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# Retention of specific protein kinase C isozymes following chronic phorbol ester treatment in BC3H-1 myocytes

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Since insulin effects on glucose transport persist in phorbol ester "desensitized" or "down-regulated" BC3H-1 myocytes, we reexamined the evidence for protein kinase C (PKC) depletion. After 24 hrs of 5 μM 12-0-tetradecanoyl phorbol-13-acetate (TPA) treatment, PKC -directed histone phosphorylation and acute TPA effects on glucose transport were lost, but PKC-dependent vinculin phosphorylation was still evident. Hydroxylapatite (HAP) chromatography revealed loss of a type III, but not a type II, PKC-dependent vinculin phosphorylation. Immunoblots of cytosolic preparations of PKC-"depleted" myocytes confirmed the retention of PKC. Our findings indicate that TPA "down-regulated" BC3H-1 myocytes contain immunoreactive and functionally active PKC. The latter may explain the continued effectiveness of both insulin and diacylglycerol (DiC8) for stimulating glucose transport in "down-regulated" cells.


PKC-mediated phosphorylation of lysine-rich histone is abolished after long term or "chronic" (24-48 hr) phorbol ester treatment of numerous cell types, including the BC3H-1 myocyte (1,2). This effect presumably reflects TPA-induced activation and proteolytic turnover of PKC (3-5). The "down-regulation" or "depletion" of PKC is usually associated with loss of TPA-induced effects on biological processes (6-8). The loss of histone phosphorylation and biological effects have commonly served as major criteria for determining if PKC has been depleted, and persistent biological effects of agonists in "down-regulated" cells are generally taken as evidence that the agonist operates through a PKC-independent mechanism. In some cases, loss of immunoreactive 80kDa PKC has been documented as supportive evidence for validity of the experimental paradigm (2,9),

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Abbreviations used: Protein kinase C, PKC; hydroxylapatite, HAP; 12-0-tetradecanoyl phorbol-13-acetate, TPA; 2-deoxyglucose, 2-DOG; diacylglycerol, DAG; sn-1,2-dioctanoylglycerol, DiC8; dimethylsulfoxide, DMSO.

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but this loss is not always complete, and other immunoreactive proteins possibly derived from PKC, may still be present (2,8,10,11).

In BC3H-1 myocytes and rat-1 cells (12), PKC-dependent histone phosphorylation is no longer detected after chronic TPA treatment, but vinculin, another substrate for PKC, is still phosphorylated by PKC. Several isozymes of PKC have now been recognized (13), and these differ in their (a) activation by diacylglycerol (DAG) vis-a-vis TPA (14-16), and (b) immunoreactivity and recognition by specific antibodies (17,17a,18). Thus, TPA treatment may selectively deplete some, but not necessarily all, PKC isozymes, and data obtained from PKC "depleted" cells may be hazardous to interpret, particularly if "depletion" is based merely on histone phosphorylation and biological effects of TPA.

In BC3H-1 myocytes, we have previously reported that chronic TPA treatment results in loss of TPA, but persistence of insulin effects on glucose transport (1,19). Although these findings may be construed to suggest that insulin stimulates glucose transport independently of PKC, we now report that (a) DAG (DiC8) effects on glucose transport, which appear to be mediated by PKC, are not abolished after chronic TPA treatment, and (b) TPA treatment provokes a loss of some but not all PKC isozymes.

## Materials and Methods

**Cell culture and 2-deoxyglucose uptake:** BC3H-1 myocytes were cultured as described previously (19), and where indicated treated for 24 hr with TPA (Sigma) in 0.25% dimethylsulfoxide (DMSO), or DMSO alone. The uptake of [$^3$H]2-deoxyglucose (2-DOG)(50 mCi/mmol, Dupont/NEN) was measured as described (19). For PKC studies, cells were collected by centrifugation, sonicated in 20 mM Tris-HCL, pH 7.5, 0.25 M sucrose, 1.2 mM EGTA, 0.1 mM phenylmethylsulfonylfluoride (Sigma), and centrifuged at 105,000 x g for 30 min. **PKC chromatography:** Cytosolic fractions (3 mg protein) were chromatographed by fast protein liquid chromatography (FPLC system, Pharmacia) on a Mono Q column (Pharmacia HR 5/5) as described (20). Fractions containing PKC activity were pooled and then applied to a HAP column (Bio-Gel HT, 1 x 10 cm, Pharmacia HR 10/10) and PKC isozymes were eluted with an 84-ml linear concentration gradient of potassium phosphate [20 to 280 mM potassium phosphate (pH 7.5), 0.5 mM EGTA, 0.5 mM EDTA, 10 % glycerol, and 10 mM 2-mercaptoethanol.] PKC was assayed as previously described (1). **Immunoblot analysis:** Polyclonal antibodies were raised to rat brain PKC which contained isozyme types I, II and III (3); characterization of this antiserum is described (21). Cytosolic extracts were subjected to 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to nitrocellulose. Peptide blots were incubated with antibody for 16 hr at room temperature, washed with anti-rabbit IgG-horseradish peroxidase conjugate (Bio-Rad) and developed with diaminobenzidine tetrahydrochloride and $H_2O_2$. Antiserum to type II PKC (22) was a gift from Drs. Bryan L. Roth and John P. Mehegan.

## Results

In non-desensitized myocytes, [$^3$H]2-DOG uptake was stimulated by insulin, and the cell permeant DAG, sn-1,2-dioctanoylglycerol ($DiC_8$) or TPA (Figure 1, top panel).

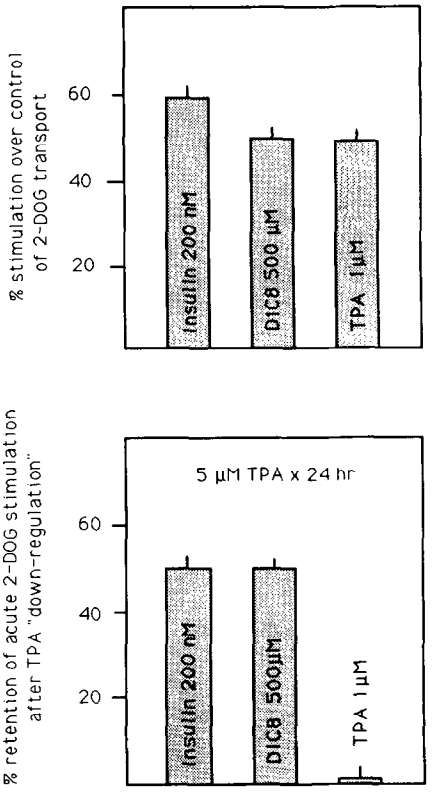


Fig. 1 Retention of insulin effects after phorbol ester-induced "down-regulation" in BC3H-1 myocytes. BC3H-1 myocytes were grown to confluence in 24-well dishes as described in "Materials and Methods". Glucose (25 mM) was added during the final 24 hr incubation period with TPA (5µM in 0.25% DMSO) or DMSO (0.25% final) for the control cells. Cells were rinsed in Dulbecco's phosphate buffered saline with 0.1% BSA and preincubated for 20 min prior to the addition of insulin (200 nM, Elanco), $DiC_8$ (500 µM, Molecular Probes), or TPA (1 µM) for 20 min before the end of the incubation. Results are expressed as % increase over control (top panel) or retention of acute 2-DOG stimulation (bottom panel). Data are from triplicate determinations in an experiment which was repeated on three other occasions.

After cells were chronically treated with 5 µM TPA for 24 hrs, insulin and $DiC_8$ continued to significantly stimulate [$^3$H]2-DOG uptake (albeit at slightly less relative effectiveness due to elevation of basal transport), but acutely added TPA was no longer effective (Fig. 1, bottom panel). In other experiments where chronic TPA treatment was varied by using 1-16 µM for periods of 24-72 hrs, identical results were observed. Moreover, staurosporine, a PKC-inhibitor (23), inhibited insulin-stimulated [$^3$H]2-DOG uptake after chronic TPA treatment (data not shown), suggesting that PKC was still involved.

As reported earlier, PKC-dependent histone phosphorylation was no longer found in HAP column effluents (Figs. 2A and B), of cytosols from chronic TPA-treated cells.

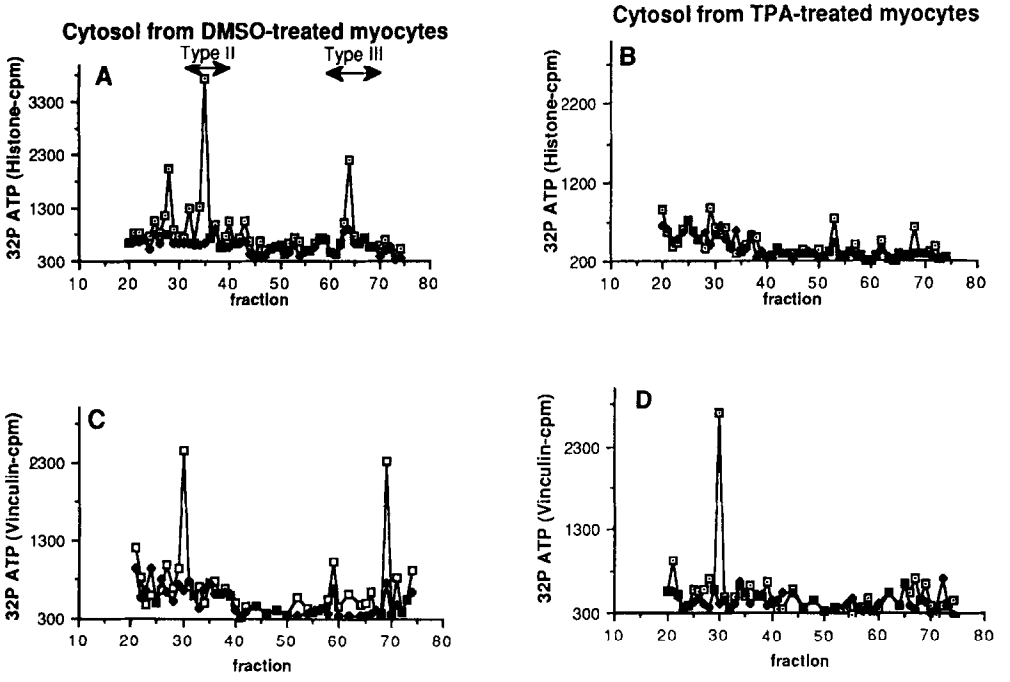


Fig. 2 HAP chromatography of BC3H-1 myocyte PKC isozymes. BC3H-1 myocytes were treated with 5 μM TPA or DMSO for 24 hrs. Cytosolic extracts prepared as described in "Materials and Methods" were chromatographed on Mono Q prior HAP chromatography. Aliquots (80 μl) of 1 ml fractions were assayed for PKC activity in the presence of 10 μg PtdSer and 0.5 mM $CaCl_2$ (□-□)or 0.5 mM EGTA(◆-◆), and 50 μg histone III-S (Sigma) or 40 μg vinculin purified from chicken gizzards as described (29), 2.5 nM [$\gamma$-$^{32}$P]ATP , and 1.25 μmol magnesium acetate in 0.25 ml. PKC from control (panel A) or TPA treated (panel B) myocytes assayed with histone as substrate. PKC from control (panel C) or TPA-treated (panel D) myocytes assayed with vinculin as substrate. Profiles shown were repeated in two separate experiments.

These results, coupled with [$^3$H]2-DOG uptake results could be interpreted to suggest that PKC is depleted and insulin and $DiC_8$ stimulate glucose transport independently of PKC. However, using two different polyclonal antibodies, one raised to rat brain PKC and the other raised to a synthetic peptide sequence from the variable region of type II PKC (22), immunoblot analysis (Fig. 3) of cytosolic extracts revealed that PKC immunoreactivity persisted after chronic TPA treatment, being, in fact, equal to that observed in control cells. Similar immunoblots (data not shown) were also obtained using 16 μM TPA treatment for 48 hrs. Moreover, the failure to deplete PKC by chronic TPA treatment contrasts to decreases observed with acute TPA treatment of these cells (21).

We (1), and others (12), previously reported that PKC-dependent vinculin phosphorylation, unlike histone phosphorylation, persists in cytosolic extracts of cells

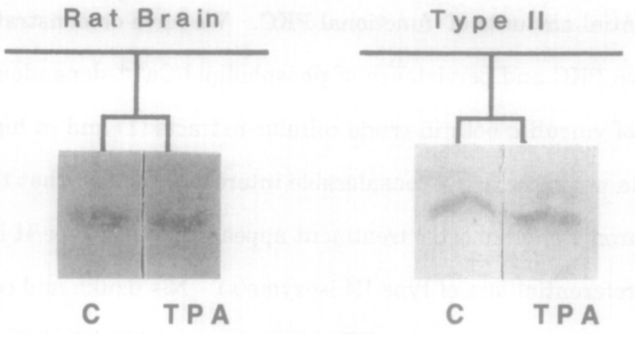


Fig. 3 Immunoreactive PKC from BC3H-1 myocytes treated with insulin or phorbol ester. Myocytes were pre-treated with TPA (5 μM) or DMSO (control). Cytosolic extracts were separated by SDS-PAGE prior to the immunoblot procedure. Results are shown for the same samples developed with an antiserum to rat brain PKC (left) and an antiserum to a peptide sequence specific to type II or β PKC (right). Similar results were obtained in 4 separate experiments.

treated acutely or chronically with TPA. To evaluate the possibility that chronic TPA treatment may selectively deplete only certain PKC isozymes (10), HAP column effluents were analyzed for PKC-dependent phosphorylation of vinculin (Fig. 2C and D) as well as histone (Fig. 2A and B). Two major peaks of PKC mediated-histone phosphorylation were detected in control cells corresponding to types II and III (or β and α) rat brain PKC isozymes (14-16,20,24). Using vinculin as the substrate, enzyme activity of control cells also eluted as types II and III PKC (Fig. 2C). Thus, both types II and III fractions of HAP-purified PKC contained isozymes which recognized vinculin, as well as histone, as substrates, and it appears that heterogeneity within these types (15) may be responsible for differences in specific fractions which were found to catalyze the phosphorylation of these substrates. In myocytes treated with 5 μM TPA for 24 hrs, on the other hand, although PKC-dependent histone phosphorylating activity was no longer detectable (Fig. 2B), PKC-dependent vinculin phosphorylating activity was still evident in type II PKC (Fig. 2D). The presence of type II PKC in these HAP column fractions was verified by immunoblot (data not shown).

## Discussion

Although chronic TPA treatment resulted in losses of both phospholipid/$Ca^{++}$-dependent histone phosphorylation and ability of acute TPA treatment to stimulate glucose transport, it seems clear that BC3H-1 myocytes, which would classically be considered to be "TPA-desensitized", "PKC-down-regulated" or "PKC-depleted", still

contained substantial amounts of functional PKC. We have demonstrated the presence of immunoreactive PKC and persistence of phospholipid/$Ca^{++}$-dependent phosphorylation of vinculin, both in crude cellular extracts (1) and in highly purified PKC preparations (data not shown). Of considerable interest is the fact that the PKC which persisted after chronic phorbol ester treatment appeared to be a type II isozyme, and there appeared to be preferential loss of type III isozyme(s). Nishizuka and coworkers have shown in another cell type that chronic TPA-treatment initially depletes a type II isozyme (25). That observation was based, however, on histone phosphorylation.

The persistance of a type II PKC activity observed in the present work after chronic phorbol ester treatment of BC3H-1 myocytes may also be relevant to other studies in which immunoreactive PKC is evaluated in putative "down-regulated" cells. Clearly, simple loss of histone phosphorylation or acute biological effects of TPA cannot serve as the criteria for true PKC depletion. Also, since polyclonal antibodies are variable in their recognition of specific PKC isozymes (17,18,26-28), the apparent absence or deficiency of immunoreactive material may also be misleading. In addition, it appears that "down-regulation"-resistant forms of PKC which were previously thought to be enzymatically inactive (10) may be inactive only with respect to histone phosphorylation as vinculin was not tested as a substrate in those studies.

The presence of a staurosporine-sensitive glucose transport activity in TPA "down-regulated" cells suggests that the residual PKC is still functional and required in the action of insulin. Indeed, studies with purified rat brain PKC isozymes have shown that type III isozymes respond better than type II isozymes to phorbol ester activation at higher $Ca^{++}$ concentrations (16), and type II isozymes are less dependent on $Ca^{++}$ for full activation than type III isozymes (14,15). Whether these *in vitro* differences in PKC activation are relevant to *in vivo* findings, however, is presently uncertain.

The depletion of some, but not all, PKC isozymes by chronic TPA treatment may reflect differences in the ability of TPA to translocate each of the various isozymes to the plasma membrane, and thereby result in proteolysis and eventual degradation of the isozyme (4,5,10,26). The selective persistance of certain PKC isozymes could also be explained by postulating that the rates of synthesis of these isozyme(s) are greater than those of other isozymes during chronic TPA treatment.

In summary, the present findings suggest that chronic TPA treatment, despite causing losses of both histone phosphorylation and ability of acute TPA treatment to provoke biological effects, does not result in full depletion of all PKC isozymes, at least in the BC3H-1 myocyte. Accordingly, persistence of certain PKC isozymes after chronic TPA treatment may explain continued effectiveness, not only of insulin, but also of exogenous DAG to stimulate glucose transport in these cells. Similarly, the inability of TPA to provoke biological effects in cells which are deficient in certain PKC isozymes suggests that some isozymes may be more important than other isozymes in the stimulation of specific biological processes in intact cells by TPA. Obviously, gaps remain in our understanding of the mechanisms involved in the activation of PKC by phorbol esters and DAG. Until these gaps are filled, experimental findings after chronic TPA treatment must be interpreted with caution.

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