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Inhibition of Connexin43 Gap Junctional Intercellular Communication by TPA Requires ERK Activation

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Abstract The phorbol ester, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), is a potent inhibitor of gap junctional intercellular communication (GJIC). This inhibition requires activation of protein kinase C (PKC), but the events downstream of this kinase are not known. Since PKC can activate extracellular signal regulated kinases (ERKs) and these also downregulate GJIC, we hypothesized that the inhibition of GJIC by TPA involved ERKs. TPA treatment (10 ng/ml for 30 min) of WB-F344 rat liver epithelial cells strongly activated p42 and p44 ERK-1 and -2, blocked gap junction-mediated fluorescent dye-coupling, and induced connexin43 hyperphosphorylation and gap junction internalization. These effects were completely prevented by inhibitors of PKC (bis-indolylmaleimide I; 2 μM) and ERK activation (U-0126; 10 μM). These data suggest that ERKs are activated by PKC in response to TPA treatment and are downstream mediators of the gap junction effects of the phorbol ester. J. Cell. Biochem. 83: 163–169, 2001.

Key words: cell–cell communication; signal transduction; liver

Gap junction channels are conduits for the direct cell-to-cell exchange of small molecules and ions [Simon and Goodenough, 1998]. This is known as gap junctional intercellular communication (GJIC) and functions in electrical coupling, homeostasis, growth regulation, cell coordination, and many other physiological processes. Reduced GJIC has been associated with several human diseases that include cancer, teratogenesis, cataracts, hereditary deafness, and peripheral neuropathy.

Gap junction channels are formed by protein subunits known as connexins. At least 13 mammalian connexins exist and are expressed in a cell- and tissue-specific manner [Simon and Goodenough, 1998]. A widely studied member, connexin43 (Cx43), was first identified as the major connexin expressed in rat heart, but is also expressed in many other tissues [Beyer et al., 1987]. Gap junction assembly, channel permeability, and stability are regulated by phosphorylation, pH_I, pCa_I, reactive oxygen species, prostanoids, and other factors in a cell and connexin-specific manner. Growth factors, oncogenes, tumor promoters, teratogens, inflammatory cytokines, steroids, retinoids, carotenoids, and other agents can enhance or decrease GJIC by altering connexin expression and gap junction assembly, permeability, or stability [Ruch, 2000].

One of the most widely studied inhibitors of GJIC is the tumor promoting phorbol ester, 12-*O*-tetradecanoylphorbol-13-acetate (TPA) [Murray and Fitzgerald, 1979; Yotti et al., 1979]. Inhibition of GJIC by TPA likely contributes to alterations of growth, differentiation, and apoptosis during tumor promotion. TPA rapidly inhibits GJIC by closing gap junction channels [Oh et al., 1991] and this is dependent upon the activation of protein kinase C (PKC) [Ren et al., 1998]. In the case of Cx43-containing gap junctions, channel closure was accompanied by hyperphosphorylation of Cx43 and gap junction internalization [Matesic et al., 1994]. Although PKC activity was critical for these effects, it is still unknown whether the kinase is directly responsible or if downstream

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molecules are involved. PKC can activate many other kinases that might be responsible for the TPA effects.

The extracellular signal regulated kinases (ERKs; also known as mitogen-activated protein kinases, MAPKs) are a family of kinases that might be involved in the inhibition of GJIC by TPA. ERKs are activated by mitogens and stress and are involved in growth, differentiation, stress responses, and other cellular functions [Tian et al., 2000]. Downstream targets of ERKs include nuclear transcription factors (e.g., c-myc, ATF2, c-fos, c-jun, and Elk-1) and cytosolic proteins. Cx43 is also a substrate of ERKs in cells treated with epidermal growth factor (EGF) and this causes loss of GJIC [Warn-Cramer et al., 1998].

ERKs are also activated by PKC in phorbol ester-treated cells [El-Shemerly et al., 1997; Stadheim and Kucera, 1998]. We therefore hypothesized that the inhibition of GJIC in TPA-treated cells was due to PKC-activated ERKs. To test this, we have used specific inhibitors of PKC and ERKs and demonstrate that activated ERKs are essential for the inhibition of GJIC, Cx43 hyperphosphorylation, and gap junction internalization in TPA-treated WB-F344 rat liver epithelial cells.

MATERIALS AND METHODS

Cell Culture and Treatment With Inhibitors

WB-F344 rat liver epithelial cells are diploid and nontumorigenic [Tsao et al., 1984]. The cells were cultured in Richter's Improved MEM (Irvine Scientific, Irvine, CA) supplemented with fetal bovine serum (5% v/v) and gentamicin sulfate (40 μg/ml) as described by Ren et al. [1998].

Confluent cell monolayers were treated with TPA (Sigma Chemical Co., St. Louis, MO) (10 or 100 ng/ml final concentration) for 30 min. TPA was predissolved in dimethylsulfoxide (DMSO; Sigma) and control cultures were treated with DMSO (1 μl/ml). In some cases, the cells were pretreated with U-0126 (10 μM; Calbiochem-Novabiochem; San Diego) or BIM I (also known as GF109203X; 2 μM; Calbiochem-Novabiochem) for 1 h prior to TPA or DMSO.

Dye-Coupling Assay of GJIC

GJIC was detected by microinjection of fluorescent Lucifer Yellow CH dye and enumeration of dye spreading to neighboring cells (dye-coupling assay) as described by Ren et al. [1998]. For each treatment condition, three replicate cultures were sampled and 10 cells were microinjected per culture. The percentage of cells directly adjacent to microinjected cells (first-order neighbors) that took up dye was determined. Statistical analyses were performed using 2×2 Chi-squared tests.

Western Blotting

Levels and phosphorylation status of Cx43 and p42 and p44 ERKs (ERK-1 and -2) were analyzed by Western blotting. For Cx43, membrane-enriched cell extract (40 μg of protein per sample) was separated by 12% SDS–PAGE and transferred to Hybond N+ membrane, then Cx43 was detected using a mouse monoclonal anti-Cx43 antibody (Zymed, South San Francisco, CA; cat. 03-6900) that recognizes phosphorylated and nonphosphorylated forms of Cx43 as described by Guan et al. [1995]. Equal sample loading was determined by staining the membranes with Ponceau S.

Both total and phosphorylated ERK-1 and -2 were evaluated by Western blotting. Fifty microgram of total cellular protein per sample was separated by 10% SDS–PAGE and transferred to supported nitrocellulose membranes. Equal loading of the samples was verified by staining the membranes with Ponceau S. Total and phosphorylated ERK-1 and -2 were detected using specific antibodies and the enhanced chemiluminescence (ECL) detection kit (Amersham-Pharmacia Biotech, Piscataway, NJ) as described previously [Fischer et al., 1999]. Phosphorylation state-specific ERK antibody (New England Biolabs Inc., Beverley, MA) was used at a dilution of 1:1,000. The antibody only recognizes serine and tyrosine phosphorylated ERK-1 and -2. Phosphorylation state-independent ERK antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) was used at a dilution of 1:5,000. This antibody recognizes ERK-1 and -2 independent of phosphorylation state and indicates their total levels.

Immunofluorescence Staining of Cx43

Cx43 was detected in WB-F344 cells grown on glass coverslips by immunofluroescence staining using a mouse monoclonal anti-Cx43 antibody (Zymed #03-6900) as described by Guan et al. [1995].

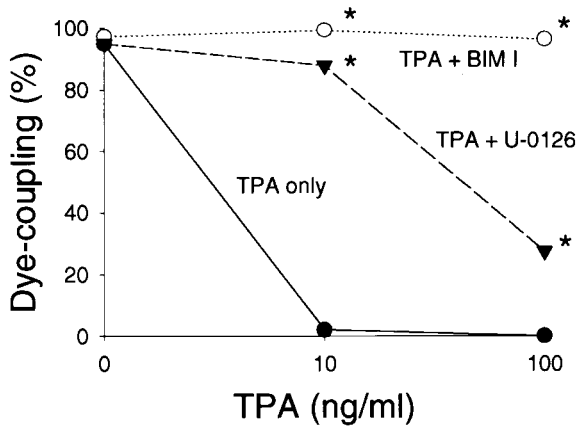

Fig. 1. Inhibition of GJIC (dye-coupling) by TPA and protection by U-0126 and BIM I in WB-F344 cells. The cells were pretreated with U-0126 (10 μM), BIM I (2 μM), or solvent vehicle (ethanol or DMSO; 1 μl/ml) for 1 h and then with TPA (T; 10 or 100 ng/ml) or solvent vehicle (DMSO; 1 μl/ml) for 30 min. GJIC was then determined by microinjection of fluorescent Lucifer Yellow CH dye. Three cultures were sampled per treatment and the data pooled. The asterisk (*) indicates a significant difference ($P < 0.01$) compared to the TPA only group (C).

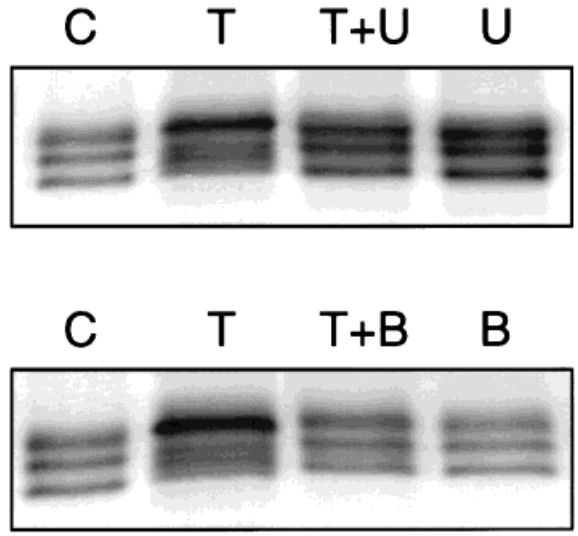

Fig. 2. Western blot analyses of Cx43 in control WB-F344 cell cultures (C) or cells treated with TPA (T; 10 ng/ml), U-0126 (U; 10 μM), or BIM I (B; 2 μM) as described in Figure 1.

RESULTS

Control WB-F344 cells exhibited a high level of GJIC (94.8%) determined by fluorescent dye microinjection (Fig. 1). Treatment of these cells with TPA (10 ng/ml for 30 min) strongly decreased dye-coupling (2.1%) as previously reported by Matesic et al. [1994]. Pretreatment of the cells with U-0126 (10 μM) or BIM I (2 μM) for 1 h followed by subsequent treatment with TPA (10 ng/ml) completely prevented the loss of dye-coupling. However, if the cells were treated with a higher concentration of TPA (100 ng/ml), U-0126 could only partially prevent the loss of dye-coupling (Fig. 1). BIM-I was still completely protective. Treatment with U-0126 and BIM I alone did not affect dye-coupling. In subsequent experiments, 10 ng/ml TPA was used since U-0126 was most effective with this concentration.

Western blot analyses of Cx43 revealed three major forms of the protein in control cells (Fig. 2). In previous studies, treatment of the cellular extract with alkaline phosphatase before SDS–PAGE resulted in the migration of all Cx43 at the lowest band position [Guan et al., 1995]. This indicates that the lowest band was nonphosphorylated and the upper bands were phosphorylated. Treatment of WB-F344 cells with TPA (10 ng/ml) increased the amount of the upper phosphorylated Cx43 species and decreased the lower isoforms. This upward shift of Cx43 bands has been referred to as Cx43 hyperphosphorylation [Matesic et al., 1994]. Co-treatment of TPA-treated cultures with U-0126 or BIM I prevented this (Fig. 2).

Immunostaining revealed prominent Cx43-positive gap junction plaques located between adjacent cells in control cultures (Fig. 3). Treatment with TPA (10 ng/ml) induced the internalization of these into the cytoplasm and fewer plaques at the plasma membrane. This was prevented by co-treatment with U-0126 and BIM I (Fig. 3).

Western blot analyses using an antibody for ERK-1 and -2 that recognizes only the activated (phosphorylated) forms revealed that TPA (10 ng/ml) strongly activated the ERKs and that co-treatment with U-0126 or BIM I completely suppressed this (Fig. 4, top panel). Western blotting using a phosphorylation-state independent ERK antibody revealed that the total content of p42 and p44 ERKs were similar after each treatment (Fig. 4, bottom panel).

DISCUSSION

We have provided evidence that the inhibition of GJIC and induction of Cx43 hyperphosphorylation and gap junction internalization by TPA in WB-F344 rat liver epithelial cells is due to PKC-activated ERKs. Previous studies

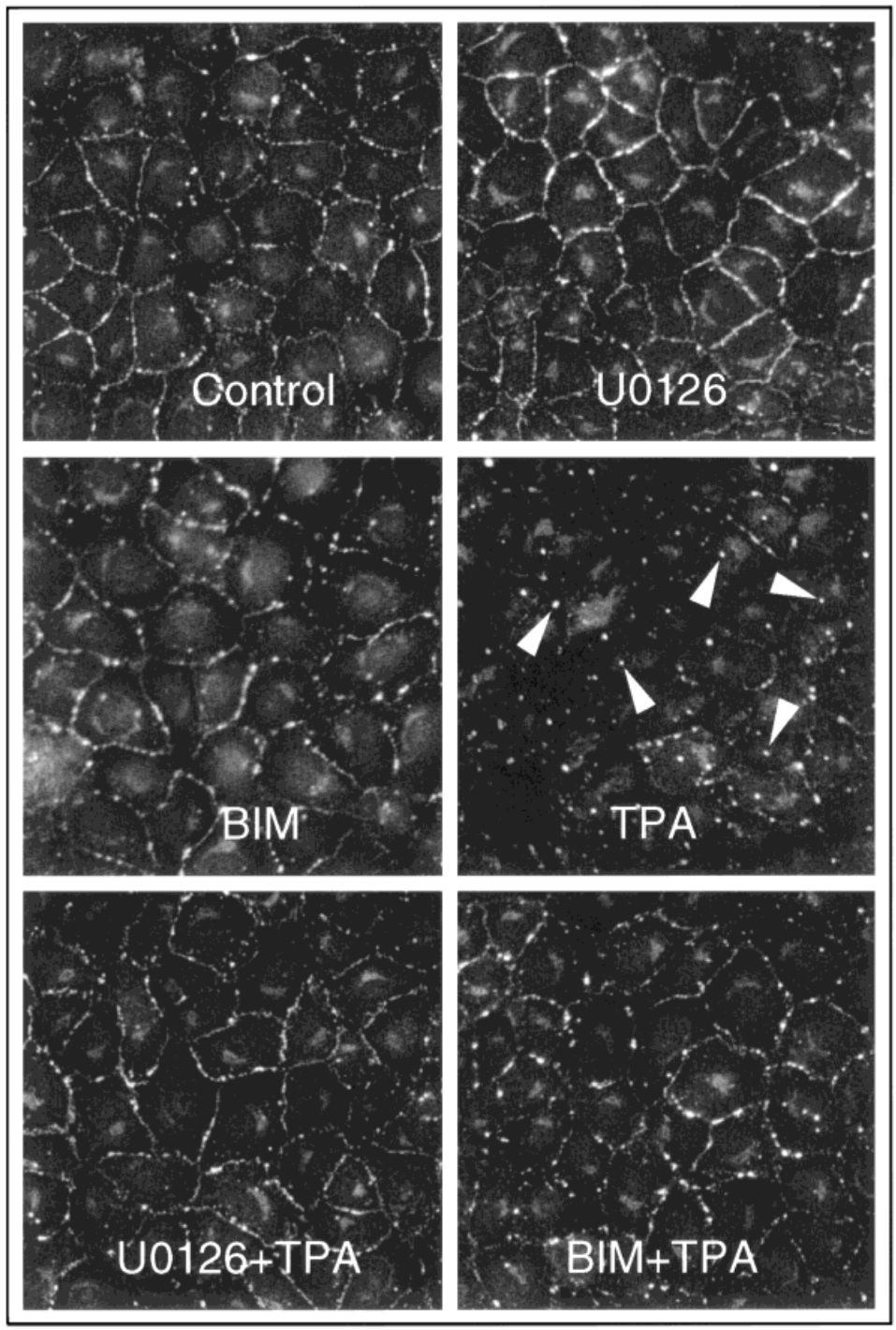

Fig. 3. Cx43 immunostaining of WB-F344 cells treated with TPA (10 ng/ml), U-0126 (10 μM), or BIM I (2 μM) as described in Figure 1. The arrowheads indicate internalized Cx43 gap junctions.

have shown that activated PKC in turn activates ERKs [El-Shemerly et al., 1997; Stadheim and Kucera, 1998] and that ERKs activated by epidermal growth factor phosphorylate Cx43 resulting in the loss of GJIC [Warn-Cramer et al., 1998]. Additionally, there is evidence that PKC can directly phosphorylate Cx43 in viable cells and is responsible for the loss of dye-coupling [Lampe et al., 2000]. Our study, however, is the first to demonstrate that the inhibition of GJIC by TPA results from PKC-activated ERKs.

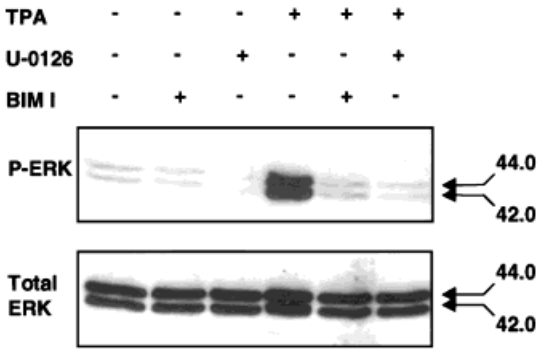

Fig. 4. Western blot analyses of phosphorylated (active) p42 and p44 ERKs (P-ERK; top panel) and total ERK (bottom panel) in WB-F344 cells treated with TPA (10 ng/ml), U-0126 (10 μM), or BIM I (2 μM) as described in Figure 1.

The ability of the PKC inhibitor, BIM I, to prevent the effects of TPA on WB-F344 cell gap junctions reiterates that classical or novel PKC(s) were involved. Loss of dye-coupling, Cx43 hyperphosphorylation, and gap junction internalization were completely prevented by BIM I in cells treated with 10 or 100 ng/ml TPA. There are 12 known mammalian isozymes of PKC that are divided into three subgroups [Dempsey et al., 2000]. The classical PKCs (α, βI, βII, and γ) are activated in a Ca^{2+}-dependent manner by diacylglycerol (DAG). The novel PKCs (δ, ϵ, η, and θ) are activated by DAG independently of Ca^{2+}. Lastly, the atypical PKCs (ζ and λ) are Ca^{2+}-independent and not activated by DAG. TPA mimics DAG and thus activates only classical and novel PKCs. BIM I is a potent and highly specific inhibitor of these, but not the atypical PKCs [Toullec et al., 1991; Gschwendt et al., 1996]. Thus, the preventive actions of BIM I implicate classical and/or novel PKC(s) as the mediators of the effects of TPA on GJIC and Cx43 in agreement with other data [Husøy et al., 2001].

The results also demonstrate that PKC-activated ERKs are essential for the effects of TPA on WB-F344 gap junctions. TPA (10 ng/ml) activated ERK-1 and -2 and this was completely inhibited by BIM I which also completely suppressed the gap junction effects of TPA at this concentration. Like BIM I, the ERK kinase inhibitor, U-0126 [Favata et al., 1998], also completely suppressed ERK activation and the gap junction effects of 10 ng/ml TPA. A second highly specific inhibitor of ERK activation, PD98059, produced similar results (data not shown). The simplest interpretation of these results is that TPA activates ERKs via PKC and that ERKs cause the gap junction effects. It is not clear, however, whether the activated ERKs are directly responsible for the TPA effects or whether downstream signals or kinases are involved. Warn-Cramer and co-workers [Warn-Cramer et al., 1998] provided evidence that ERKs were directly responsible for the loss of dye-coupling and Cx43 phosphorylation in EGF-treated cells, but such evidence is lacking for TPA-activated ERKs. Clearly, however, when cells are treated with TPA at a concentration of 10 ng/ml, PKC is not directly responsible because U-0126 completely protected the cells. This contrasts with a recent study in T51B rat liver epithelial cells where a higher dose of TPA (100 ng/ml) blocked dye-coupling and induced the direct phosphorylation of Cx43 by PKC on Ser-368 [Lampe et al., 2000]. However, Cx43 phosphorylation by other unidentified kinase(s) was also apparent in these cells. A study in neonatal rat cardiac myocytes, however, did not find evidence that TPA-activated PKC was directly responsible for Cx43 phosphorylation [Saez et al., 1997]. We found that U-0126 was only partially protective when cells were treated with a high concentration (100 ng/ml) of TPA (Fig. 1). Together, these studies suggest that PKC-activated ERKs are responsible for the loss of GJIC and Cx43 phosphorylation at low concentrations of TPA (e.g., 10 ng/ml), but at higher concentrations (e.g., 100 ng/ml), PKC may have a direct effect on the gap junctions. Perhaps high concentrations of TPA activate more PKC types that can directly act on Cx43.

A correlation between the loss of dye-coupling, Cx43 hyperphosphorylation, and gap junction internalization was observed in TPA-treated WB-F344 cells [this study and Matesic et al., 1994]. It is unknown whether these processes are interdependent. Hyperphosphorylation could induce a conformational change in Cx43 that closes the gap junction channels and triggers internalization of the plaques. However, loss of dye-coupling can occur in the absence of Cx43 hyperphosphorylation in TPA-treated cells [Husøy et al., 1993]. Similarly, the pesticides, DDT and lindane, close gap junction channels and induce gap junction internalization in the absence of Cx43 hyperphosphorylation [Guan and Ruch, 1996]. Densitometric analyses of Cx43 bands in TPA-treated cells indicated no net loss of Cx43

following gap junction internalization which suggests that this internalized Cx43 is recycled to the cell membrane [Matesic et al., 1994]. In contrast, the internalized gap junctions induced by DDT and lindane are degraded in lysosomes [Guan and Ruch, 1996].

TPA was also reported to inhibit the assembly of Cx43 gap junctions during the reaggregation of dissociated Novikoff rat hepatoma cells [Lampe, 1994]. This inhibition was correlated with increased Cx43 phosphorylation, but not with reduced plasma membrane or total Cx43 content. Interestingly, TPA (50 nM) had no effect on preexisting gap junctions in monolayer cultures of these cells. These data illustrate that TPA affects Cx43 gap junctions at multiple levels and that its actions are cell type-specific.

TPA also inhibited GJIC mediated by other connexins (Cx26, Cx32, and Cx40), but the mechanisms are less well characterized than for Cx43. The inhibition of Cx32-mediated GJIC was demonstrated in a Cx32-transduced variant of WB-F344 cells and likely involved PKC [Ren et al., 1998]. Co-treatment of the cells with BIM I or downregulation of PKC by prolonged pretreatment with TPA prevented the inhibition of dye-coupling by the phorbol ester. Interestingly, internalization of Cx32-containing gap junctions was not stimulated by TPA. The phorbol ester also inhibited GJIC in HeLa cells that were transfected with Cx26, Cx32, or Cx40 [Mazzoleni et al., 1996]. Chronic treatment with TPA made Cx32- and Cx40-, but not Cx26-transfected cells, refractory and again suggests that PKC was involved. The role of ERKs in these systems has not been investigated.

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