The Involvement of Novel Protein Kinase C Isozymes in Influencing Sensitivity of Breast Cancer MCF-7 Cells to Tumor Necrosis Factor-α

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ABSTRACT

Protein kinase C (PKC) has been implicated in tumor necrosis factor-α (TNF) signaling. Structurally and functionally distinct PKC activators and selective inhibitors of PKC were used to investigate the involvement of PKC isozymes in influencing TNF sensitivity in MCF-7 cells. Activators of PKC, such as phorbol-12,13-dibutyrate (PDBu) (1.0 μM), indolactam V (10 μM), and bryostatin 1 (1.0 μM) decreased the sensitivity of MCF-7 cells to TNF by 5-, 10-, and 1.7-fold, respectively. The PKC-specific inhibitor bisindolylmaleimide II (BIM) ($\geq$1 μM) antagonized the effect of PDBu in protecting MCF-7 cells against TNF cytotoxicity. High concentrations of BIM ($\geq$10 μM) also significantly enhanced the sensitivity of MCF-7 cells to TNF. In contrast, Gö 6976, a specific inhibitor of cPKCs, did not potentiate TNF sensitivity and failed to reverse the effect of PDBu. In addition, BIM but not Gö 6976 blocked PDBu-mediated down-regulation of TNF receptors. There was no correlation between down-regulation of PKCα, -δ, and -ϵ, and protection against TNF cytotoxicity by PKC activators. A 6-hr exposure to 1.0 μM PDBu, 10 μM indolactam V, and 1.0 μM bryostatin 1 caused a 1.8-, 3.5- and 1.2-fold induction, respectively, of nPKCη in MCF-7 cells. Similar exposure to BIM but not Gö 6976 led to a significant down-regulation of nPKCη. This novel regulation of PKCη implicates this isozyme in PDBu-mediated protection of MCF-7 cells against TNF cytotoxicity.

TNF, a pleotropic cytokine, exhibits cytotoxic/cytolytic activity against several tumors (Beyaert and Fiers, 1994; Heller and Kronke, 1994). TNF mediates its action by binding to its cell surface receptors, and two receptors, with molecular masses of 55–60 kDa (TNFRI) and 70–80 kDa (TNFRII), have been identified (Lewis *et al.*, 1991; Schall *et al.*, 1990; Smith *et al.*, 1990). Most cells, including TNF-resistant cells, express TNFRI, which is believed to be the major mediator of TNF cytotoxicity (Lewis *et al.*, 1991; Schall *et al.*, 1990; Smith *et al.*, 1990). The binding of TNF to its receptors is necessary but not sufficient for its cytotoxic action (Creasey *et al.*, 1987; Lewis *et al.*, 1991).

Unlike growth factor receptors, TNF receptors do not possess any intrinsic kinase activity (Schall *et al.*, 1990; Smith *et al.*, 1990). Nevertheless, the interaction of TNF with its receptors induces phosphorylation of several proteins, and inhibitors of protein kinases influence TNF sensitivity significantly, suggesting that protein phosphorylation plays a critical role in TNF signaling (Beyaert and Fiers, 1994; Vil-

cek and Lee, 1991). Several protein kinases, including PKC, have been implicated in mediating TNF responses (Galeotti *et al.*, 1993; Hamamoto *et al.*, 1990; Johnson and Baglioni, 1988; Sampson *et al.*, 1993; Schutze *et al.*, 1990; Zhang *et al.*, 1994). It has been demonstrated that TNF can resemble a PKC activator. It can cause rapid production of DAG, activation of PKC, and phosphorylation of proteins (Kronke *et al.*, 1992; Pusztai *et al.*, 1993; Sampson *et al.*, 1993; Schutze *et al.*, 1990). TNF-stimulated protein phosphorylation could be blocked by PKC inhibitors (Sampson *et al.*, 1993). In addition, PKC-dependent protein phosphorylation induced resistance to TNF-mediated cytotoxicity, and inhibition of PKC potentiated the cytotoxicity of TNF (Sampson *et al.*, 1993). The regulation of TNF sensitivity by PKC, however, varied significantly among cell types. TNF caused translocation of PKC in some but not all cells (Schutze *et al.*, 1990). TNF-induced DAG production was not accompanied by an increase in cellular calcium (Heller and Kronke, 1994). Nonspecific PKC inhibitors, such as staurosporine, did not influence TNF sensitivity at concentrations required to block PKC activity *in vitro* and in intact cells (Beyaert *et al.*, 1993). Finally, inhibition and/or down-regulation of PKC by 12-*O*-tetradeca-

ABBREVIATIONS: TNF, tumor necrosis factor; PKC, protein kinase C; DAG, diacylglycerol; aPKC, atypical protein kinase C; cPKC, conventional protein kinase C; nPKC, novel protein kinase C; NF-κB, nuclear factor κB; PDBu, phorbol-12,13-dibutyrate; ILV, indolactam V; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; BIM, bisindolylmaleimide II; Me$_2$SO, dimethyl sulfoxide; PKA, cAMP-dependent protein kinase.

noylphorbol-13-acetate failed to inhibit TNF-mediated activities (Beyaert *et al.*, 1993; Pusztai *et al.*, 1993).

These apparent anomalies can be attributable, in part, to the differential expression, complex regulation, and distinct functions of PKC isozymes, a family of 12 closely related proteins [reviewed in Basu (1993)]. Based on structural variations and biochemical properties, the PKC isozymes can be categorized into three groups: group A or cPKC (α, βI, βII, and γ); group B or nPKC (δ, ϵ, η, θ, and μ); and group C or aPKC (ζ and λ/ι) (Basu, 1993; Johannes *et al.*, 1994). The isozymes differ in biochemical properties, tissue specific distribution, and intracellular localization. Whereas cPKCs are calcium- and phospholipid-dependent, nPKCs and aPKCs do not require any calcium for their activities. Both cPKCs and nPKCs can be activated by DAG and tumor-promoting phorbol esters, whereas aPKCs are insensitive to phorbol ester/DAG. The expression and regulation of PKC isozymes vary significantly with cell types.

Several studies have suggested a role for phorbol ester-insensitive PKC, namely aPKCζ, in TNF signaling. First, interaction of TNF with its receptors generates second messengers, ceramide, and arachidonic acid that can regulate PKCζ (Muller *et al.*, 1995). Second, overexpression of PKCζ in fibroblasts activated NF-κB, a critical mediator of TNF signaling (Diaz-Meco *et al.*, 1993). Third, the expression of a dominant negative mutant of PKCζ inhibited NF-κB activation (Diaz-Meco *et al.*, 1993). Another group was, however, unable to demonstrate NF-κB activation by overexpression of PKCζ in NIH 3T3 cells (Montaner *et al.*, 1995), thus questioning the importance of PKCζ in NF-κB activation. In addition, although the observations that TNF can generate DAG in the absence of an increase in cellular calcium and that tumor promoting phorbol esters influence TNF sensitivity suggest a strong role for DAG/phorbol ester-dependent but calcium-independent PKC in TNF signaling, there have been no reports on the involvement of nPKCs in TNF signaling. In the present study, I have used several activators and inhibitors of PKC to examine the role of PKC isozymes in influencing TNF sensitivity in breast cancer MCF-7 cells.

Experimental Procedures

Materials. TNF was purchased from R & D Systems (Minneapolis, MN). PDBu and ILV were from LC Service Corporation (Woburn, MA) and protein kinase inhibitors from CalBiochem (San Diego, CA). MTT was purchased from Sigma (St. Louis, MO) and Alamar Blue from Accumed International (Westlake, OH). Monoclonal antibodies to PKC isozymes were purchased from Transduction Laboratories (Lexington, KY). Polyclonal antibody to PKCζ was from GIBCO-BRL (Grand Island, NY), and PKCη and -ϵ were from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-conjugated goat anti-mouse and donkey anti-rabbit antibodies were obtained from JacksonImmuno Research (West Grove, PA). Enhanced chemiluminescence detection kit was from Amersham (Arlington Heights, IL). ^{125}I-TNF (specific activity, 44.6 μCi/μg) was from DuPont-New England Nuclear (Wilmington, DE).

Cell culture. Cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum and 2 mM glutamine, and kept in a humidified incubator at 37° with 95% air and 5% CO$_2$.

Assessment of cell viability. Exponentially growing cells were plated in microtiter plates and incubated at 37° in 5% CO$_2$. The following day, cells were pretreated without or with protein kinase modulators and then with different concentrations of TNF. After 6–15 hr, the medium was replaced with fresh medium containing 10% fetal bovine serum. The number of viable cells was determined after 48–96 hr using the dye MTT as described previously (Basu *et al.*, 1990). Recently, I have adopted an Alamar Blue assay instead of MTT assay because of its simplicity. Very similar results were obtained using either method. In the Alamar Blue assay, cells in the microtiter plate were incubated with 20 μl per well (0.1 of the volume of the culture medium) dye at 37° for 4–6 hr, and fluorescence was determined using a Cytofluor II fluorescence plate reader (PerSeptive Biosystems, Cambridge, MA) using an excitation wavelength of 530 nm and emission wavelength of 590 nm.

Immunoblot analysis. Cells were treated with TNF or PKC modulators as described in Results and in the legend to Fig. 1. At the end of the incubation, cells were harvested and washed with cold phosphate-buffered saline. Briefly, cells were homogenized in buffer A (20 mM Tris·HCl, pH 7.5, 0.25 M sucrose, 2 mM EDTA, 2 mM EGTA, 10 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin and aprotinin) and centrifuged at $100,000 \times g$ for 1 hr, and the pellet was homogenized in buffer A. Equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred electrophoretically to a polyvinylidene difluoride membrane. Immunoblot analyses were performed with PKC isozyme-specific antibodies as described previously (Basu *et al.*, 1996). The blots were visualized using the enhanced chemiluminescence detection reagents and the manufacturer's protocol. Intensities of immunoreactive proteins were quantified by laser densitometry. Because the abundance of PKCζ and -ι was not altered by any of the treatments, the level of these isozymes was used as an internal control to account for any variability associated with the amount of protein loaded in each lane during electrophoresis.

Binding assay. Cells (1×10^5) were treated with or without PKC activators and/or inhibitors as described in the text, washed, and then incubated with different concentrations of ^{125}I-TNF in complete medium at 4° for 2 hr. Cells were then washed extensively with phosphate-buffered saline containing 0.1% BSA, solubilized in 0.25 N NaOH, and counted in a γ-counter. Nonspecific binding was determined in the presence of 100-fold excess of unlabeled TNF. Specific binding was defined as the difference between total binding and nonspecific binding. The maximum binding sites (B_{max}) and binding affinity (K_d) were calculated from the nonlinear regression analysis of saturation binding isotherms using the Prism computer program (GraphPad Software, San Diego, CA).

Results

Effects of protein kinase modulators on the sensitivity of MCF-7 cells to TNF. I have compared the ability of structurally and functionally distinct PKC activators, namely PDBu, ILV, and bryostatin 1, to influence the sensitivity of MCF-7 cells to TNF. Continuous exposure to TNF for several hours was necessary for the cytotoxic action of TNF. Because PKC modulators by themselves may affect cell growth, I pretreated cells with PKC modulators for 1 hr, exposed them to TNF for an additional 6 hr, and then incubated them in fresh medium for 2–4 days. Under that condition, contribution of PKC activators and inhibitors on cell growth was significantly reduced. Fig. 1 shows that 1 μM PDBu and 10 μM ILV decreased TNF sensitivity by approximately 5- and 9-fold, respectively. One micromolar ILV was slightly less effective than 1 μM PDBu in protecting cells against TNF cytotoxicity (data not shown). In contrast, 1 μM bryostatin 1 had only a modest effect (1.7-fold) on TNF sensitivity.

Fig. 2 shows the effects of three PKC inhibitors on the sensitivity of MCF-7 cells to TNF. It has been shown before that the nonspecific PKC inhibitor staurosporine potentiates
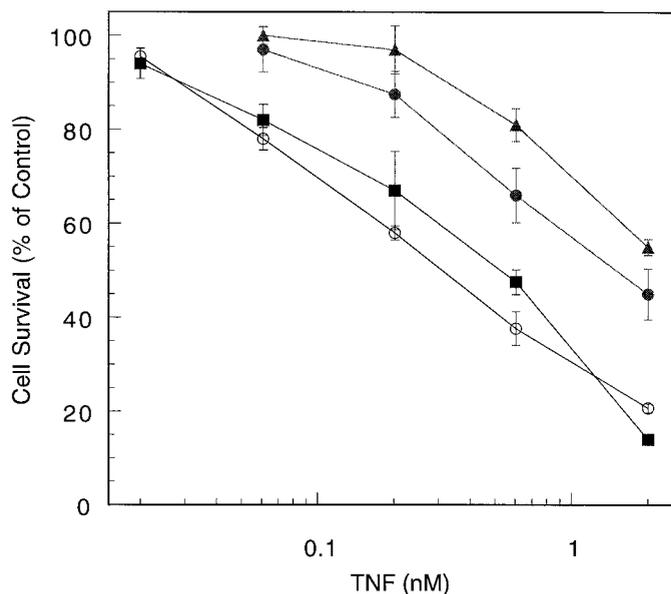
Fig. 1. Effects of PKC activators on the sensitivity of MCF-7 cells to TNF. Cells were pretreated with the vehicle Me$_2$SO (○) or with 1.0 μM PDBu (●), 10 μM ILV (▲), or 1.0 μM bryostatin 1 (■) for 1 hr and then treated with various concentrations of TNF for 6 hr. The medium was replaced with fresh medium, and cell survival was determined after 72 hr by a colorimetric assay as described in Experimental Procedures. Results are representative of three experiments. The mean values and standard error ranges are shown for quadruplicate determinations of a representative experiment. Control values are based upon the number of cells survived in the presence of either vehicle Me$_2$SO, PDBu, or ILV but in the absence of any TNF.
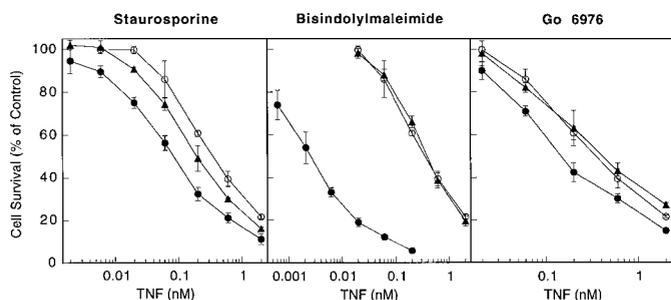

Fig. 2. Effects of PKC inhibitors on the sensitivity of MCF-7 cells to TNF. Cells were pretreated without (○) or with different concentrations of PKC inhibitors (●, ▲) for 1 hr and then treated with various concentrations of TNF for 6 hr. Cell survival was determined after 72 hr as described in Experimental Procedures. Results are representative of 3–12 experiments. The mean values and standard error ranges are shown for quadruplicate determinations of a representative experiment. A, ▲, 0.01 μM staurosporine; ●, 0.025 μM staurosporine. B, ▲, 1.0 μM BIM; ●, 10 μM BIM. C, ▲, 1.0 μM Gö 6976; ●, 10 μM Gö 6976. Control values are based upon the number of cells survived in the presence of either vehicle Me$_2$SO, staurosporine, BIM, or Gö 6976 but in the absence of any TNF.

TNF cytotoxicity in several tumor cells (Beyaert *et al.*, 1993; Hamamoto *et al.*, 1990; Zhang *et al.*, 1994). As shown in Fig. 2, staurosporine caused a concentration-dependent increase in TNF sensitivity; a 4-fold sensitization was achieved with 25 nM staurosporine. Higher concentrations of staurosporine (≥100 nM) were extremely toxic to MCF-7 cells, causing more than 50% cell kill, presumably due to inhibition of several kinases. I also investigated the effects of two PKC inhibitors that are specific for PKC but exhibit distinct selectivity toward PKC isozymes. BIM inhibits all PKC isozymes, whereas Gö 6976 inhibits only cPKCα and -β1 (Martiny-Baron *et al.*, 1993; Toullec *et al.*, 1991). BIM (10 μM) by itself had little

effect on cell growth (<20%), but it enhanced the sensitivity of MCF-7 cells to TNF by approximately 100-fold, whereas 1 μM BIM had no effect (Fig. 2). In contrast, the cPKC inhibitor Gö 6976 caused only a 2-fold increase in TNF sensitivity even at 10 μM concentrations. Fig. 3 shows that cells treated with 10 μM BIM (Fig. 3B) retained morphology similar to untreated control cells (Fig. 3A), whereas treatment with 1.0 nM TNF (Fig. 3C) or combination of TNF and BIM (Fig. 3D) induced chromatin condensation indicative of cell death by apoptosis.

To further evaluate the involvement of PKC, I tested the ability of PKC inhibitors to reverse PDBu-mediated protection against TNF cytotoxicity. As shown in Fig. 4, both staurosporine and 10 μM BIM not only sensitized MCF-7 cells to TNF but also antagonized the effect of PDBu. Because the IC$_{50}$ values of 10 μM BIM-treated cells were extremely low (0.005 nM and 0.007 nM in the absence and presence of PDBu, respectively), it was difficult to see the columns. Although 1 μM BIM did not sensitize cells to TNF, it was able to counteract the effect of PDBu in a statistically significant manner ($p < 0.005$). In contrast, the effect of Gö 6976 on TNF sensitivity was statistically insignificant in both the presence and the absence of PDBu.

Effects of PKC modulators on PKC isozyme expression. To investigate the possible involvement of a PKC isozyme in influencing TNF sensitivity, I monitored the level of PKC isozymes after treatment with TNF or PKC modulators (Fig. 5). The intensity of PKC isozymes was quantified by scanning immunoblots with a laser densitometer. A 6-hr exposure to 1.0 nM TNF had little effect on the expression of any of the PKC isozymes. In addition, the expression of PKCμ, -ζ (Fig. 5A), and -ι (data not shown) was not altered significantly by any of the treatments. Fig. 5B shows the changes in expression of PKCα, -δ, -ε, and -η by PKC modulators. A 6-hr exposure to 1 μM PDBu and 1 μM bryostatin 1 led to a 25 and 60% down-regulation of PKCα, respectively. They also caused approximately 50% down-regulation of PKCε. In contrast, 10 μM ILV had no effect on the expression of PKCα and -ε. All three PKC activators caused a substan-
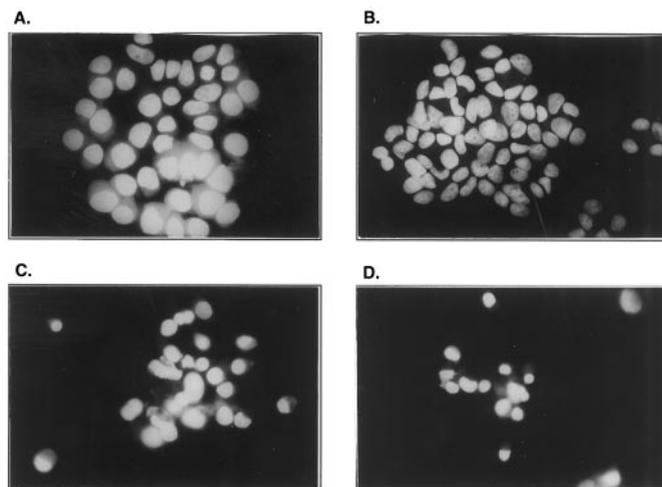

Fig. 3. Morphological appearance of TNF-induced apoptosis. Cells were treated with or without 10 μM BIM for 1 hr and then treated with or without TNF (1.0 nM) for 6 hr. The following day, cells were scraped, washed, fixed with 4% paraformaldehyde, stained with Hoechst 33258, and examined under a fluorescent microscope (×40). A, Untreated control; B, BIM; C, TNF; D, BIM and TNF.
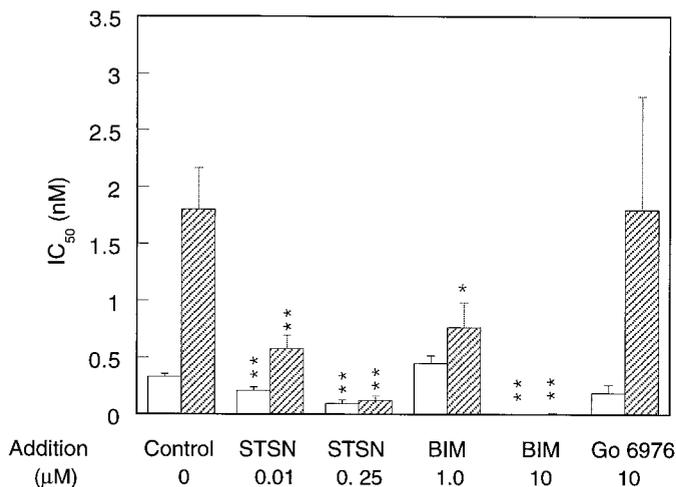
Fig. 4. Effects of PKC inhibitors on the protection of TNF sensitivity by PDBu. Cells were pretreated with Me$_2$SO (○) or PKC inhibitors for 1 hr before exposure to 1.0 μM PDBu for 1 hr. Cells were then treated with different concentrations of TNF for 6 hr, and cell survival was determined as described in Experimental Procedures. The IC$_{50}$ values were determined from the cell survival curves. □, −PDBu; ▨, +PDBu. Values are the means ± standard error of three to five independent experiments. ∗∗, $p < 0.01$ versus controls (Student's t test); ∗∗∗, $p < 0.001$ versus controls (Student's t test). Ninety-five percent confidence limits ($p < 0.05$) were considered significant. *STSN*, staurosporine.

tial down-regulation of PKCδ. Thus, there was no correlation between the effects of PKC activators on the expression of PKCα, -δ, and -ε, and protection against TNF cytotoxicity. PKCη appeared as a doublet, and the intensities of both bands were altered to a similar extent by PKC modulators. PDBu, ILV, and bryostatin 1 caused approximately 180, 350, and 120% increase in the abundance of PKCη, respectively (Fig. 5B). Thus, there was a good correlation between PKCη up-regulation and protection against TNF cytotoxicity by PKC activators. In addition, the PKC inhibitors, such as BIM and staurosporine, caused a significant down-regulation of PKCη. A 6-hr exposure to 1 μM BIM, 10 μM BIM, and 0.01 μM staurosporine caused approximately 60, 90, and 70% decrease in PKCη expression, respectively. A similar exposure to 1 μM BIM and 0.01 μM staurosporine had little effect on the expression of PKCα, -δ, and -ε, but 10 μM BIM also caused a 50% decrease in PKCδ and a 35% decrease in PKCε. In contrast, 10 μM Gö 6976 did not affect the expression of PKC isozymes except for a 25% decrease in PKCη expression.

Because TNF has been shown to cause translocation of PKC in several cell lines (Schutze *et al.*, 1990), I compared the distribution of PKC isozymes after treatment with TNF or PKC activators in MCF-7 cells (Fig. 6). Whereas cPKCα was primarily cytosolic, nPKCη was essentially particulate. The majority of nPKCδ and -ε fractions were membrane-bound, and aPKCζ was distributed equally in both fractions. A 6-hr exposure to 1 μM PDBu, 10 μM ILV, or 1 μM bryostatin 1 caused translocation of PKCα, -δ, and -ε from the cytosol to the membrane fraction. The decrease in nPKCδ from the cytosol was not accompanied by an increase in the membrane fraction, presumably due to rapid down-regulation of this isozyme after translocation to the membrane fraction. Bryostatin 1 was more effective than PDBu or ILV in inducing down-regulation of cPKCα and nPKCδ. PDBu and ILV caused a significant increase in nPKCη in the membrane fraction, whereas bryostatin 1 had no effect. PKC activators

did not influence the subcellular distribution of aPKCζ. A 6-hr exposure to 1.0 nM TNF had little effect on the distribution of any of the PKC isozymes.

Effects of PDBu and BIM on binding of TNF to its receptor. PDBu has been shown to cause down-regulation of TNF receptors (Aggarwal and Eessalu, 1987; Johnson and Baglioni, 1988). Fig. 7 shows that pretreatment of MCF-7 cells with 1 μM PDBu for 1 hr significantly reduced binding of ^{125}I-TNF to its receptors. PDBu caused a significant decrease in maximum binding sites (B_{max}) with little change in binding affinity (K_d); B_{max} was decreased from 3681 ± 151 to 608 ± 33 cpm and K_d from 1.48 ± 0.11 to 1.0 ± 0.11 nM. To examine whether the effect of PDBu on TNF binding was mediated by cPKCs or nPKCs, I compared the effects of BIM and Gö 6976 on PDBu-mediated receptor down-regulation (Fig. 7). Although incubation of MCF-7 cells with 10 μM BIM alone for 2 hr had little effect on TNF binding, it abolished the decrease in TNF binding elicited by PDBu. In addition, 1 μM BIM that had no effect on TNF cytotoxicity by itself also blocked TNF receptor down-regulation by PDBu completely. Gö 6976 at 10 μM did not influence ^{125}I-TNF binding to cell surface receptors either alone or in combination with PDBu.

Discussion

Binding of TNF to its receptors triggers two competing signals—activation of cell death and induction of cellular protective responses to abrogate its own cytotoxicity. The ultimate effect is a balance between these two pathways. PKC activators have been shown to protect cells against TNF cytotoxicity but the isozyme of PKC that is responsible for the protection against TNF cytotoxicity has not been documented. The results of our present study indicate that nPKCs but not cPKCs are important in regulating sensitivity of breast cancer MCF-7 cells to TNF.

I have compared the effects of three PKC activators, PDBu, ILV, and bryostatin 1 that are structurally and functionally distinct, on the sensitivity of MCF-7 cells to TNF and on the expression of PKC isozymes. Although PDBu and ILV caused a significant protection against TNF cytotoxicity, bryostatin 1 had little effect. PKC activators had no major effect on the abundance of PKCμ, -ζ, or -ι. In addition, there was no correlation between down-regulation of PKCα, -δ, and -ε, and protection against TNF cytotoxicity by PKC activators. Interestingly, both PDBu and ILV caused a significant induction of PKCη, whereas bryostatin 1 had only a little effect. Similar up-regulation of PKCη by PDBu was also noted in EL4 mouse thymoma cells (Resnick *et al.*, 1997). There was a good correlation between PKCη up-regulation and fold-protection by PKC activators (Fig. 1 versus Fig. 5B).

I also compared the effects of three PKC inhibitors that exhibit differential specificity toward PKC isozymes. Staurosporine is a non-specific kinase inhibitor and inhibits both Ca^{2+}-dependent and -independent PKCs in the nanomolar range but does not inhibit aPKCζ at concentrations up to 30 μM (Seynaeve *et al.*, 1994). BIM demonstrates significant specificity toward PKC but inhibits all PKC isozymes, albeit with different potencies (Martiny-Baron *et al.*, 1993). cPKCs are the most sensitive and their IC$_{50}$ values are in the nanomolar range. nPKCs can be inhibited by submicromolar concentrations of BIM in *in vitro* kinase assays, whereas aPKCs are least sensitive to BIM (IC$_{50}$ = 5.8 μM). In contrast, Gö
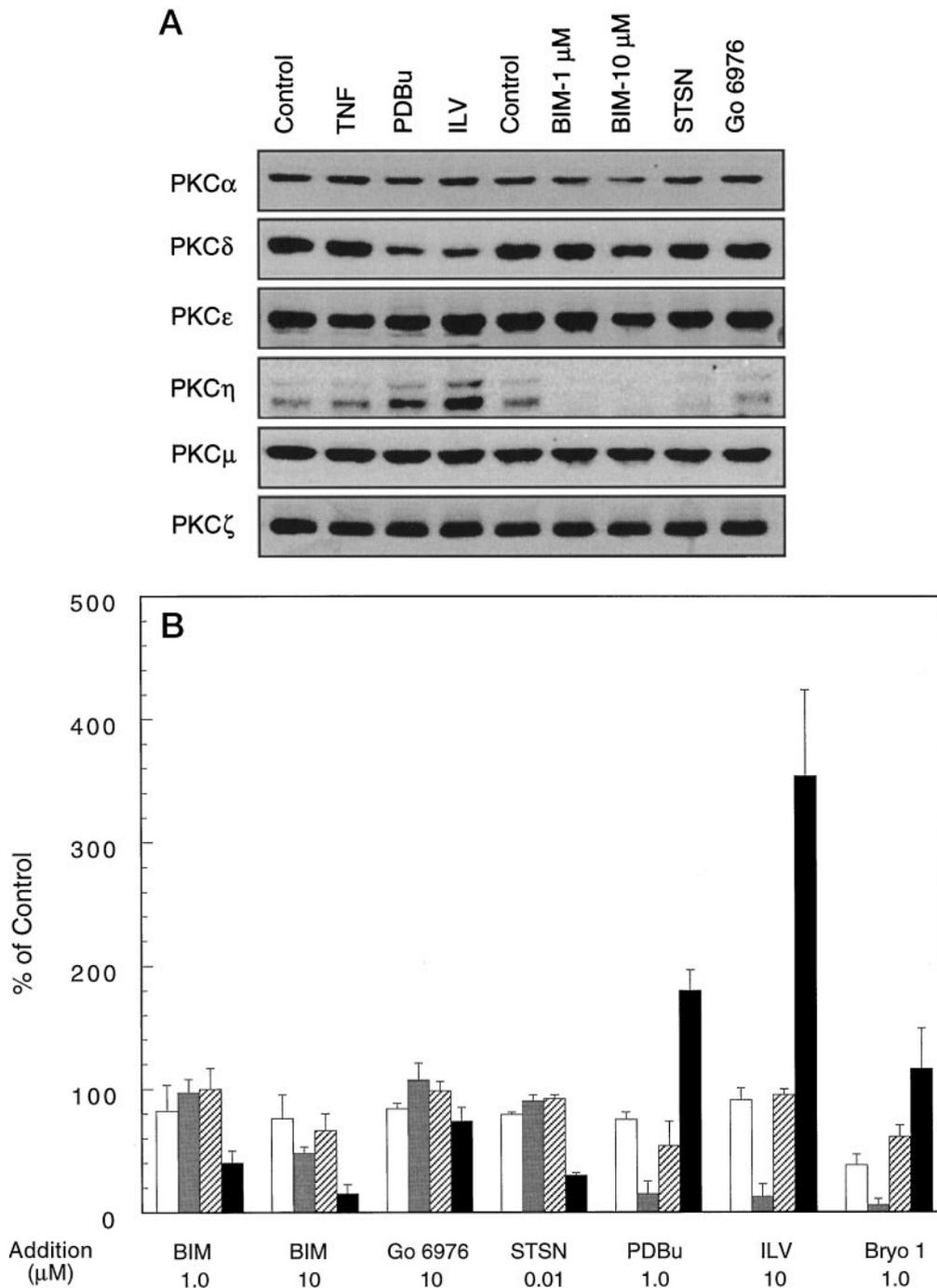
Fig. 5. Effects of PKC modulators on the expression of PKC isozymes in MCF-7 cells. A, MCF-7 cells were treated with or without 1.0 nM TNF, 1.0 μM PDBu, 10 μM ILV, 1.0 μM BIM, 10 μM BIM, 10 nM staurosporine (*STSN*), or 10 μM Gö 6976 for 6 hr as indicated. Western blot analyses were performed with total cellular extracts using PKC isozyme-specific antibodies. The same blot was probed with different antibodies. Results are representative of three to five experiments. B, The abundance of PKC isozymes was quantified by scanning immunoblots with a laser densitometer, and the values are the means ± standard error of three to five individual experiments. □, PKCα; ▨, PKCδ; ▨, PKCε; and ■, PKCη.

6976 selectively inhibits cPKCα and -β1 ($IC_{50} < 10$ nM) but has little effect on Ca^{2+}-independent PKCs at micromolar levels (Martiny-Baron *et al.*, 1993).

The studies with PKC inhibitors also support the notion that nPKCs are important in TNF signaling. For example, the broad PKC inhibitors BIM and staurosporine influenced TNF sensitivity, whereas cPKC inhibitor Gö 6976 had no effect (Fig. 4). Although low concentrations of BIM (1 μM) and staurosporine (10 nM) had little effect on TNF sensitivity by themselves, they reversed the effect of PDBu in protecting cells against TNF cytotoxicity (Figs. 2 and 4). Because aPKCs are PDBu-insensitive, these results also indicate that nPKCs influence the protection against TNF cytotoxicity by PDBu.

Interestingly, both BIM and staurosporine caused significant down-regulation of nPKCη, suggesting that nPKCη level may be regulated by a phosphorylation-dephosphorylation mechanism. Based on this novel regulation of PKCη by PKC activators and inhibitors, it is tempting to speculate that this isozyme was responsible for protection of MCF-7 cells against TNF cytotoxicity by PKC activators. Similar results were observed with another breast cancer BT-20 cell line, which also expresses nPKCη (A. Basu, unpublished observation). The generality of this observation, however, must await study in transfected model systems and a wider spectrum of PKCη-expressing nontransfected cells.
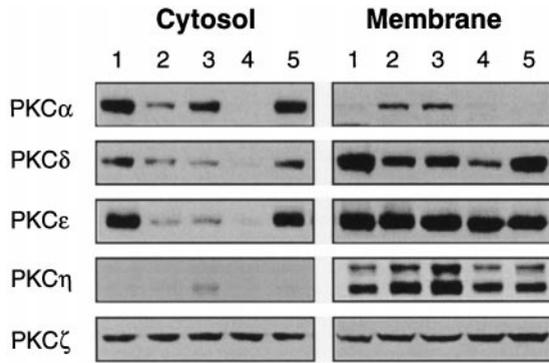
Our results suggest that PKC inhibitors act at more than

Fig. 6. Effects of TNF and PKC activators on the subcellular distribution of PKC isozymes. Cells were incubated without (*lane 1*) or with 1.0 μM PDBu (*lane 2*), 10 μM ILV (*lane 3*), 1.0 μM bryostatin 1 (*lane 4*), or 1.0 nM TNF (*lane 5*) for 6 hr. Cytosol and membrane fractions were separated, and Western blot analyses were performed with PKC isozyme-specific antibodies.
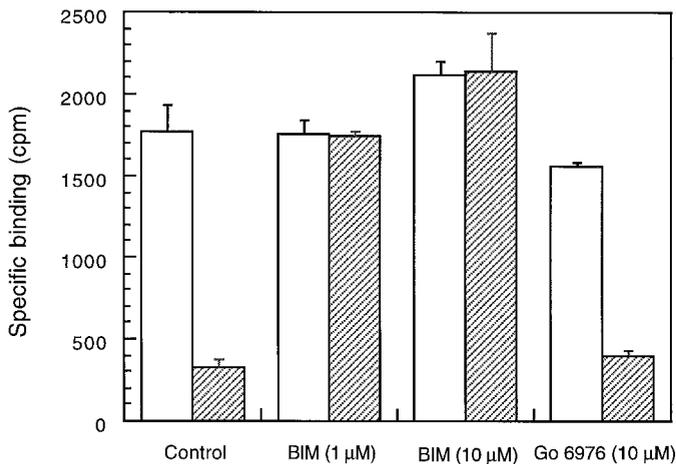

Fig. 7. Effects of PKC inhibitors on down-regulation of TNF receptors by PDBu. Cells were pretreated with or without 1.0 μM BIM, 10 μM BIM, or 10 μM Gö 6976 for 1 hr and then treated with or without 1.0 μM PDBu for 1 hr. Specific binding of ^{125}I-TNF was determined as described in Experimental Procedures. □, −PDBu; ▨, +PDBu. Results are means of two to three independent experiments performed in duplicate. *Bars*, mean ± standard error.

one step in the TNF signal transduction pathway. BIM completely blocked TNF receptor down-regulation by PDBu, suggesting that receptor down-regulation can partly explain the mechanism of protection by PDBu. In contrast, the cPKC inhibitor Gö 6976 had no effect on PDBu-mediated reduction in TNF binding. Although both 1 and 10 μM BIM blocked receptor down-regulation, only 10 μM BIM enhanced TNF cytotoxicity, suggesting that inhibition of receptor down-regulation was not responsible for TNF sensitization. It has been reported earlier that TNF cytotoxicity does not correlate with receptor number and/or affinity (Creasey *et al.*, 1987; Lewis *et al.*, 1991; Sugarman *et al.*, 1985). The differential effects of 1 and 10 μM BIM on TNF cytotoxicity, in fact, dissociates receptor binding from TNF sensitization and emphasizes that the postreceptor signaling event was responsible for potentiation of TNF cytotoxicity by high concentrations of BIM. In addition, inasmuch as 1 μM BIM caused significant down-regulation of nPKCη but did not sensitize cells to TNF, it is unlikely that down-regulation of PKCη was associated with the enhancement of TNF cytotoxicity. Thus, high concentrations of BIM may trigger a parallel pathway

that may involve some as yet unidentified kinase or aPKCζ, which has also been implicated in TNF signaling (Diaz-Meco *et al.*, 1993; Muller *et al.*, 1995). Furthermore, the regulation of PKC isozymes depends on the cellular context. Cytosolic translocation of nPKCδ and -ε has been associated with ceramide-induced apoptosis (Sawai *et al.*, 1997). I have shown that overexpression of nPKCε in rat fibroblasts prevented apoptosis induced by the chemotherapeutic drug cisplatin (Basu and Cline, 1995). Future studies are needed to determine the contribution of these isozymes in TNF signaling.

Recent studies suggest that the activation of NF-κB provides protection against TNF cytotoxicity and inhibition of NF-κB sensitizes cells to TNF (Beg and Baltimore, 1996; Van Antwerp *et al.*, 1996; Wang *et al.*, 1996). BIM by itself had no effect on NF-κB activation (A. Basu, unpublished observation). Although both TNF and PDBu caused induction of NF-κB in MCF-7 cells, BIM inhibited NF-κB induction by PDBu but not by TNF. In fact, like staurosporine (Beyaert *et al.*, 1993; Hohmann *et al.*, 1992), BIM enhanced TNF-mediated NF-κB induction by almost 2-fold, suggesting that the mechanism of TNF sensitization may not involve inhibition of NF-κB. Because many tumor cells are resistant to TNF, an understanding of the mechanism(s) by which BIM enhances TNF cytotoxicity will greatly facilitate development of novel approaches to cancer therapy.

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