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Phorbol Ester Stimulation of the Type I and Type III Adenylyl Cyclases in Whole Cells†

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ABSTRACT: Phorbol esters and activators of protein kinase C have been reported to either facilitate or inhibit increases in intracellular cAMP caused by activators of adenylyl cyclase. The variable responses to activators of protein kinase C may reflect, in part, the existence of distinct adenylyl cyclases present in animal cells. There are a family of adenylyl cyclases with different regulatory properties, and clones for six distinct types of adenylyl cyclase have been reported. Two of these enzymes, the type I and type III adenylyl cyclases, are stimulated by calcium and calmodulin whereas the others are not. In this study, we examined the effect of phorbol esters on the activity of the type I and type III adenylyl cyclases in whole cells. TPA markedly enhanced the forskolin responsiveness of the type I and type III adenylyl cyclases expressed in kidney 293 cells. The effect of TPA on the activity of the calmodulin-sensitive adenylyl cyclases was not mediated through increases in intracellular free calcium. These data suggest that activation of protein kinase C can elevate intracellular cAMP in animal cells that contain the type I or type III adenylyl cyclase.

Cross-talk between the cAMP and the phosphoinositide/calcium signal transduction pathways can occur by several different mechanisms. For example, muscarinic agonists can indirectly increase intracellular cAMP through mobilization of free Ca^{2+} which activates the type I calmodulin- (CaM-)[1] sensitive adenylate cyclase (Choi et al., 1992a). Phorbol esters and other activators of protein kinase C (PKC) can also affect intracellular cAMP levels in various tissues and cultured cells. Activation of protein kinase C in intact cells can either facilitate (Bell & Brunton, 1987; Choi & Toscano, 1988; Johnson & Toews, 1990; Quilliam et al., 1989; Rozengurt et al., 1987; Anand-Srivastava & Srivastava, 1990) or inhibit (Bell & Brunton, 1987; Bushfield et al., 1987; Summers & Cronin, 1988; Dixon et al., 1988; Yamashita et al., 1988) cAMP accumulations caused by forskolin or receptor stimulation of adenylyl cyclases. The response of various cell types and membrane preparations to phorbol esters is complex and varied. For example, treatment of a rat osteosarcoma cell line with 12-*O*-tetradecanoylphorbol 13-acetate (TPA) increased parathyroid hormone-stimulated adenylyl cyclase activity and inhibited prostaglandin E2-responsive enzyme activity (Freyaldenhoven et al., 1992). Activation of PKC in PC12 cells increased the response of the cAMP-generating systems whereas PKC activation in NCB20 cells and NIH 3T3 caused inhibition of cAMP-generating systems (Gusovsky & Gutkind, 1991). These multiple and contradicting effects of phorbol esters on hormone-sensitive adenylyl cyclase can occur within the same cell and may be due to multiple PKC phosphorylation sites within the adenylyl cyclase system, different forms of

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[1] Abbreviations: CaM, calmodulin; CDM8(I-AC), the plasmid containing the mammalian expression vector CDM8 and the complete coding sequence of type I adenylyl cyclase; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; IBMX, 3-isobutyl-1-methylxanthine; BAPTA/AM, 1,2-bis(2-amino-5,5'-difluorophenoxy)-ethane *N,N,N',N'*-tetrakis(acetoxymethyl) ester; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; PKC, protein kinase C; 293(I-AC), 293 cells that express type I adenylyl cyclase; 293(III-AC), 293 cells that express type III adenylyl cyclase.

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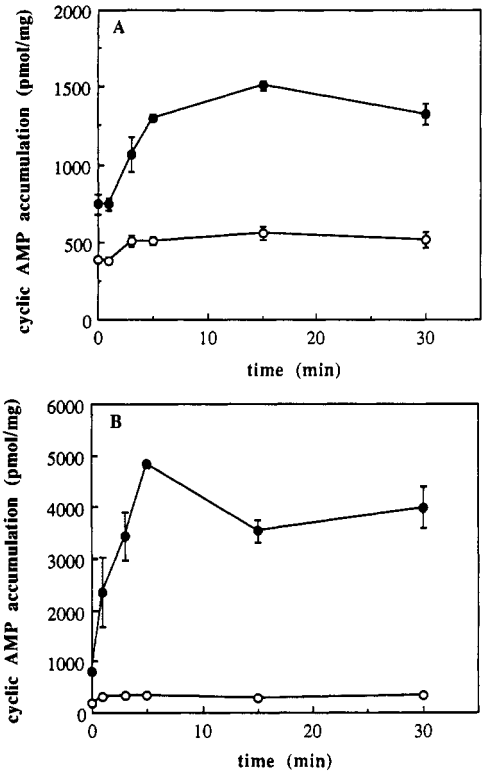

FIGURE 1: Kinetics for the effect of TPA on intracellular cAMP levels in 293 cells expressing type I or type III adenylyl cyclase. Confluent cultures of 293 cells were exposed to 100 nM TPA for the indicated times, rinsed once with phosphate-buffered saline solution, and then incubated with 1 mM IBMX in the presence of 5 μM forskolin for 30 min. The amount of intracellular cAMP was assayed as described in Experimental Procedures. Panel A: (○) control 293 cells; (●) 293(I-AC) cells. Panel B: (○) control 293 cells; (●) 293-(III-AC) cells.

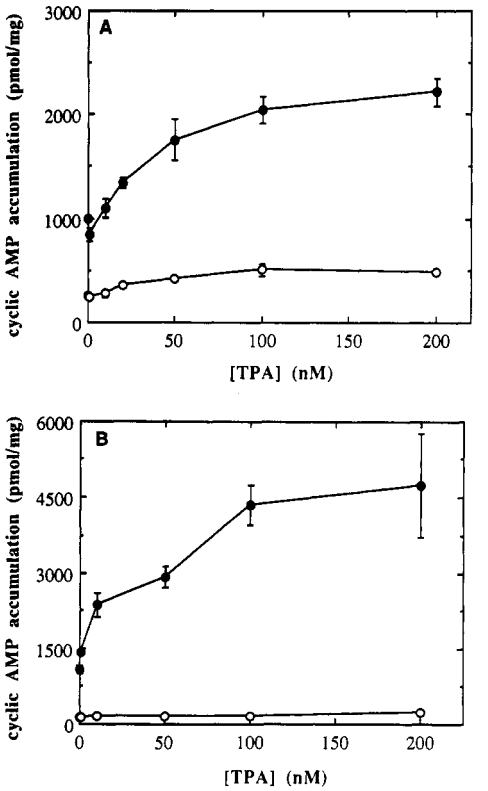

FIGURE 2: TPA concentration dependence for stimulation of cAMP levels in 293 cells expressing type I or type III adenylyl cyclase. Confluent cultures of 293 cells were exposed to TPA at the indicated concentrations for 15 min (A) or 5 min (B), prior to incubation with 5 μM forskolin and 1 mM IBMX for 30 min. The reaction was terminated by adding 5% trichloroacetic acid, and the cAMP accumulation in 293 cells was measured as described in Experimental Procedures. Panel A: (○) control 293 cells; (●) 293(I-AC) cells. Panel B: (○) control 293 cells; (●) 293(III-AC) cells.

adenylyl cylase present in various cells, or different isozymes of PKC present.

The availability of cDNA clones for different adenylyl cyclases (Krupinski et al., 1989; Feinstein et al., 1991; Bakalyar & Reed, 1990; Gao & Gilman, 1991; Ishikawa et al., 1992; Yoshimura & Cooper, 1992) makes it possible to examine the sensitivity of distinct adenylyl cyclases to direct or indirect regulation by activators of PKC in whole cells. The type I (Tang et al., 1991) and type III (Choi et al., 1992b) adenylyl cyclases are both stimulated by Ca^{2+} and CaM whereas the type II and type IV–VI adenylyl cyclases are not (Feinstein et al., 1991, Gao & Gilman, 1991; Ishikawa et al., 1992; Yoshimura & Cooper, 1992). The type I adenylyl cyclase is neurospecific and has been strongly implicated in neuroplasticity (Xia et al., 1993) whereas the type III enzyme was originally cloned from an olfactory library (Bakalyar & Reed, 1990) but is expressed in a number of other tissues including brain (Xia et al., 1992). In this study, we report that both of the two CaM-sensitive adenylyl cyclases are stimulated by phorbol esters in whole cells. These data suggest that activation of PKC may indirectly modulate cellular cAMP levels in mammalian tissues, particularly in specific regions of brain which express type I or type III adenylyl cyclases.

EXPERIMENTAL PROCEDURES

Cell Culture and DNA Transfection. Human embryonic kidney 293 cells were routinely grown at 36 °C in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum in a humidified 95% air/5% CO_2 incubator. The 293 cells were stably transfected with an expression vector, CDM8, that contained cDNA for either type I adenylyl cyclase (CDM8(I-AC)) or type III adenylyl cyclase (CDM8(III-AC)), as previously described (Choi et al., 1992a,b). Briefly, cells in 100-mm dishes at a 20–30% confluency were transfected with the CDM8 control vector, CDM8(I-AC) or CDM8(III-AC) (10 μg of DNA/plate), and pKo-Neo (1 μg of DNA/plate) by the calcium phosphate method (Chen & Okayama, 1987). Neomycin-resistant cells were selected in culture medium containing G418 (500 μg/mL). Each clone of G418-resistant cells was examined for adenylyl cyclase activity to detect cells that express either type I or type III adenylyl cyclase. Particular clones, 293(I-AC43) (Choi et al., 1992a) and 293(III-AC3), were used in the present study for 293 cells expressing either type I or type III adenylyl cyclase, respectively. G418-resistant 293 cells that were transfected with the CDM8 vector were used as a control.

Cyclic AMP Accumulation. Confluent cultures of 293-(I-AC), 293(III-AC), or control 293 cells in 60-mm dishes were rinsed with phosphate-buffered saline and exposed to TPA or other phorbol esters in Dulbecco's modified Eagle's medium for indicated times. After treatment with phorbol esters, cells were incubated with 5 μM forskolin and 1 mM IBMX in an incubation buffer (118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 0.5 mM EDTA, 10 mM glucose, and 20 mM HEPES, pH 7.4) for 30 min. The reaction was terminated by rinsing the cells twice with 150 mM NaCl and adding 3 mL of 5% trichloroacetic acid. The amount of cAMP in samples was assayed by the method of Gilman (1970). Protein concentrations in samples were determined by the bicinchoninic acid method (Pierce).

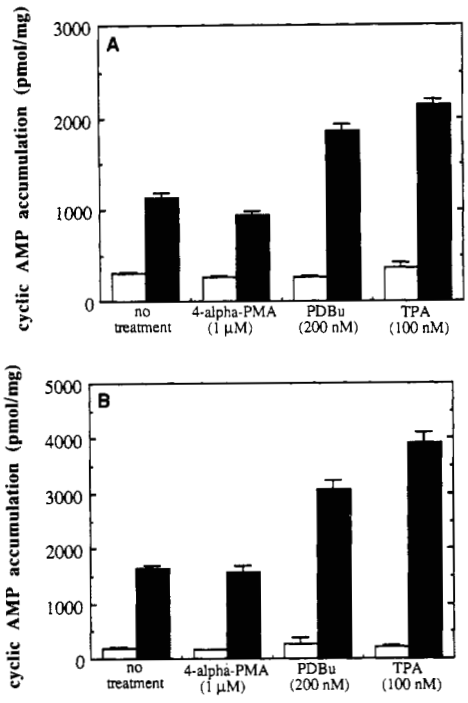

FIGURE 3: Effects of phorbol ester derivatives on the cAMP levels of 293 cells expressing type I or type III adenylyl cyclase. Cultured 293 cells were exposed to various phorbol esters for 15 min (A) or 5 min (B), followed by incubation with 5 μM forskolin and 1 mM IBMX for 30 min. Cyclic AMP accumulation was determined as described in Experimental Procedures. Panel A: (□) control 293 cells; (■) 293(I-AC) cells. Panel B: (□) control 293 cells; (■) 293-(III-AC) cells.

Quantitation of Intracellular Free Ca^{2+}. Cells grown on glass cover slips were examined for intracellular free Ca^{2+} using fura-2 by the method of Hassid (1986).

RESULTS AND DISCUSSION

In order to determine if phorbol esters can modulate the activity of the calmodulin-sensitive adenylyl cyclases in vivo, we examined the effect of TPA on intracellular cAMP levels in human kidney 293 cells expressing type I adenylyl cyclase (293(I-AC) cells) or cells expressing type III enzyme (293-(III-AC) cells). All experiments in this study were performed in the presence of 1 mM IBMX in order to inhibit endogenous cyclic nucleotide phosphodiesterase activities.

When cultured 293 cells were exposed to TPA for various times, TPA alone did not affect basal cAMP levels in control, 293(I-AC), or 293(III-AC) cells (data not shown). Furthermore, TPA did not affect cAMP levels in these cells when they were treated with 1,9-dideoxyforskolin, a structural analogue of forskolin that does not stimulate adenylyl cyclase activity but does exhibit other activities associated with forskolin including inhibition of glucose transporter activity (Joost et al., 1988). TPA did, however, markedly enhance the cAMP accumulation stimulated by forskolin in 293(I-AC) cells (Figure 1A). At a TPA concentration of 100 nM, a maximal 2-fold increase in forskolin-stimulated cAMP accumulation occurred within 15 min after exposure to TPA.

Treatment of 293(III-AC) cells with 100 nM TPA resulted in a 5-fold increase in intracellular cAMP levels within 5 min (Figure 1B), and the increase in cAMP remained for at least 30 min after the addition of TPA. Although the kinetics for the TPA-stimulated cAMP increases were similar in 293(I-AC) and 293(III-AC) cells, cells containing the type III enzyme exhibited significantly larger increases in cAMP (3–5-fold) compared to those expressing the type I adenylyl cyclase (2-fold). The control cells, which do not express type I adenylyl cyclase activity (Xia et al., 1993), showed a 10–20% increase in cAMP in response to TPA. The small increase in cAMP seen when control cells were treated with TPA is likely due to the presence of a low level of endogenous type III adenylyl cyclase in these cells (Xia et al., 1992).

The elevation of cyclic AMP levels in either 293(I-AC) or 293(III-AC) cells after TPA treatment was dependent upon the concentrations of TPA applied to the cultured cells (Figure 2). Maximal stimulation of cAMP levels was observed at 100 nM TPA in both 293(I-AC) and 293(III-AC) cells.

In order to evaluate the specificity of the cAMP increase caused by TPA, we examined the effects of two other phorbol esters on intracellular cAMP levels in 293(I-AC) and 293-(III-AC) cells. TPA and PDBu are potent activators of protein kinase C. Both of these two compounds enhanced cAMP increases caused by forskolin in 293(I-AC) and 293(III-AC) cells, and TPA was somewhat more effective than PDBu (Figure 3). The inactive phorbol ester, 4-α-PMA, did not affect cAMP increases in 293(I-AC) or 293(III-AC) cells. These data are consistent with the conclusion that the cAMP increases caused by TPA or PDBu were due to activation of protein kinase C and direct or indirect stimulation of both type I and type III adenylyl cyclase activities.

We were also interested in determining if TPA affected hormone stimulation of type I adenylyl cyclase in 293 cells. Unfortunately, the type I adenylyl cyclase expressed in 293-(I-AC) cells is poorly coupled to endogenous Gs, making it difficult to examine the effect of TPA on hormone-stimulated cAMP accumulation. Similar observations have been reported by other laboratories (Bakalyar & Reed, 1990; Yoshimura & Cooper, 1992).

Because both the type I and type III adenylyl cyclases are stimulated by calcium (Choi et al., 1992a,b; Tang et al., 1991) and activators of protein kinase C may elevate intracellular calcium levels in some cells (Yamaguchi et al., 1987), the increases in cAMP caused by TPA could be due to increases in free calcium. In order to evaluate this possibility, we examined the effect of the intracellular calcium chelator BAPTA/AM on the TPA-stimulated cAMP increases. BAPTA/AM has been shown to block cAMP increases caused by the calcium stimultaion of type I adenylyl cyclase in 293 cells (Choi et al., 1992a). Exposure of 293(I-AC) or 293-(III-AC) cells to BAPTA/AM, prior to TPA treatment, did not block the elevations in cAMP caused by TPA (Figure 4). Furthermore, TPA had no effect on intracellular free Ca^{2+} levels in 293(I-AC) or 293(III-AC) cells (data not shown). These results indicate that the stimulatory effect of TPA on type I and type III adenylyl cyclases in whole cells was not due to an increase in intracellular free calcium.

Another possible mechanism for the effect of phorbol esters on the adenylyl cyclase activities would be the direct phosphorylation of type I and type III adenylyl cyclase catalytic subunits by protein kinase C in vivo. This possibility was examined by incubation of 293(I-AC) with [^{32}P]inorganic phosphate followed by immunoprecipitation of the adenylyl cyclase. Our preliminary data suggested no significant increase in the incorporation of ^{32}P into polypeptides corresponding to the size of the type I adenylyl cyclase (120 kDa) when 293(I-AC) cells were exposed to TPA. However, we cannot rule out the possibility that the expression level of type I adenylyl cyclase in 293(I-AC) cells may be too low to detect in vivo phosphorylations.

In conclusion, activators of protein kinase C enhanced cellular cAMP levels in cells expressing type I or type III

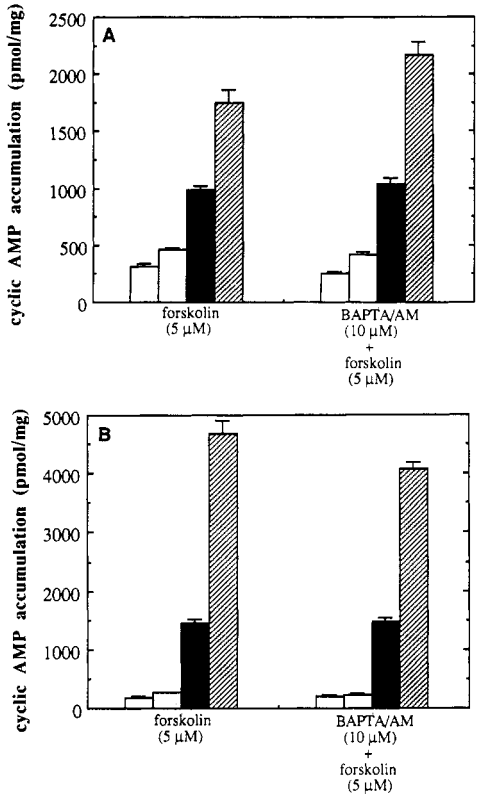

FIGURE 4: BAPTA/AM does not inhibit TPA-stimulated increases in intracellular cAMP levels. A confluent culture of cells was preincubated for 10 min with 10 μM BAPTA/AM prior to exposure of cells to 100 nM TPA for 15 min. Cells were then rinsed once with PBS and incubated with 5 μM forskolin and 1 mM IBMX for 30 min. cAMP accumulation was determined as described in Experimental Procedures. Panel A: (□) control cells without TPA treatment; (⧈) control cells with TPA treatment; (■) 293(I-AC) cells without TPA treatment; (◪) 293(I-AC) cells with TPA treatment. Panel B: (□) control cells without TPA treatment; (⧈) control cells with TPA treatment; (■) 293(III-AC) cells without TPA treatment; (◪), 293(III-AC) cells with TPA treatment.

calmodulin-sensitive adenylyl cyclase in vivo. Considering that both type I and type III adenylyl cyclases are expressed in brain, modulation of the cAMP metabolism in brain by protein kinase C may be an important mechanism for a variety of neuronal functions including long-term adaptive responses. Protein kinase C activation of the type I adenylyl cyclase during long-term potentiation is a particularly interesting hypothesis because this enzyme has been strongly implicated in neuroplasticity (Xia et al., 1991, 1993; Levin et al., 1992).

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