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PHORBOL ESTERS DOWN-REGULATE PROTEIN KINASE C IN RAT BRAIN CEREBRAL CORTICAL SLICES

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The effect of phorbol esters on cyclic AMP production in rat cerebral cortical slices was studied using a prelabelling technique to measure cyclic nucleotide accumulation. Cholera toxin-stimulated cyclic AMP accumulation was enhanced approximately 2-fold by phorbol 12-myristate, 13-acetate (PMA) which alone had no effect on cyclic AMP production. The augmentation by PMA was maximal within the first hour of incubation, decreasing progressively thereafter. Protein kinase C activity was decreased 80-90% during a 3 hr exposure to PMA, as was ^{3}H-phorbol 12,13-dibutyrate binding. Both phosphatidyl serine and arachidonic acid were found to enhance protein kinase C activity in a concentration-dependent manner, an effect that was attenuated by prolonged incubation of the brain tissue with PMA. The results indicate that exposure of brain slices to phorbol esters causes a down-regulation of rat brain protein kinase C, and that this modification corresponds with a decrease in the ability of PMA to augment cyclic AMP production, suggesting a functional relationship between the two systems in rat brain.

A number of components are associated with receptor-mediated changes in cyclic AMP production (1). Certain neurotransmitter receptors are directly coupled to adenylate cyclase by way of guanine nucleotide binding proteins (G), with some activating (through Gs) and others inhibiting (through Gi) adenylate cyclase activity (1). Other receptors are indirectly associated with second messenger production (2-4). In this case, receptor activation alone does not modify cyclic AMP production, although the response obtained during stimulation of other sites coupled to Gs is augmented (2-5). For example, while neither γ-aminobutyric acid B ($GABA_B$) nor α-adrenergic receptor agonists alter basal levels of cyclic AMP in brain slices, both increase the amount of cyclic AMP accumulated during exposure of the tissue to β-adrenergic agonists, adenosine or vasoactive intestinal peptide (VIP). This augmenting action requires extracellular calcium ions (3-5), and is associated with the calcium-dependent enzyme, protein kinase C (6,7). This hypothesis was supported by the discovery that phorbol esters known to directly activate protein kinase C also augment transmitter-stimulated cyclic AMP

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accumulation (8-11) and by the finding that the augmenting action of phorbol esters correlates with protein kinase C-stimulated phosphorylation of brain proteins (10). Furthermore, prolonged exposure to PMA resulted in a time-dependent attenuation of the augmenting effect of PMA on cyclic AMP production.

The aim of the present study was to examine the effect of prolonged exposure to phorbol esters on cellular protein kinase C activity and PMA-mediated augmentation of cyclic AMP accumulation in rat brain slices. The results indicate that a 3 hr incubation of brain tissue with phorbol esters decreases protein kinase C activity, phorbol ester binding, and PMA-mediated augmentation of cyclic AMP accumulation. The findings point to the possibility that protein kinase C is down-regulated under these conditions and suggest that this enzyme contributes to the regulation of cyclic AMP production in brain.

MATERIALS AND METHODS

^{3}H-Adenine (29 Ci/mmole) and ^{14}C-cyclic AMP (44 mCi/mmole) were purchased from ICN, whereas ^{3}H-phorbol 12,13-dibutyrate (10 Ci/mmole) and γ-^{32}P-ATP (3000 Ci/mmole) were obtained from Amersham Corporation. Phorbol 12-myristate, 13-acetate (PMA), phosphatidyl serine, diolein, and histone IIIs were purchased from Sigma Chemical Co, DEAE-cellulose DE-52 from Whatman, histone H_1 from Worthington Biochemicals, and cholera toxin from Calbiochem.

Cyclic AMP accumulation was measured using the prelabelling procedure of Shimizu et al (12). Rat brain cerebral cortical slices (350 x 350 μm) were incubated in an oxygenated (95% 0_2/ 5% $C0_2$) Krebs-Ringer bicarbonate buffer (4) containing 0.1 mM ^{3}H-adenine for 1 hr at 37^{o}C. The labelled tissue was rinsed twice and portions (15-20 mg wet weight) placed into vials prior to incubation with PMA (10 μM) and/or cholera toxin (50 μg/ml). The reaction was terminated by homogenizing the samples in 10% (w/v) trichloroacetic acid and the samples centrifuged at 13,000 x g for 10 min. ^{3}H-Cyclic AMP present in the supernatant was estimated by the double columm method of Salomon et al (13), using ^{14}C-cyclic AMP to measure recovery. The results are expressed as the percentage of total tritium present as ^{3}H-cyclic AMP (i.e. % conversion). Control samples were exposed to solvent (DMSO) instead of PMA.

In parallel experiments, unlabelled tissue slices that had been incubated in the presence or absence of PMA were homogenized in 50 mM Tris-HCl buffer (pH 7.5), containing 250 mM sucrose, 5 mM EGTA, 1 mM dithiothreitol, and 0.1% Triton X-100. The homogenate was centrifuged at 100,000 x g for 45 min and the supernatant (2.5 mg protein) applied to a DEAE-cellulose column (1 x 3 cm) equilibrated in 10 mM Tris-HCl buffer (pH 7.5), containing 2 mM EDTA and 50 mM 2-mercaptoethanol. The column was washed extensively with the same buffer prior to developing with a linear gradient of buffer containing 0 to 0.2 M NaCl (total volume 25 ml). Protein kinase C activity was measured using histone IIIs and histone H_1 as substrates. Protein kinase C activity and ^{3}H-phorbol dibutyrate (PDB) binding were measured using established procedures (14). The protein kinase C assays were routinely carried out in the presence of excess heat-stable protein inhibitor of cyclic AMP dependent protein kinase.

RESULTS

A 3 hr exposure of rat brain cortical slices to cholera toxin (50 μg/ml) resulted in a 14-fold increase in cyclic AMP accumulation (Table 1). Inclusion of PMA (10 μM) during the final 15 min of incubation significantly increased the amount of cyclic AMP accumulated as

TABLE 1. Cyclic AMP accumulation in rat brain cerebral cortical slices during incubation with cholera toxin and PMA

Incubation Condition	Cyclic AMP Accumulation (% Conversion)
Cholera toxin alone (3 hr)	0.68 ± 0.07
Cholera toxin + PMA (15 min)	1.05 ± 0.11*
Cholera toxin + PMA (30 min)	1.40 ± 0.10*
Cholera toxin + PMA (1 hr)	1.38 ± 0.08*
Cholera toxin + PMA (2 hr)	0.96 ± 0.09*
Cholera toxin + PMA (3 hr)	0.76 ± 0.05

In all cases, rat brain cerebral cortical slices were incubated with cholera toxin (50 μg/ml) for 3 hr. When present, PMA (10 μM) was added for the last 15 or 30 min, 1 hr, 2 hr, or during the entire 3 hr incubation period. Basal cyclic AMP accumulation was 0.05% throughout the 3 hr period. Each value represents the mean ± s.e.m. of 3 separate experiments, each of which was performed in duplicate. * $p \leq 0.05$ compared to cholera toxin alone (two-tailed Student's t-test).

compared to cholera toxin alone. PMA-induced augmentation was concentration-dependent (EC_{50} = 1 μM), with 10 μM PMA yielding a maximal response (data not shown). Augmentation was observed only with those phorbol esters known to stimulate protein kinase C (data not shown), and was found to be maximal during the first hour of incubation, decreasing over the next 2 hr to the level obtained with cholera toxin alone (Table 1). The addition of PMA during a 1 or 2 hr exposure to cholera toxin also caused an augmentation of cyclic AMP accumulation, although the phorbol ester was much less effective when present for a 3 hr incubation period (Table 2).

TABLE 2. Influence of PMA on cholera toxin-induced accumulation at cyclic AMP in rat brain cerebral cortical slices

Incubation Condition (Time)	Cyclic AMP Accumulation (% Conversion)	
	Without PMA	With PMA
Cholera Toxin (1 hr)	0.12 ± 0.02	0.30 ± 0.03*
Cholera Toxin (2 hr)	0.38 ± 0.04	0.69 ± 0.05*
Cholera Toxin (3 hr)	0.59 ± 0.03	0.63 ± 0.04

Rat brain cerebral cortical slices were incubated with cholera toxin (50 μg/ml) alone or in the presence of PMA (10 μM) for 1, 2, or 3 hr, after which cyclic AMP accumulation was measured. Basal accumulation of cyclic AMP was 0.05% in all cases. Each value represents the mean ± s.e.m. of 3 separate experiments, each of which was performed in duplicate. * $p \leq 0.05$ compared to corresponding value obtained in the absence of PMA (two-tailed Student's t-test).

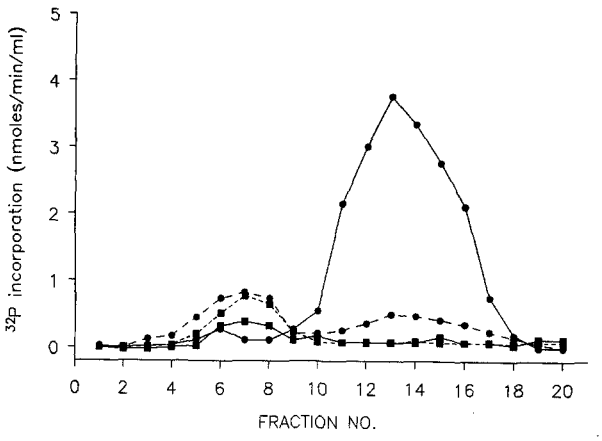

Figure 1. Fractionation of rat brain protein kinase C by DEAE-cellulose chromatography

The brain cytosol (100,000 x g supernatant) was applied to DEAE-cellulose as described in Methods. Portions (10 μls) of each fraction were assayed for protein kinase C activity by examining phosphorylation of histone IIIs in the presence (solid line) and absence (dotted line) of calcium, diolein and phosphatidyl serine. Protein kinase activity in control tissue extract (circles) and extracts of tissue exposed to PMA for 3 hr (squares) are indicated.

Protein kinase C activity could be detected only after chromatography of the tissue cytosol (100,000 x g supernatant) on DEAE-cellulose (Figure 1). The ability of the enzyme to catalyze the phosphorylation of histone IIIs was increased 15- to 30-fold in the presence of calcium (1 mM), diolein (20 μg/ml) and phosphatidyl serine (200 μg/ml). Control tissue displayed two peaks of histone kinase activity, one of which eluted at approximately 0.11 M NaCl. This fraction was sensitive to calcium and phosphatidyl serine, as expected for protein kinase C. However, only the first histone kinase peak was detected after a 3 hr exposure of the brain slices to PMA. As opposed to that found in 0.11 M NaCl, the activity of this enzyme (eluted at 0.05 M NaCl) was inhibited approximately 50% by calcium (1 mM) and phosphatidyl serine (200 μg/ml). Thus, whereas a 3 hr exposure to PMA reduced protein kinase C activity by 80-90%, the activity detected in the first peak of histone kinase was essentially unchanged.

Phosphatidyl serine stimulated protein kinase C activity in the eluted fractions in a concentration-dependent manner (Figure 2). The protein kinase activity from control tissue was stimulated maximally by concentrations of phospholipid greater than 200 μg/ml using histone H_1 or histone IIIs as substrates. The extent of activation in the presence of 1 mM $CaCl_2$, diolein (20 μg/ml) and phosphatidyl serine (200 μg/ml) was greater using histone H_1, being approximately 55-fold, which was similar to that observed with 1 μM PMA alone (data not shown). Under these conditions, two Ka values (5 μg/ml and 45 μg/ml) were found for

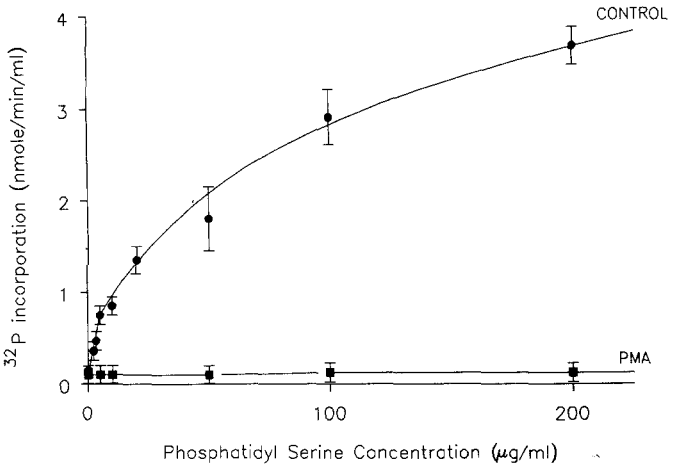

Figure 2. The activation of rat brain protein kinase C by phosphatidyl serine

Peak fractions of protein kinase C eluting at 0.11 M NaCl from DEAE-cellulose were assayed for activation of histone H_1 phosphorylation by varying concentrations of phosphatidyl serine in the presence of 1 mM $CaCl_2$ and diolein (20 μg/ml). Each point represents the mean ± s.e.m. of 3 experiments. The control fraction is represented by closed circles and the PMA treated fraction by the solid squares.

phosphatidyl serine. In contrast to control tissue, fractions obtained from PMA-treated tissue phosphorylated histone H_1 (with a 1.5 to 1.8-fold stimulation by Ca^{2+}/phospholipid) at maximal concentrations of phosphatidyl serine.

Like phosphatidyl serine, arachidonic acid also stimulated protein kinase C in control tissue, having an apparent K_a of 0.13 mM (Figure 3). Moreover, in comparison to the findings with phosphatidyl serine, enzyme from PMA-treated tissue failed to respond to arachidonic acid.

When ^{3}H-phorbol dibutyrate (PDB) binding was examined in the presence of phosphatidyl serine (200 μg/ml), radioligand binding was found in precisely the same fractions as protein kinase C activity following ion-exchange chromatography (data not shown). The binding of ^{3}H-PDB was stimulated by phosphatidyl serine in the absence of calcium ions, increasing approximately 5-fold in the peak fractions at saturating concentrations of the phospholipid. In contrast, ^{3}H-PDB binding fractions obtained from PMA-treated tissue were unaffected by phosphatidyl serine, being identical to that observed in control slices in the absence of the phospholipid. Moreover, PMA (10 μM) displaced very little (< 20%) of the total isotope bound to fractions from tissue exposed for 3 hr to the phorbol ester (Figure 4).

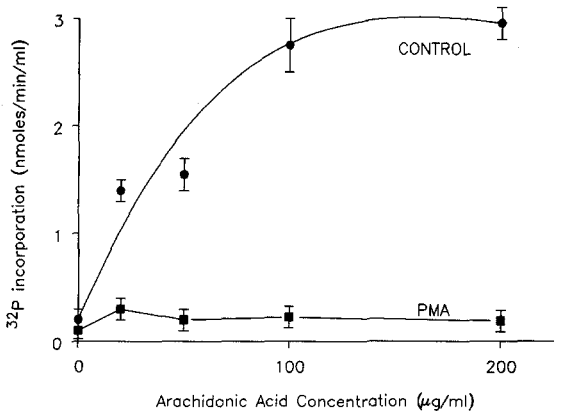

Figure 3. Activation of rat brain protein kinase C by arachidonic acid

Histone H_1 phosphorylation was activated by various concentrations of arachidonic acid in the presence of 1 mM $CaCl_2$ and diolein (20 μg/ml). Each point represents the mean ± s.e.m. of 3 experiments. Closed circles represent the activation of the control fraction and squares represent the fraction from PMA-treated tissue.

DISCUSSION

Recent reports have indicated that PMA enhances the ability of a variety of receptor agonists to increase intracellular levels of cyclic AMP, suggesting that PMA modifies a post-receptor constituent of the adenylate cyclase system. The fact that PMA augments cyclic

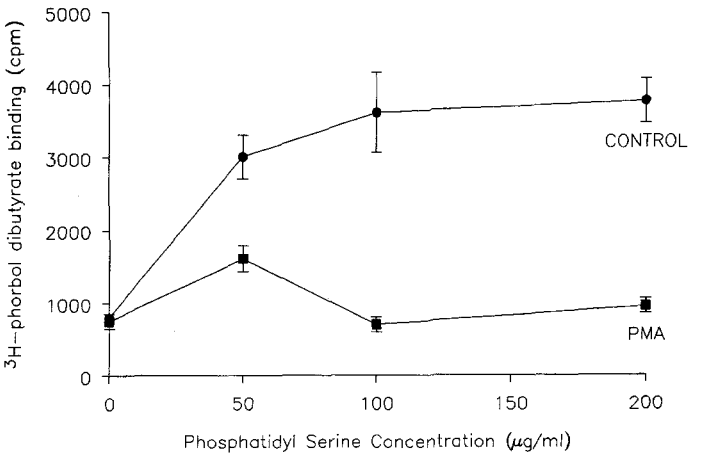

Figure 4. Effect of PMA exposure on ^{3}H-Phorbol 12,13-dibutyrate binding to rat brain protein kinase C

Rat brain cerebral cortical slices were incubated with PMA for 3 hr after which the peak fractions of protein kinase C activity obtained from DEAE-cellulose were analysed for phosphatidyl serine-stimulated ^{3}H-PDB binding. Portions (50 μls) of the peak fraction, representing approximately 5.0 μg protein, were incubated for 15 min at 30°C with ^{3}H-PDB (10 nM). Specific binding was defined as the difference between total binding and that observed in the presence of a saturating (10 μM) concentration of unlabelled PMA. Each point represents the mean ± s.e.m. of 3 experiments, each of which was performed in duplicate. Control fractions (closed circles) and fractions from the PMA-treated tissue (squares) were assayed.

AMP production in response to forskolin (10, 14), a diterpine that directly stimulates the catalytic subunit of adenylate cyclase and perhaps Gs (15,16), would seem to support this conclusion. Moreover, as demonstrated in the present study, PMA also augments the cyclic AMP response to cholera toxin, an agent that promotes second messenger accumulation by ADP-ribosylating Gs, lending further support to the notion that PMA influences some component of the adenylate cyclase system beyond the receptor recognition site. The potency of PMA (EC_{50} = 1 μM) in this regard was similar to that observed previously with intact tissue (10, 17). The capacity of the partially purified rat brain protein kinase C to be fully activated in vitro by 1 μM PMA suggests that the higher concentration required with intact tissue may be due to a limited penetration of the phorbol into the slice preparation (10).

The major finding of the present study was that prolonged exposure of rat brain slices to PMA reduces the ability of the phorbol ester to augment cholera toxin-stimulated cyclic AMP accumulation. The decline in the augmenting response to PMA does not appear to be due to a decrease in the capacity of adenylate cyclase to synthesize cyclic AMP since cholera toxin-stimulated second messenger accumulation was unaffected by prolonged exposure to PMA. Moreover, previous work has demonstrated that the cyclic AMP response to 2-chloroadenosine is not modified by a long-term incubation of the rat brain tissue slice with phorbol esters (10). Prolonged exposure of cells to phorbol esters has been reported to diminish cellular protein kinase C or total phorbol binding (18-22), and a down-regulation of brain protein kinase C following a 3 hr incubation with PMA has been previously suggested (10).

In the present study, a maximal extraction of protein kinase C was achieved by homogenizing brain tissue with 5 mM EGTA and 0.1% Triton X-100. Extensive washing of the particulate fraction with 1% Triton X-100 failed to yield additional protein kinase C, indicating a complete liberation of the enzyme by this treatment. Analysis of the extract fractionated on DEAE-cellulose suggested a selective time-dependent decrease in calcium- and phosphatidyl serine-dependent protein kinase activity following prolonged exposure to PMA. Tissue treated with PMA retained less than 20% of its protein kinase C activity when assayed at all concentrations of phosphatidyl serine or arachidonic acid, indicating that the loss of activity was not due to an alteration in the K_a of the allosteric regulators of the enzyme. The finding that phosphatidyl serine-stimulated ^{3}H-PDB binding was reduced to a similar extent as protein kinase C activity confirms that the primary cellular receptor for the phorbol esters is no

longer available (13, 21). Immunological analysis of the absolute amount of protein kinase C, as undertaken by Ballester and Rosen (25), will be required to establish whether this change is due to a loss of enzyme (20-24) or to a modification in enzyme activity.

The present findings represent the first demonstration of a phorbol ester-stimulated down-regulation of protein kinase C in a tissue preparation. The results indicate a key role for this enzyme in the augmentation of neurotransmitter-stimulated cyclic AMP accumulation in brain, implying an association between protein kinase C and the adenylate cyclase system in the regulation of receptor-mediated responses.

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