0026-895X/91/020124-06$03.00/0

MOLECULAR PHARMACOLOGY, 39:124-129

Selective Effects of Activation of Protein Kinase C Isozymes on Cyclic AMP Accumulation

FABIAN GUSOVSKY and J. SILVIO GUTKIND

Laboratory of Bioorganic Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases (F.G.), and Laboratory of Cellular Development and Oncology, National Institute of Dental Research (J.S.G.), National Institutes of Health, Bethesda, Maryland 20892

Received May 22, 1990; Accepted November 14, 1990

SUMMARY

Activation of protein kinase C (PKC) in intact cells can induce significant changes, either facilitatory or inhibitory, in cyclic AMP accumulation elicited either by receptor activation or by the activator of adenylate cyclase, forskolin. Such interaction represents an example of "cross-talk" between second messenger systems and may underlie the biochemical basis of synchronization between external stimuli and biological responses. PKC is now known to comprise a variety of subspecies. Although differences among the PKC subspecies are apparent in terms of their enzymological properties, no functional differences among them have been described. In PC12 cells, where both α and γ isozymes of PKC are present, activation of PKC causes enhancement of the responses of cyclic AMP-generating systems. In NCB20 cells and NIH 3T3 cells, where only the α isozyme is expressed, activation of PKC causes inhibition of cyclic AMP-generating systems. In NIH 3T3 cells after transfection of γ-PKC, activation of the enzyme was no longer inhibitory; instead, a facilitation of cyclic AMP accumulation was observed. Thus, the α and γ isozymes of PKC appear to have opposite actions, facilitatory for γ-PKC and inhibitory for α-PKC, on the responses of cyclic AMP-generating systems in NIH 3T3 cells. Such opposing actions represent a remarkable functional distinction between two PKC subspecies.

Calcium/phospholipid-dependent protein kinase, PKC, is present in most cells, and its activity is stimulated by the second messenger DAG. Because DAG levels are increased upon stimulation of phosphoinositide breakdown, PKC belongs to the group of enzymes whose activity can be modulated by extracellular stimuli through signal transduction pathways. Activation of PKC results in phosphorylation of numerous protein substrates, and such action has been linked to a wide variety of biological responses (1, 2).

PKC represents, in fact, a family of enzymes. PKC isozymes α, βI, βII, and γ are abundant and have heterogeneous tissue distribution. Other subspecies (δ, ϵ, ζ) appear to be present in smaller amounts (2).

PKC activation can significantly influence another second messenger system, namely cyclic AMP generation (1). In some cells PKC activation results in potentiation of receptor- or forskolin-mediated cyclic AMP formation (3–6), and in other cases PKC activation results in inhibition of such responses (7–14). Examples of opposite PKC actions on cyclic AMP-generating systems are those observed in two cell lines, rat pheochromocytoma (PC12) cells (5) and neuroblastoma hybrid (NCB20) cells (15). In PC12 cells, adenosine receptor- or forskolin-mediated generation of cyclic AMP is potentiated when PKC activity is stimulated either with a phorbol ester, PMA (5), which directly activates PKC, or with the marine toxin MTX (16), which stimulates phosphoinositide breakdown and, therefore, increases intracellular levels of DAG. In contrast, in NCB20 cells, treatment with PMA (15) or MTX (16) induces inhibition of receptor-mediated cyclic AMP accumulation. In brain preparations, similar to PC12 cells, PMA induces a potentiation of receptor-mediated cyclic AMP formation (3). We have now studied the possible relationship between the expression of PKC isozymes and the responses observed in cyclic AMP formation in cells.

Materials and Methods

Cell Cultures

NCB-20 and NIH 3T3 cells were cultured in DMEM containing 10% fetal calf serum and antibiotics. PC12 rat pheochromocytoma cells were cultured in DMEM containing 5% horse serum, 5% fetal calf serum, and antibiotics. Selection of transfected cells was performed in DMEM containing 10% fetal calf serum, antibiotics, and 750 μg/ml G418 (GIBCO, Grand Island, NY).

Immunoblots for PKC Isozymes

Whole-cell lysates of cells were obtained by treatment of 10-cm confluent plates with lysing buffer (20 mM Tris, pH 7.5, 2 mM EDTA,

ABBREVIATIONS: PKC, protein kinase C; DMEM, Dulbecco's modified Eagle's medium; EGTA, ethylene glycol bis (β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PMA, phorbol-12-myristate-13-acetate; DAG, diacylglycerol; MTX, maitotoxin; cAMP, cyclic AMP.

0.5 mM EGTA, 0.2 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1% NP-40). After centrifugation (30 min, 15,000 × *g*), the supernatant was partially purified through a DE-52 (DEAE) column, as described (16, 17). Eluates were concentrated with Centricon 30 filters, and aliquots of the concentrated extract were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 10% polyacrylamide gels and electroblotted onto nitrocellulose filters (16). Nonspecific binding was prevented by an overnight preincubation of sheets with Blotto (Advanced Biotechnologies, Columbia, MD). The nitrocellulose sheets were then incubated with the indicated antibodies. If a polyclonal antibody was used, the nitrocellulose sheets were incubated with the antibody for 2 hr at room temperature, washed with Blotto three times, and subsequently incubated with ^{125}I-Protein A (Amersham) for 45 min. If a monoclonal antibody was used, the nitrocellulose sheets were incubated for 4 hr at room temperature. After three washes, a second antibody, anti-mouse IgG from rabbit (Cappel, Malvern, PA), was applied (1:200) for 2 hr. After three washes, the nitrocellulose sheets were incubated with ^{125}I-Protein A (Amersham) for 45 min. With both antibodies, blots were washed, dried, and subjected to autoradiography at −70° for the times indicated in the figures. A mixture of ^{14}C-labeled protein standards (BRL, Gaithersburg, MD) was run in every gel for determination of molecular weights.

Translocation and Immunodetection of PKC Isozymes

Confluent plates of cells were harvested in 5 ml of translocation buffer (20 mM Tris·HCl, pH 7.5, 2 mM EDTA, 0.5 mM EGTA, 0.2 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 0.33 M sucrose). Cells were disrupted in a glass-glass homogenizer, and the homogenate was centrifuged at 15,000 × *g* for 20 min. This supernatant represents the cytosolic fraction. Cytosolic fractions were partially purified and concentrated, and PKC isozymes were analyzed by immunoblotting (see above).

Preparation of and Transfection with γ-ZipNeo Construct

cDNA for rat γ-PKC (18) was subcloned into the *Bam*HI site of the expression vector ZipNeoSV(X) (19), utilizing standard cloning procedures. NIH 3T3 cells were transfected with γ-ZipNeo (γ$^+$) or with the expression vector ZipNeoSV(X) (control) by the calcium phosphate procedure.

Determination of Cyclic AMP Levels in Cells

NCB20 and PC12 cells. Cells were harvested from culture flasks and washed twice with buffer A (118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 0.5 mM EDTA, 2.5 mM $CaCl_2$, 10 mM glucose, 20 mM HEPES, pH 7.4). Cells were then resuspended in buffer A (about 2 × 10^6 cells/ml). Aliquots of the cell suspensions were then transferred to 1.5-ml Microfuge tubes that contained the agents in buffer A prewarmed at 37°. The final volume was 500 μl. Incubations were for 10 min and were stopped by transfer of 400 μl to Microfuge tubes preheated at 95°. After 3 min at 95°, the tubes were centrifuged for 2 min at 12,000 × *g*, and cyclic AMP was determined in the supernatants using a commercial [^{3}H]cAMP assay kit (Amersham, Arlington Heights, IL).

NIH 3T3 and transfected cells. Cells were cultured in 12-well multiwell plates. Medium was aspirated and cells were washed with buffer A. Fresh buffer (1 ml) was added. After a 10-min preincubation, compounds were added as indicated and the incubations were continued for 15 min. Incubations were stopped by removal of the buffer and addition of 0.2 N HCl (0.5 ml). After 30 min, the acid was removed and neutralized, and cyclic AMP levels were determined as described above.

Materials

Initial characterization of isozymes was performed using anti-PKC isozyme antisera (20) provided by Dr. Ora M. Rosen (Sloan Kettering Cancer Research Center, New York). Subsequent immunoblots were performed using monoclonal anti-PKC isozyme antibodies (Seikagaku America, St. Petersburg, FL). Rat γ-PKC cDNA was provided by Dr. John Knopf (Genetics Institute, Boston, MA). Expression vector pZip Neo SV(X) was provided by Dr. Richard Mulligan (Whitehead Institute, Boston, MA). Other reagents were from standard commercial sources.

Results

Cyclic AMP accumulation in NCB20 and PC12 cells. Stimulation of PKC with the phorbol ester PMA in NCB20 cells induced a significant inhibition of forskolin-mediated cAMP accumulation (Table 1). Conversely, in PC12 cells PMA induced a significant potentiation of forskolin-mediated cAMP accumulation (Table 1). No phosphodiesterase inhibition was induced in either cell line, which explains the lower values obtained compared with those previously reported for forskolin-mediated cAMP accumulation in these cells (5, 15).

PKC isozyme expression in NCB20 and PC12 cells. Both cell lines expressed α-PKC, as determined by immunoblots performed with anti-α-PKC antibodies (Fig. 1a). The identity of the bands in both cell lines was confirmed by the recording of their disappearance in cytosolic extracts of cells treated with PMA (Fig. 1b). Such response is interpreted as translocation of α-PKC from cytosol to membranes, an indication of PKC activation (16, 17). In PC12 cells, but not in NCB20 cells, γ-PKC was present, as determined by immunoblot (Fig. 1c). The identity of the band in PC12 cells was confirmed by the recording of its disappearance in cytosolic extracts after treatment with PMA (Fig. 1d). Neither NCB20 nor PC12 cells expressed β-PKC (as detectable by immunoblot; data not shown).

Transfection of NIH 3T3 cells with γ-ZipNeo construct. Control and transfected (γ$^+$) NIH 3T3 cells expressed comparable levels of α-PKC, as determined by immunoblot (see Fig. 2). Control cells had no detectable γ-PKC (Fig. 2, *left*) or β-PKC (data not shown). γ$^+$ cells had significant amounts of γ-PKC, as determined by immunoblot (Fig. 2, *right*). Translocation of α-PKC in control cells and of α-PKC and γ-PKC in γ$^+$ cells was observed after treatment with PMA (1 μM) or MTX (1 ng/ml) for 10 min (Fig. 3).

Modulation of cyclic AMP levels by PKC isozymes. In control NIH 3T3 cells, forskolin- but not 2-chloroadenosine-induced cyclic AMP accumulation was inhibited by treatment with PMA (Fig. 4A). The marine toxin MTX also inhibited forskolin-induced cyclic AMP formation, presumably through phosphoinositide breakdown and subsequent DAG formation (16). NIH 3T3 cells transfected with γ-PKC cDNA showed a

TABLE 1

Effects of PMA on forskolin-mediated cyclic AMP accumulation in NCB20 and PC12 cells

Suspensions of NCB20 cells and PC12 cells were incubated for 10 min at 37° in the presence of the indicated agents. Incubations were terminated by heating of the cell suspensions at 95° for 2 min. After centrifugation, cAMP levels were determined in the supernatants. Values are means ± standard errors of three or four independent experiments performed in duplicate.

Treatment	cAMP	
	NCB20 cells	PC12 cells
	pmol/10^6 cells	
Control	24 ± 3	12 ± 2
Forskolin (10 μM)	57 ± 3[a]	313 ± 58
PMA (1 μM)	21 ± 3	13 ± 7
Forskolin + PMA	37 ± 7[b]	617 ± 71[b]

[a] $p < 0.05$ versus control.
[b] $p < 0.05$ versus forskolin alone.

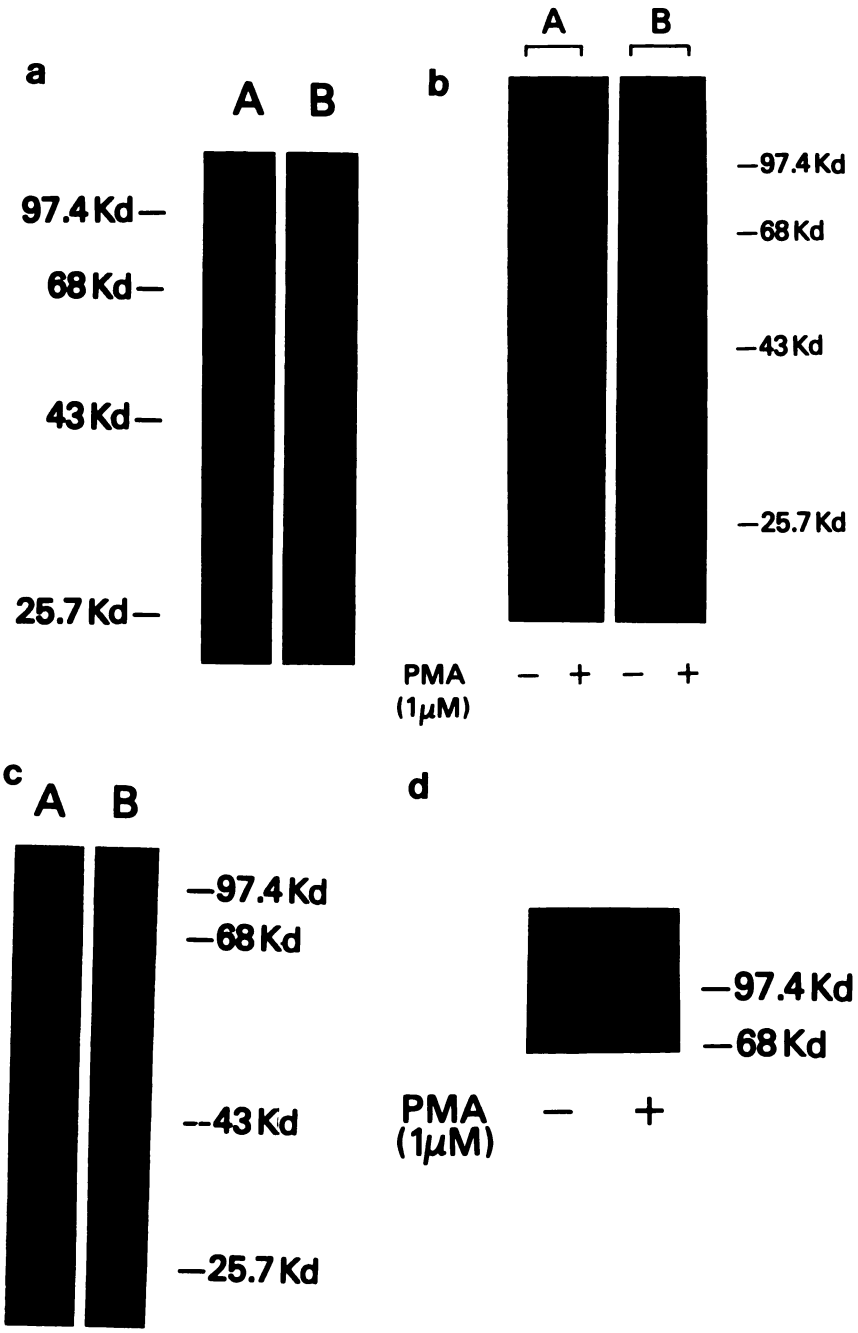

Fig. 1. a, Expression of α-PKC in NCB20 and PC12 cells. Whole-cell lysates of NCB20 (A) and PC12 (B) cells were obtained and sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting were performed as described in Materials and Methods. An anti-α-PKC monoclonal antibody was used (see Materials and Methods). b; Translocation of α-PKC in NCB20 and PC12 cells. NCB20 and PC12 cells were treated with medium or 1 μM PMA for 10 min. Cytosolic extracts were obtained and processed as in a. An anti-α-PKC monoclonal antibody was used (see Materials and Methods). c, Expression of γ-PKC in PC12 cells. Cell lysates were obtained and analyzed as described in a, except that an anti-γ-PKC polyclonal antibody was used (see Materials and Methods). d, Translocation of γ-PKC in PC12 cells. Cytosolic extracts were obtained as described in b. Immunoblots were performed as described in c. In all cases, between 50 and 100 μg of protein/lane were loaded. In translocation experiments, equal amounts of protein were loaded for control and stimulated cells.

remarkably different profile. In these cells, PMA induced a potentiation of forskolin- and 2-chloroadenosine-induced cyclic AMP accumulation (Fig. 4B), and MTX did not inhibit the forskolin response.

Discussion

Interactions between PKC and cyclic AMP-generating systems are observed in a wide variety of cells. The nature of this interaction is usually of one of two types; PKC activation results in either (i) potentiation or (ii) inhibition of cyclic AMP formation. Generally, the type of interaction observed is dependent on the cell studied, although in a few cases both effects can be detected, depending on the conditions of the experiment (21–24). PC12 and NCB20 cells represent two cell lines in which activation of PKC has opposite effects on cyclic AMP generation. Thus, in PC12 cells a phorbol ester, PMA, which activates PKC, induces potentiation of cyclic AMP formation elicited by forskolin and 2-chloroadenosine (5), and in NCB20 cells PMA induces inhibition of receptor agonist-mediated cyclic AMP formation (15).

Because γ-PKC seems to be expressed only in brain and neurally derived tissues (2) and potentiation of cyclic AMP formation is observed with phorbol esters in brain and neurally derived preparations, we tested the possibility that activation of γ-PKC mediates the potentiation of cyclic AMP accumulation. Immunoblots of PC12 and NCB20 cell extracts showed immunoreactivity when tested with an anti-α-PKC antibody. Immunoblots of cell extracts of PC12 cells, but not of NCB20

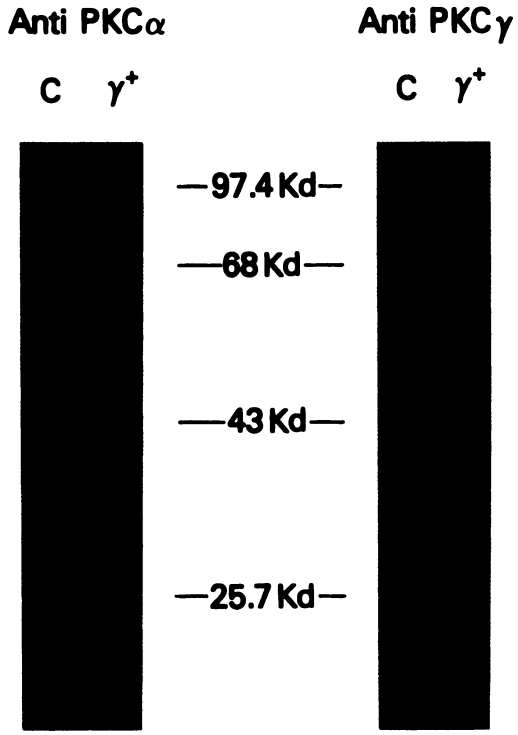

Fig. 2. Expression of γ-PKC in transfected NIH 3T3 cells. NIH 3T3 cells were transfected with the γ-ZipNeo construct, using the calcium phosphate procedure. Lysates from confluent cells grown in 10-cm plates were processed as described in Fig. 1a. Immunoblots were obtained using an anti-α-PKC monoclonal antibody (*left*) or an anti-γ monoclonal antibody (*right*). Both antibodies were purchased from Seikagaku America (St. Petersburg, FL). One hundred micrograms of protein/lane were loaded. *C*, control.

cells, showed immunoreactivity when tested with an anti-γ-PKC antibody (Fig. 1c).

In order to test whether the expression of γ-PKC was responsible for the observed potentiation of cyclic AMP accumulation, NIH 3T3 cells, which only express the α isozyme, were transfected with γ-PKC cDNA that was introduced into the ZipNeo SV(X) expression vector (Fig. 2). The presence of γ-PKC did not affect the expression levels of the native isozyme, α-PKC (Fig. 2). Moreover, α-PKC and γ-PKC in γ^+ cells were translocated from cytosol to membranes upon stimulation with PMA or MTX (Fig. 3). The latter indicates that, in the transfected cells, γ-PKC is functional and responsive to agents that affect PKC activity, namely a phorbol ester tumor promoter, which directly activates the enzyme(s), and DAGs, which are generated with MTX (16). The presence of γ-PKC had a dramatic effect on the regulation of cyclic AMP formation. In γ^+ cells PMA potentiated forskolin- and 2-chloroadenosine-mediated formation of cyclic AMP, whereas in control cells, which only express α-PKC, inhibition by PMA of forskolin-mediated (but not 2-chloroadenosine-mediated) stimulation was observed. Such difference between control and γ^+ cells can only be attributed to the presence of γ-PKC, because these control cells had been transfected with ZipNeo SV(X) with no insert. The marine toxin MTX, which induces PKC translocation through DAG formation, inhibited forskolin-mediated cyclic AMP formation in control cells but failed to do so in γ^+ cells. However, in contrast to the potentiative effect of the phorbol ester on forskolin-stimulated cyclic AMP accumulation in γ^+ cells, treatment with MTX did not potentiate cyclic AMP accumulation. Thus, it seems that stimulation by MTX could only reverse the inhibitory action of α-PKC on cyclic AMP formation in γ^+ cells. Perhaps DAG (or the increase in intracellular calcium) generated after MTX stimulation is more readily available for α-PKC than for γ-PKC activation. PKC isozymes isolated from brain have distinguishable properties *in vitro* (25, 26). γ-PKC (PKC type I) appears to be less sensitive than α-PKC (PKC type III) to calcium *in vitro* (26). Because MTX induces increases in intracellular calcium (16), α-PKC may be more responsive than γ-PKC to MTX-induced stimulation.

Several mechanisms have been proposed to explain the interactions between PKC and cyclic AMP generation systems at the molecular level. In platelets, which show potentiation of cyclic AMP formation with phorbol esters, Katada *et al.* (27) showed that PKC can phosphorylate $G_{i\alpha}$, i.e., the α subunit of the guanine nucleotide-binding protein involved in inhibition

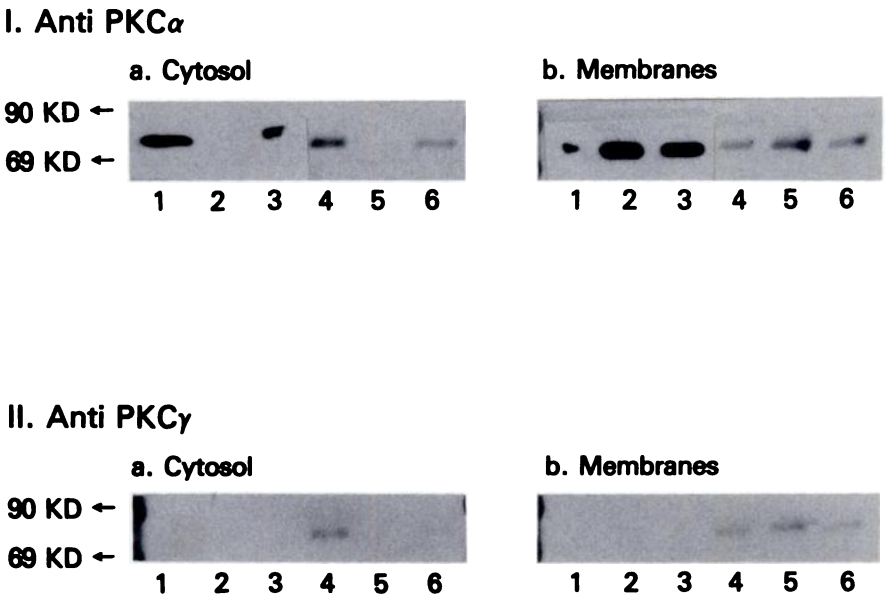

Fig. 3. Translocation of PKC isozymes in control and transfected NIH 3T3 cells. Control and γ^+ (*lanes 1–3*)/(*lanes 4–6*) cells were treated with medium (*lanes 1 and 4*), 1 μM PMA (*lanes 2 and 3*), or 1 ng/ml MTX (*lanes 3 and 6*) for 10 min. Cytosolic and membrane extracts were obtained and processed as described in Materials and Methods. Twenty micrograms μg of protein/lane or 100 μg of protein/lane were loaded for cytosolic or membrane extracts, respectively.

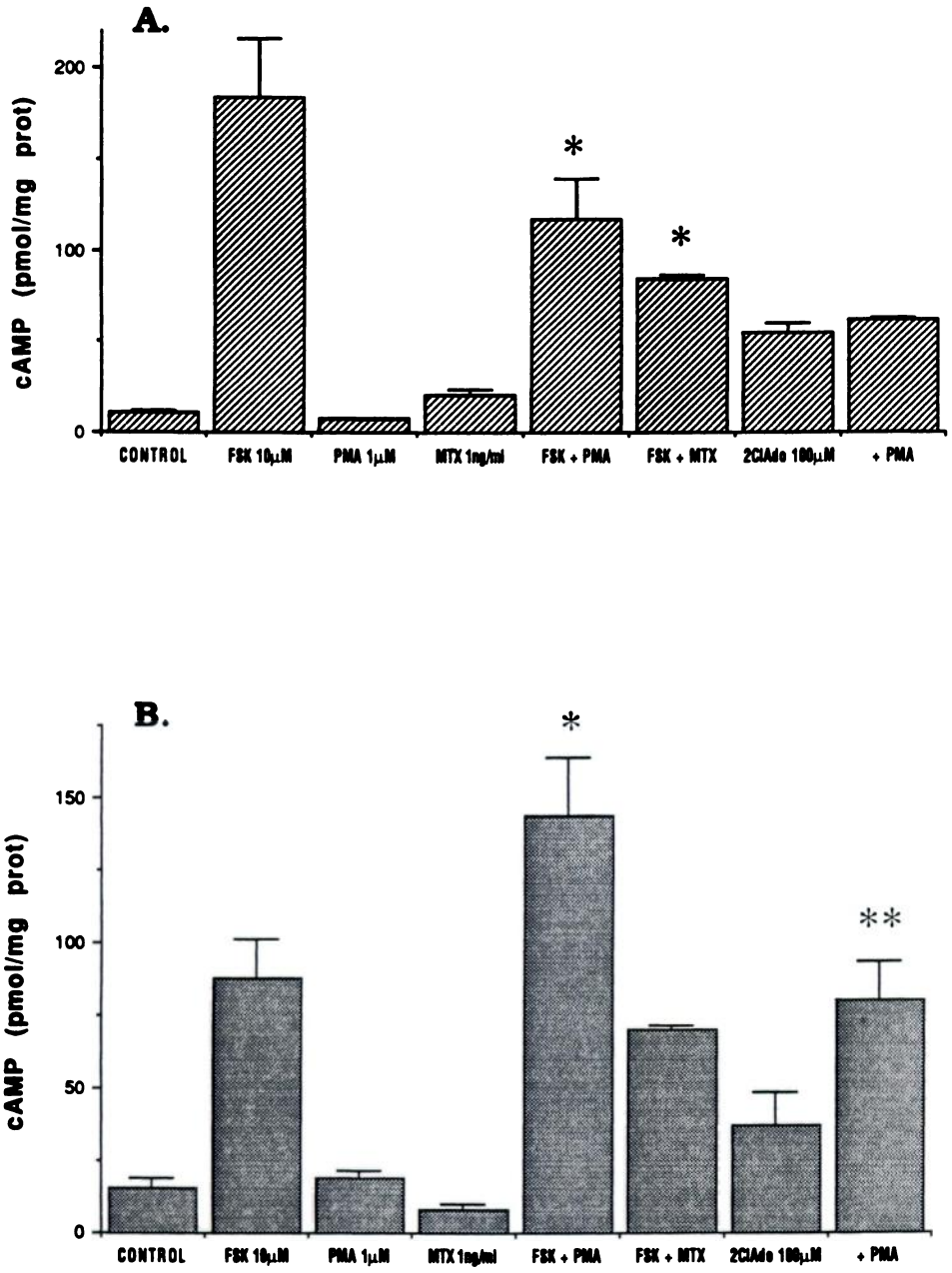

Fig. 4. Effects of stimulation of PKC on cyclic AMP accumulation in control NIH 3T3 cells (A) and in γ^+ NIH 3T3 cells (B). Cells were cultured in 12-well multiwell plates and incubated with the agents for 15 min, as described in Materials and Methods. Results correspond to an experiment performed in triplicate that was repeated three times with similar results. *, $p < 0.05$ versus forskolin (*FSK*) alone. **, $p < 0.05$ versus 2-chloroadenosine (*2ClAdo*) alone.

of adenylate cyclase, thereby supressing its inhibitory input to cyclase. In frog erythrocytes, which also show potentiation of cyclic AMP with phorbol esters, Yoshimasa *et al.* (28) demonstrated that PKC activation results in phosphorylation of adenylate cyclase. The inhibitory effects of PKC on cyclic AMP formation are thought to occur through phosphorylation of stimulatory receptors or G_s, the guanine nucleotide-binding protein involved in activation of adenylate cyclase (15).

This study indicates a clear functional difference between effects of activation of PKC α and γ isozymes. Expression of γ-PKC seems to be directly related to potentiation of cyclic AMP formation, whereas lack of γ-PKC and possibly the presence of α-PKC seem to be involved in inhibition of cyclic AMP accumulation, as observed with phorbol esters. Because γ-PKC is abundant in brain, it may be inferred that potentiation of cyclic AMP formation by phorbol esters is a manifestation of this interaction in the nervous system. In this regard, Otte *et al.* (29) recently demonstrated that cross-talk between PKC and cyclic AMP pathways mediates neural induction, a crucial step in the differentiation of the central nervous system. The distribution of PKC mRNA in the cerebellum suggests that γ-PKC is predominantly postsynaptic (30). Because cyclic AMP-accumulating entities are located postsynaptically (31), this distribution could explain the synergistic effects observed between activation of PKC and receptors coupled to adenylate cyclase (3, 31, 32).

In most cells, more than one PKC isozyme is present. Therefore, the net effect observed on cyclic AMP formation will depend on the functional balance of the different isozymes and the compartmental availability of the substrates involved in each case. In fact, in some cases the changes observed are bimodal, i.e., potentiation and inhibition of cyclic AMP for-

mation after PKC stimulation. It might be inferred from the present study that those cells probably express both α- and γ-PKC.

The identification of the PKC isoforms involved in potentiation and inhibition responses in cyclic AMP accumulation provides new insight into the cross-talk mechanisms between second messengers. With this information, a clearer picture of the interaction between PKC and cyclic AMP formation may emerge.

Acknowledgments

We acknowledge the generous help of the following individuals in providing the indicated materials: Dr. Ora M. Rosen (Sloan Kettering Cancer Research Center) provided polyclonal anti-PKC isozyme antibodies, Dr. John Knopf (Genetics Institute) provided γ-PKC cDNA, and Dr. Richard Mulligan (Whitehead Institute) provided the expression vector pZip Neo SV(X). F.G. gratefully acknowledges the support and invaluable counsel from Dr. John W. Daly.

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Send reprint requests to: Fabian Gusovsky, LBC, NIDDK, Building 8, Room 1A-15, NIH, Bethesda, MD 20892.