was observed after ~12 h (data not shown). We next wanted to investigate the possible role of the proteasome in the TPA-induced transient inhibition of GJIC. Co-incubating the cells with TPA and the specific proteasomal inhibitor MG132 did not affect the initial drop in GJIC. However, under these conditions the re-establishment of GJIC was completely abolished (Figure 2A and B). Similar results were obtained with the proteasomal inhibitor lactacystin (not shown). Thus, proteasome activity appears to be essential for the recovery of functional cell communication after exposure to TPA. The lysosomal inhibitors leupeptin (Figure 2C) and chloroquine (not shown) did not affect the recovery of GJIC after TPA treatment.

Proteasomal inhibition prevents TPA-induced degradation of PKCα, δ and ε and causes prolonged PKC activation

Proteasomal inhibitors have previously been shown to inhibit TPA-induced PKC degradation (26). We wanted to determine if the block in GJIC recovery caused by proteasomal inhibitors could be due to sustained activation of PKC. We first examined the role of PKC in the TPA-induced inhibition of GJIC in IAR20 cells, by co-incubating TPA and the general PKC inhibitor GF109203X for 30 min. Under these conditions, the TPA-induced inhibition of GJIC was prevented, indicating that TPA inhibits GJIC via PKC in IAR20 cells (Figure 3A). The PKC isoform α was recently proposed to play a major role in TPA-induced inhibition of GJIC in various cell types (39,40). To investigate the involvement of PKCα in the TPA-induced inhibition of GJIC in IAR20 cells, cells were co-incubated with TPA and the PKCα inhibitor Gö6976 for 30 min. As shown in Figure 3A, this treatment reduced the TPA-induced inhibition of GJIC. Quantification of dye coupling revealed that GJIC was increased to 90% by 3 μM GF109203X (Figure 3B) and to 70% by 3 μM Gö6976 (Figure 3C), relative to control. These results indicate that PKCα has an important role in mediating the TPA-induced inhibition of functional cell communication in IAR20 cells, but also suggest that other PKC isoenzymes are involved. These results are compatible with previous studies in other cell types (39–42).

Subsequently, the effect of TPA on the different PKC isoenzymes in IAR20 cells was studied. As shown in Figure 4, the protein level of PKC isoform α was strongly reduced by 1 h and was not detected after 4 h of TPA treatment. Isoform δ was substantially reduced by 1 h and only a weak band was detected at 4 h. Similarly, isoform ε was reduced at 1 h and barely visible at 4 h after TPA treatment. The isoform ι was present in IAR20 cells, but was not affected by TPA treatment. Similar results were obtained with isoform β (not shown). These data support an association between the down-regulation of PKCα, δ and ε and the recovery of GJIC in response to prolonged TPA exposure (Figure 2A). We next investigated the effect of proteasomal inhibition on the TPA-induced PKC depletion. The proteasomal inhibitor MG132 counteracted the TPA-induced depletion of the isoforms α...
and ε (Figure 4). In contrast, the lysosomal inhibitor leupeptin did not affect the TPA-induced degradation of PKCα or ε. MG132 also partly counteracted the TPA-induced down-regulation of isoform δ, while leupeptin did not affect the depletion. Treatment of IAR20 cells with MG132 or leupeptin alone did not appear to alter the protein level of the PKC isoforms. Thus, co-incubating TPA with the proteasomal inhibitor MG132 counteracted the TPA-induced degradation of PKCα, δ and ε observed when TPA was added alone, which could be related to the prevention of recovery of GJIC (Figure 2A).

To further examine the role of PKC degradation in the recovery of GJIC, we measured the effect of TPA on total PKC activity in IAR20 cells. As shown in Figure 5, TPA treatment for 4 h caused a significant decrease in PKC activity to ~60% compared with control cells. In contrast, co-incubating TPA with MG132 prevented the down-regulation of PKC activity observed with TPA alone. On the other hand, leupeptin did not significantly affect the TPA-induced reduction in PKC activity. MG132 or leupeptin alone did not significantly alter PKC activity in IAR20 cells. These data indicate that the proteasome is required for the TPA-induced down-regulation of PKC activity and that this process is associated with the recovery of GJIC.

Proteasomal inhibitors do not affect the recovery of GJIC when co-incubated with PKC inhibitors

Several different types of proteins with diverse functions, including Cx43, are degraded by the ubiquitin–proteasome system and could potentially be involved in the prolonged TPA-induced inhibition of GJIC caused by proteasomal inhibition. If the prolonged TPA-induced inhibition of GJIC caused by MG132 was due to sustained activation of PKC, then PKC inhibition should abolish this effect. As seen in Figure 6, cells incubated with TPA and MG132 for 1 h showed a strongly reduced GJIC, as previously shown in Figure 2. Cells treated with TPA and MG132 for 1 h and then co-incubated with vehicle (DMSO) for 3 h showed nearly complete inhibition of GJIC. In contrast, when cells were treated with TPA and MG132 for 1 h and then co-incubated with the PKC inhibitor GF109203X for 3 h, GJIC increased to ~60% compared with control cells. This value is close to the level of GJIC following exposure to TPA alone for 4 h (~70%). These results further support the above results, indicating that the sustained inhibition of functional cell communication after TPA exposure caused by MG132 is due to prolonged activity of PKC.

Discussion

Transient variation in the communication between neighboring cells is regulated by a variety of signaling pathways and is necessary for normal cell homeostasis. While most normal cells have functional GJIC, this communication is dysfunctional in most tumor cells (3,43,44). Many different classes of tumor-promoting chemicals can reversibly down-regulate GJIC, and it has been suggested that this reversible down-regulation of gap junction function is an important step during tumor promotion (30,31,45). Nevertheless, our knowledge of the molecular mechanisms involved in the inhibition and recovery of GJIC is fragmentary. Previous studies have shown that the tumor promoter TPA induces a reversible inhibition of GJIC in a number of different cell types. It is thought that TPA-induced activation of PKC induces phosphorylation of Cx43, which occurs concomitantly with modification of the gap junction channel. This PKC-mediated Cx43 phosphorylation is thought to occur directly or via extracellular signal-regulated kinases (ERKs) (17,42,46). Several mechanisms have been suggested to underlie the TPA-induced inhibition of GJIC. It has been demonstrated that TPA treatment results in a change in single channel behavior that contributes to the decrease in intercellular communication (16,17). Other studies indicated that TPA inhibited GJIC by blocking the assembly of Cx43 gap junction plaques at the plasma membrane, independent of channel permeability (18).
In the present study we have focused on the regulation of GJIC recovery after TPA treatment. TPA was previously suggested to modify GJIC via PKC, and we asked whether dysfunction in PKC degradation would affect the reversible TPA-induced inhibition of GJIC. The PKC superfamily comprises at least 12 related isoforms, and usually several isoforms are present in a single cell. These isoforms often have overlapping functions. Only the classical (α, βI, βII and γ) and the novel (δ, ε, η and θ) PKC isoforms have been found to be sensitive to phorbol esters (19). Different PKC isoenzymes are involved in TPA-induced inhibition of GJIC, depending on the cell types studied (39,40). Gø6976, a potent inhibitor of PKCa, strongly reduced the TPA-induced inhibition of GJIC in IAR20 cells, indicating that PKCa plays a predominant role in TPA-induced inhibition of GJIC in IAR20 cells. Moreover, TPA induced complete down-regulation of the PKCa protein level by 4 h after TPA exposure, which was associated with the recovery of GJIC seen at this time point. However, Gø6976 did not completely counteract the TPA-induced inhibition of GJIC, these results indicate that the PKC isoforms δ and ε might also be involved in the TPA-induced inhibition of GJIC. Indeed, PKCδ was recently suggested to be

![Fig. 4](image1.png)

**Fig. 4.** Effect of proteasome inhibition on TPA-induced depletion of PKC. IAR20 cells were treated with 100 ng/ml TPA alone or with 100 ng/ml TPA in combination with 10 μM MG132 or 100 μM leupeptin for the indicated times. The levels of PKCa, δ, ε and θ were determined by western blotting. Equal amounts of protein (15 μg) were applied in each lane.

![Fig. 5](image2.png)

**Fig. 5.** Effect of proteasome inhibition on TPA-induced down-regulation of total PKC activity. IAR20 cells were incubated with 100 ng/ml TPA alone or with the combination of 100 ng/ml TPA with 10 μM MG132 or 100 μM leupeptin for 4 h. The PKC activity was measured using the Protein Kinase C Assay System according to the manufacturer’s instructions. The error bars represent standard deviations between three independent experiments.

![Fig. 6](image3.png)

**Fig. 6.** Inhibition of PKC restores GJIC in cells treated with TPA and MG132. IAR20 cells were co-incubated with 100 ng/ml TPA and 10 μM MG132 for 1 h before addition of vehicle (DMSO) or 10 μM GF109203X for an additional 3 h incubation period in the continued presence of TPA and MG132. GJIC was then measured by scrape loading. GJIC in cells treated with 100 ng/ml TPA alone for 4 h is also shown. Statistical analysis revealed that there was a significant inhibitory effect of MG132 on the recovery of GJIC (∗∗ P < 0.05, one-way ANOVA with Tukey–Kramer multiple comparisons test), but that this effect was significantly reduced by GF109203X (∗ P < 0.05).
involved in the TPA-induced inhibition of GJIC in the Chinese hamster lung fibroblast cell line V79, while PKCζ plays a role in TPA-induced inhibition of GJIC in the rat embryo fibroblast cell line R6-C1 and Syrian hamster embryo cells (39.40).

The activity of PKC isoenzymes is closely regulated by their subcellular localization. Upon TPA treatment, classical and novel PKCs are recruited to membranes and activated (47,48). Prolonged TPA stimulation leads to PKC proteolysis, which in the case of the PKC isoforms α, δ and ε occurs by dephosphorylation, ubiquitination and degradation by the proteasome (26). Whether it is the TPA-induced activation or degradation of PKC that plays the most central role in long-term responses to TPA, such as tumor promotion, is still an open question. To investigate the role of the proteasome-dependent degradation of PKCα, δ and ε on the transient inhibition of GJIC induced by TPA, we co-incubated TPA with the proteasomal inhibitor MG132. Under these conditions the TPA-induced down-regulation of PKCα, δ and ε was counteracted and the isoforms were still present after 4 h of TPA treatment. Moreover, total PKC activity at 4 h of TPA exposure was strongly reduced, but was similar to control values when MG132 was added together with TPA. These results suggest that MG132 counteracted both the TPA-induced reduction in PKCα, δ and ε protein levels and the decrease in total PKC activity seen at 4 h incubation. This prolonged PKC activity was associated with a block in the recovery of GJIC, indicating a pivotal role for proteasome-mediated PKC degradation in the recovery of GJIC after TPA-induced inhibition.

Several different types of proteins with diverse functions, including plasma membrane channel proteins, are degraded by the ubiquitin–proteasome system. Among these proteins is Cx43 (49,50). It was previously suggested that assembly-efficient cells may have most of the cell–cell contact area occupied by gap junctions (51). Co-incubating TPA with the proteasomal inhibitor MG132 could therefore possibly cause an increase in defective gap junction channels at the plasma membrane. This could block the assembly of new gap junctions in the cell–cell contact area and thereby counteract the recovery of GJIC after TPA treatment. To determine whether the prolonged TPA-induced inhibition of GJIC induced by MG132 was due to a block in PKC degradation or to accumulation of defective gap junction channels at the plasma membrane, the general PKC inhibitor GF109203X was added to cells preincubated with TPA and MG132. Under these conditions, GJIC recovered to levels similar to when TPA was added alone. These observations indicate that the prolonged TPA-induced inhibition of GJIC caused by MG132 was due to sustained signaling from PKC, either directly or via ERKs.

In conclusion, our study suggests that proteasomal degradation of PKC is one mechanism by which the recovery of functional cell communication following prolonged TPA treatment is regulated. Given the substantial evidence that inhibition of GJIC is involved in tumor promotion, our results indicate that dysfunction in the ubiquitin–proteasome system could increase the carcinogenic effect of phorbol esters.

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References


