Protein Kinase C-α Activity Modulates Transepithelial Permeability and Cell Junctions in the LLC-PK₁ Epithelial Cell Line*

(Received for publication, February 18, 1997, and in revised form, March 26, 1997)

Dan Rosson‡§, Thomas G. O’Brien‡, Jennifer A. Kampherstein‡, Zoltan Szallasi¶, Krisztina Bogi¶, Peter M. Blumberg§, and James M. Mullin‡

From the ‡Lankenau Medical Research Center, Wynnewood, Pennsylvania 19096-3411 and the ¶Laboratory of Cellular Carcinogenesis and Tumor Promotion, NCI, National Institutes of Health, Bethesda, Maryland 20892-4255

Modulation of protein kinase C (PKC) by 12-O-tetradecanoylphorbol-13-acetate (TPA) disrupts the cell-cell junctions of the epithelial cell line LLC-PK₁. To examine the role of specific PKC isoforms in this process we have created modified LLC-PK₁ subclones that express wild-type and dominant negative versions of PKC-α under control of the tetracycline-responsive expression system. Overexpression of wild-type PKC-α rendered the cells more sensitive to the effects of TPA on transepithelial permeability as measured by loss of transepithelial resistance across the cell sheet. Conversely, expression of a dominant negative PKC-α rendered the cells more resistant to the effects of TPA as measured both by loss of transepithelial resistance as well as cell scattering. The properties of both subclones could be modulated by the addition of tetracycline, which suppressed the effect of the exogenous genes. These results indicate that the α isoform of PKC is at least one of the isoforms that regulate tight junctions and other cell-cell junctions of LLC-PK₁ epithelia.

Epithelial cells cover almost all internal and external body surfaces. All types of epithelia are polarized with apical and basolateral surfaces that have different membrane proteins and thereby different functions. The cells thus compartmentalize the tissues of which they are a part. The cells exist in sheets in which individual cells are interconnected by various types of cell-cell junctions, the regulation of which is at play during development, wound healing, and pathological processes such as cancer or chronic inflammation. Therefore, the control of cell-cell junctions and their disruption and reformation is of interest in several fields.

The LLC-PK₁ cell line (1) offers an advantageous model system for studying this process. The cell line grows subconfluently as islands of adherent cells. Upon reaching confluence, it forms a differentiated epithelial monolayer with functionally intact tight junctions. Treatment of subconfluent cultures with the tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA)³ causes a breakdown of the cell junctions resulting in a more scattered growth pattern. Acute exposure of LLC-PK₁ confluent cell sheets to TPA causes a rapid decrease in the transepithelial resistance (TER) to less than 15% of its initial value (2) because of its effect on one type of cell junction known as tight junctions.

We have been interested in the molecular events in this process and have concentrated much of our efforts on protein kinase C (PKC), the molecular target of TPA. PKC has been shown to be one of the process's key components. Treatment of LLC-PK₁ cells with the PKC inhibitor GF109203X inhibits the effects of TPA (3). PKC mediates calcium-induced tight junction assembly (4), and its inhibition prevents the proper distribution of tight junction-associated proteins such as ZO-1 and cingulin (5). One of the issues in PKC-mediated cell junction regulation is which specific isoform(s) is at play. PKC is not a single protein, as the name implies, but a family of proteins with at least 11 different members (6). Although each member or isoform is encoded by a separate gene, they all consist of conserved regulatory and catalytic regions. The isoforms can be divided into four classes based on regulatory properties. The first class, the conventional PKCs α, β, and γ, is regulated by calcium. The second, nonconventional or novel class, is made up of PKCs δ, ε, η, and θ, which are not regulated by calcium. The third class, the atypical PKCs ϵ, ζ, and η, is not regulated by calcium or TPA. A recently discovered isoform, PKC-μ, represents a fourth class, which is topologically related to the PKC family but possesses a kinase domain more related to calcium/calmodulin-dependent kinases. The complexity of the PKC family and the differential expression of the isoforms suggest that the members serve distinct roles in signal transduction processes. To examine this issue, as it relates to PKC-mediated cell junction regulation in LLC-PK₁ epithelial cells, we have begun to modulate the activities of individual isoforms by exogenously expressing wild type and dominant negative versions of the genes in an inducible expression system. LLC-PK₁ cells express the α, δ, ε, and ζ isoforms of PKC, and, in this study, we report that alteration of the activity of the α isoform of PKC modulates the integrity of cell-cell junctions as measured by sensitivity to TPA.

EXPERIMENTAL PROCEDURES

Construction of Expression Vectors—Expression vectors comprising the tetracycline-responsive expression system described by Gossen and Bujard (7) were used to express both wild-type and dominant negative versions of PKC-α. cDNA encoding the entire reading frame of wild-type PKC-α was excised from its original vector (8) by digestion with EcoRI and recloned into the EcoRI site of the tetracycline-responsive vector pUHD10-3 creating pUHD10-3a. A dominant negative version of the gene was created by in vitro mutagenesis replacing the conserved isoleucine in the ATP binding domain in position 368 with an alanine. The XcmI/BsuWI fragment spanning

---

* This work was supported by National Institutes of Health Grants CA36353 (to T. G. O.) and CA48121 (to J. M. M.) from the NCI. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Lankenau Medical Research Center, 100 Lancaster Ave., Wynnewood, PA 19096-3411. Tel.: 610-645-3420; Fax: 610-645-2205.

§ Present address: Dept. of Pharmacology, Uniformed Services University of the Health Sciences, Bethesda, MD 20814.

∥ The abbreviations used are: TPA, 12-O-tetradecanoylphorbol-13-acetate; TER, transepithelial resistance; PKC, protein kinase C; tTA, tetracycline-responsive transactivating protein.
the Lys → Ala mutation was then excised from its expression vector to replace the analogous fragment in pUHD10-3a vector creating pUHD10-3aDN.

Cell Cultures and Transfections—LLC-PK₁ cells were maintained in minimum essential medium α supplemented with 10% fetal bovine serum (HyClone Laboratories). When tetracycline was present, its concentration was 1 µg/ml. For transfection, cells were harvested by trypsinization, washed, and 5 × 10⁶ cells were resuspended in 0.8 ml of phosphate-buffered saline in an electroporation cuvette (0.4 cm). 20 µg of pUHD15-1, which encodes the bacterial transactivator tTA, along with 5 µg of a plasmid conferring resistance to hygromycin, were added to the suspension. This was subjected to one pulse (300 V, 250 microfarad) in a Bio-Rad electroporator. Cells were then plated onto three 10-cm tissue culture plates, incubated for 1 day, then adjusted to 0.1% sodium dodecyl sulfate and proteinase inhibitors. DNA was sheared to reduce viscosity, and the protein concentration was determined through the course of resistance decreases. Measurements were done in triplicate, and all results are representative of at least three experiments.

TPA-induced Cell Scattering—For assessment of cell scattering 3 × 10⁶ cells were seeded in 24-well plates in 1 ml of medium containing 10⁻⁸ M TPA. Growth patterns were followed by light microscopy and photographed 3 days after seeding.

RESULTS

Since we have noted previously that TPA treatment of LLC-PK₁ cells is associated with a translocation of PKC-α from the cytosolic fraction of the cells to the membrane fraction (9), we decided to modulate the activity of PKC-α by exogenous expression of the gene. Because randomly isolated sublines can vary from the parental in any given property, there is consequent difficulty in ascribing any phenotypic change to the expression of an exogenous gene. We chose to avoid the problems of clonal variation by utilizing the tetracycline-responsive expression system of Gossen and Bujard (7) to overexpress PKC-α. In the first step, we cotransfected into LLC-PK₁ cells a plasmid expressing tTA and a plasmid conferring hygromycin resistance. After screening drug-resistant clones for tetracycline-repressible expression of tTA, we selected a subline, LLC-PK₁tTA, for additional experiments. Next, pUHD10-3 PKC-α was transfected into LLC-PK₁tTA using pSVzeo (Invitrogen) as a coSelectable marker. 20 drug-resistant clones were analyzed for expression of PKC-α, and one was found to have elevated levels. Fig. 1 shows Western analysis of extracts of cells cultured with and without tetracycline, expressing exogenous PKC-α. Parental cells exhibited a single protein of approximately 75 kDa. LLC-PK₁tTA cells grown in the absence of tetracycline exhibited PKC-α levels that were 10–20 times higher. The same subline grown in the presence of tetracycline showed levels that were almost equal to levels in the parental LLC-PK₁tTA line. Phenotypically, the cells resemble the parental line, growing as islands of coherent cells. Upon reaching confluence, both the transfectant and the parental line remain a single layer and differentiate into a polar epithelial-like cell sheet with apical and basolateral surfaces. Both lines form dome-like or cystic structures as a result of the vectorial transepithelial transport and the tight junctions of the cell sheet. However, as seen in Fig. 2, not only do domes collapse when treated with TPA, but the transfected cells rapidly round up and begin to detach from the dish. When expression of the exogenous PKC-α was down-regulated by the addition of tetracycline, the normal dome collapse occurred on TPA treatment but not the cell rounding and detaching. The disruption of tight junctions was assessed quantitatively in measurements of TER decreases on TPA treatment. As seen in Fig. 3, cells overexpressing PKC-α showed a more rapid response to all concentrations of TPA tested compared with wild type LLC-PK₁. However, when these cells were grown in the presence of tetracycline, TER
PKC-α Modulates Epithelial Cell Junctions

Physiologically, the effect of PKC-αDN was the opposite of its wild-type counterpart. LLC-PK1αDN cells grown in the absence of tetracycline showed a much slower response to 10^{-8} M TPA than did parental counterparts. As seen in Fig. 5, at 1 h of treatment, TERs of LLC-PK1αDN were 80% of their initial value, whereas the parental cells had decreased to 25%. The degree of tetracycline responsiveness of the system is also seen in electrical measurements with LLC-PK1αDN cells grown in the presence of tetracycline which showed responses to TPA nearly identical to those of parental LLC-PK1tTA.

We next assessed the effects of PKC-αDN expression on growth patterns of the line. LLC-PK1, like most epithelial lines, grow as islands of coherent cells. Treatment of subconfluent cultures with TPA disrupts the cell junctions that produce this compact architecture. The result is a more fibroblastic growth pattern with individual cells migrating away from the island borders.

FIG. 4. Western analysis of LLC-PK1αDN. Whole cell extracts were prepared, quantitated, and subjected to Western analysis as described under “Experimental Procedures.” The sources of the protein extracts are as follows: lanes 1 and 2, LLC-PK1tTA; lanes 3 and 4, LLC-PK1αDN clone 1; lanes 5 and 6, LLC-PK1αDN clone 2; lanes 7 and 8, LLC-PK1αDN clone 3; lanes 9 and 10, LLC-PK1αDN clone 4. In odd numbered lanes, the extracts were prepared from cells grown in the absence of tetracycline; even numbered lanes are prepared from cells grown in the presence of tetracycline. □, wild-type.
because PKC is the major receptor for tumor-promoting phorbol esters such as TPA, the long history of tumor promotion research has led to a wealth of information indicating that the modulation of PKC activity is a key step in tumorigenesis as well. The development of an invasive tumor involves a disruption of normal cell-cell junctions that serve to maintain tissue architecture. Additionally, more recent studies have indicated that the mechanism of action of many growth factor receptors involves activation of PKC. This occurs via phospholipase C, which produces diacylglycerol, which binds to and activates the enzyme in the same manner as TPA. The receptor for scatter factor or hepatocyte growth factor, c-Met, is an example of such a receptor, indicating that PKC plays a role in producing a response to this cytokine.

Soon following the initial discovery of PKC, the activity was found to consist of more than one species. With the advent of techniques in molecular biology, the family of isoforms has since grown to include 11 members (6). Differences in tissue distribution, subcellular distribution, and substrate specificity suggest that there is a divergence of function among the isoforms. However, despite the abundance of PKC research, little information is available on the roles of individual isoforms. Since inhibitors of the enzyme are not specific for individual isoforms, we and others have begun to address this issue by the use of exogenous expression studies. This technique, which utilizes both wild-type and dominant negative versions of the gene to modulate the activity of specific individual isoforms, is beginning to indicate which isoforms are involved in particular processes.

We have begun examining the role of the α isoform in the disruption of cell junctions. Calcium plays an established role in the maintenance of various cell junctions including tight junctions. Calcium plays an established role in the maintenance of various cell junctions including tight disruption of cell junctions. Furthermore, in cells chronically treated with TPA, which form uneven cell sheets of single and multilayered areas, PKC-α stays up-regulated in areas in which there is multilayering, which also is where tight junctions are most leaky (9). Ellis et al. (13) report that an LLC-PK1 subline that decreases and then rapidly recovers its TER in response to TPA also rapidly down-regulates its PKC activity, whereas a subline whose TER does not recover in the presence of TPA does not down-regulate its PKC.

Our results show that a direct correlation exists between activity levels of PKC-α and the sensitivity of LLC-PK1 cell junctions to TPA; that is, up-regulation by expression of exogenous wild-type PKC-α enhances sensitivity, and down-regulation by expression of the dominant negative form decreases sensitivity. Although in vitro studies of PKC show a lack of specificity toward certain substrates, several literature studies have reported that the technique of exogenous expression of individual members of the PKC family produces results specific for individual isoforms. For example, Li et al. (14) expressed six different isoforms of PKC in 32D cells and reported that only the expression of the δ isoform rendered the cells sensitive to TPA-induced differentiation. In experiments with NIH 3T3 cells, the δ isoform inhibited cell growth, whereas the ε isoform rendered the cells tumorigenic (15). Similarly, Baier-Bitterlich et al. (16) reported that only the θ isoform was competent in stimulation of AP-1 activity in T-lymphocytes. This specificity might be explained in part by the fact that isoforms are compartmentalized differently in cells, conferring specificity to given substrates by spatial accessibility. For example, PKC-δ has been localized to the cytoskeleton of HL-60 cells (17), and PKC-ε has been localized by immunoelectron microscopy in Madin-Darby canine kidney epithelia to the region of the zonula occludens and/or zonula adherens (18). Transfection of endothelia with antisense PKC-β blocked the phorbol ester-induced increase in tight function permeability normally seen in these cell sheets (19). Similarly, overexpression of PKC-β yields an endothelial cell sheet with a dramatically increased effect of phorbol ester on tight junction permeability (20). We have begun to examine other isoforms of PKC in terms of the effect they have on regulating cell junctions in LLC-PK1 and are finding similar results.

Our work represents a significant step toward identifying the relevant isoforms of PKC in regulating cell junctions. More work is necessary to identify the mechanism of this regulation. Many putative targets of PKC have been identified which are associated with cell junctions. Among these are c-Raf (21), vinculin (22), talin (22), MARCKS (23), glycogen synthase kinase-3 (24) and focal adhesion kinase (25). PKC-mediated modulation of any one of these might conceivably play a role in reorganizing cytoskeletal structures during TPA-induced cell migration and disruption of cell junctions.

Acknowledgments—We thank Drs. Gossen and Bujard for plasmids making up the tetracycline-responsive expression system and Dr. Shigeo Ohno for the cDNA for wild type PKC-α.

REFERENCES

1. Hull, R. N., Cherry, W. R., and Weaver, G. W. (1976) In Vitro 12, 670–677
2. Mullin, J. M., and O’Brien, T. G. (1986) Am. J. Physiol. 251, C597–C602
3. Marano, C. W., Laughlin, K. V., Russo, L. M., and Mullin, J. M. (1995) Biochem. Biophys. Res. Commun. 209, 669–676
4. Balda, M. S., Gonzalez-Mariscal, L., Matter, K., Cereijido, M., and Anderson, J. M. (1993) J. Cell Biol. 123, 293–302
5. Nigam, S. K., Denisenko, N., Rodriguez-Boulan, E., and Citi, S. (1991) Biochem. Biophys. Res. Commun. 181, 548–553
6. Dekker, I. V., and Parker, P. J. (1994) Trends Biochem. Sci. 19, 51–58
7. Gossen, M., and Bujard, H. (1992) Proc. Natl. Acad. Sci. U.S.A. 89, 5547–5551
8. Ohno, S., Akita, Y., Konno, Y., Imajoh, S., and Suzuki, K. (1988) Cell 53, 731–741
9. Mullin, J. M., Peralta Soler, A., Laughlin, K. V., Kamphierstein, J. A., Russo, L. M., Saladik, D. T., George, K., Shurina, D. R., and O’Brien, T. G. (1996) Exp. Cell Res. 227, 12–22
10. Newton, A. C. (1995) J. Biol. Chem. 270, 28485–28498
11. Jovov, B., Lewis, S. A., Crowe, W. E., Berg, J. R., and Willis, N. K. (1994) Am. J. Physiol. 266, F775–F784
12. Diugos, A. A., Cheng, C., Williams, E. K., Dharia, A. G., Denning, M. F., and Yuasa, S. H. (1994) Cancer Res. 54, 6413–6420
13. Ellis, Š., Schneeberger, E. E., and Rabito, C. A. (1992) Am. J. Physiol. 263, F293–F300
14. Li, W., Mischak, H., Yu, J.-C., Wang, L.-M., Mushiinsky, J. F., Heidaran, M. A., and Pierce, J. H. (1999) J. Biol. Chem. 274, 2349–2352
15. Mischak, H., Goodnight, J., Kolch, W., Martiny-Baron, G., Schaechtel, C., Kazaniats, M. G., Blumberg, P. M., Pierce, J. H., and Mushiinsky, J. F. (1993) J. Biol. Chem. 268, 6090–6096
16. Baier-Bitterlich, G., Überall, F., Bauer, B., Presser, F., Wachtler, H., Grunicke, H., Utermann, G., Altman, A., Baier, G. (1996) Mol. Cell. Biol. 16, 1842–1850
17. Owen, P. J., Johnson, G. D., and Lord, T. M. (1996) Exp. Cell Res. 225, 366–373
18. Dodane, V., and Kachar, B. (1996) J. Cell. Biol. 134, 1276–1283
19. Kazanietz, M. G., Blumberg, P. M., Pierce, J. H., and Mushinski, J. F. (1996) J. Biol. Chem. 271, L223–L241
20. Nigam, S. K., Denisenko, N., Rodriguez-Boulan, E., and Citi, S. (1991) Biochem. Biophys. Res. Commun. 181, 548–553
Protein Kinase C-α Activity Modulates Transepithelial Permeability and Cell Junctions in the LLC-PK₁ Epithelial Cell Line
Dan Rosson, Thomas G. O'Brien, Jennifer A. Kampherstein, Zoltan Szallasi, Krisztina Bogi, Peter M. Blumberg and James M. Mullin

J. Biol. Chem. 1997, 272:14950-14953.
doi: 10.1074/jbc.272.23.14950

Access the most updated version of this article at http://www.jbc.org/content/272/23/14950

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 25 references, 8 of which can be accessed free at http://www.jbc.org/content/272/23/14950.full.html#ref-list-1