Vol. 136, No. 3, 1986 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 14, 1986 Pages 1007-1012

DIOCTANOYLGLYCEROL AND PHORBOL DIESTERS ENHANCE PHOSPHORYLATION OF PHOSPHOPROTEIN B-50 IN NATIVE SYNAPTIC PLASMA MEMBRANES

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Received February 17, 1986

The short chain diacylglycerol, 1,2-dioctanoylglycerol, at concentrations of 100- 300 μM stimulated phosphorylation of the nervous system-specific membrane protein B-50 (M_r 48 kDa, IEP 4.5) in isolated synaptic plasma membranes both in the presence and absence of exogenous protein kinase C. Comparable enhancement of histone phosphorylation by purified protein kinase C was achieved with 1 μM neutral lipid. Phorbol dibutyrate was 100 times more potent than the diacylglycerol in stimulating endogenous B-50 kinase in the membranes, whereas 4-α-phorbol was without effect. These results further confirm that B-50 is phosphorylated physiologically by a C kinase. Our data are consistent with a negative feedback mechanism in which generation of 1,2-diacylglycerol by enhanced phosphatidylinositol-4,5-bisphosphate hydrolysis could stimulate B-50 phosphorylation, thereby diminishing phosphatidylinositol-4-phosphate kinase activity and decreasing phosphatidylinositol-4,5-bisphosphate biosynthesis.

The modulation of synaptic activities is now generally thought to require the participation of membrane-bound phosphoproteins (1). Although the mode of their involvement is still unclear, it may be that the degree of phosphorylation alters the membrane environment in the immediate vicinity of the protein and thereby affects ion permeability or enzyme activity. We have extensively characterized one such nervous tissue-specific membrane protein, termed B-50 (M_r 48 kDa; IEP 4.5) and shown it to be located in the presynaptic structures and in the neural growth cones (2-4). The protein is phosphorylated by an endogenous kinase that resembles protein kinase C and no other kinase (5,6).

Some years ago, we found that ACTH decreased B-50 phosphorylation and simultaneously enhanced phosphatidylinositol-4,5-bisphosphate (PIP_2) formation in synaptic plasma membranes (SPM) (7,8). Recently, an increased state of phosphorylation of B-50 has been shown to directly

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0006-291X/86 $1.50

inhibit the activity of phosphatidylinositol-4-phosphate kinase, a key enzyme in the synthesis of PIP_2 (9). Agonist-induced breakdown of PIP_2 in brain and non-neural preparations is known to lead to the formation of inositol-1,4,5-trisphosphate and 1,2-diacylglycerol, substances that trigger intracellular Ca^{2+}-release into the cytosol and activate protein kinase C respectively (10). These facts led us to propose an hypothetical scheme for the negative feedback regulation of polyphosphoinositide metabolism mediated by changes in the extent of B-50 phosphorylation as brought about by B-50 kinase activity (11,12). As a step forward in evaluating this hypothesis, we have examined the effects of membrane-permeable 1,2-diacylglycerol (DOG) and phorbol-12,13-dibutyrate (PDB), agents well known to activate protein kinase C (13), on the phosphorylation of B-50 in SPM catalyzed by both exogenous and endogenous B-50 kinase.

MATERIALS AND METHODS

SPM were prepared according to Kristjansson et al. (2). Partially purified protein kinase C from rat brain was obtained by the method of Aloyo et al. (5). PDB and phosphatidylserine (PS) were purchased from Sigma Chemical Co. DOG was a product of Avanti Biochemicals and was added to all reaction mixtures from a 0.1 M stock solution in ethanol. Addition of ethanol alone at concentrations up to 0.3% had no effect on protein phosphorylation. Phosphorylation assays using purified protein kinase C and histone as substrate were performed in a reaction mixture which consisted of HEPES buffer, pH 6.5, histone 2A (10 μg), protein kinase C (1 μg protein in buffer A: 20 mM Tris-HCl, pH 7.5, 0.5 mM EGTA and 50 mM β-mercaptoethanol), 1 mM Ca^{2+}, 7.5 mM [γ-^{32}P]ATP (1 μCi, Amerham, UK, spec. act. 3000 Ci/mmol) and quantities of PS and DOG indicated in a final volume of 25 μl. The reaction was started by addition of ATP and after 30 sec at 30°C was terminated by adding trichloroacetic acid (final concentration 10 % w/v). Histone phosphorylation was quantified by the filter paper assay according to the method of Corbin and Reimann (14). Phosphorylation of SPM protein was carried out in a reaction mixture which contained 20 mM HEPES buffer, pH 7.4, 10 mM Na-acetate, 10 mM Mg-acetate, 80 mM KCl, 1 mM EGTA, 0.9 mM Ca-acetate (estimated free Ca^{2+} concentration: 300 nM), 200 μg/ml PS, 7.5 mM [γ-^{32}P]ATP (2 μCi) and 10 μg protein in a final volume of 25 μl. DOG and PDB were added as indicated. Membrane protein, PS (dried from chloroform solution) and DOG were sonicated for 1 min in an ultrasonic waterbath at 4°C. The assay was started by the addition of ATP and after 15 sec was terminated by adding a denaturing solution (15). The extent of B-50 phosphorylation was quantified by liquid scintillation counting after separation of the proteins by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Zwiers et al. (15). In some experiments, exogenous protein kinase C (1 mg in buffer A) was included in the reaction mixture. Protein was determined by the method of Lowry et al. (16) with bovine serum albumin as standard.

RESULTS

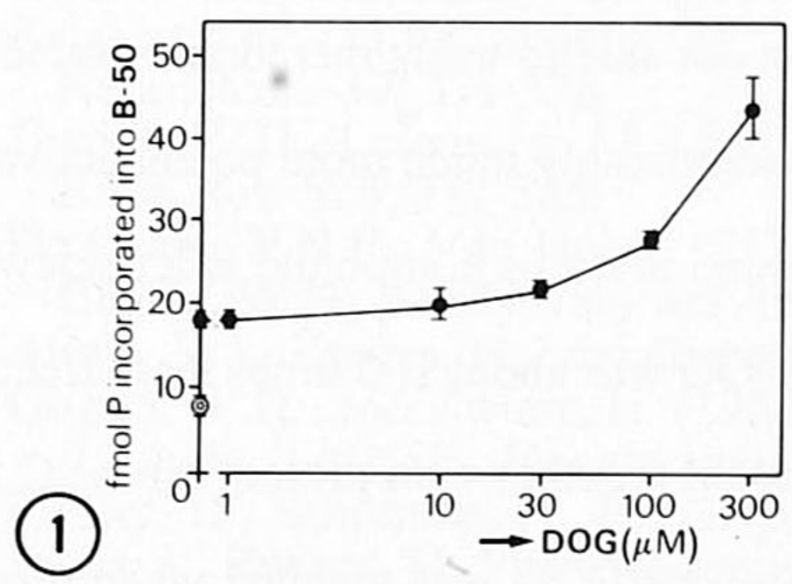
The phosphorylation of histone by purified protein kinase C was markedly stimulated by DOG in a dose-dependent manner when 1 mM Ca^{2+} was present (Table 1). As expected, the effect was observed only in the presence of PS. Raising the level of added phospholipid from 30 μg/ml to 200 μg/ml at 10 μM Ca^{2+} lowered the threshold for enhanced phosphorylation from 100 μM to 1 μM DOG (data not shown).

Table 1. DIOCTANOYLGLYCEROL (DOG) STIMULATION OF HISTONE PHOSPHORYLATION BY PROTEIN KINASE C

DOG concentration (μM)	Histone phosphorylation (%)
0	100
0.1	135
1.0	197
10	244
100	323

The addition of exogenous protein kinase C to SPM under ionic conditions approximating intracellular concentrations in the brain (10 mM Mg^{2+}; 10 mM Na^{+}; 80 mM K^{+}; 300 nM Ca^{2+}) resulted in substantial phosphorylation of B-50 and inclusion of DOG produced detectable stimulation at concentrations of 100 μM (Fig. 1). Omission of either KCl or PS reduced but did not abolish the stimulation caused by DOG (data not shown).

These findings led us to investigate whether an effect of DOG could be demonstrated on endogenous B-50 kinase activity in SPM. Initial experiments indicated that substantial stimulation was elicited when 100-300 μM DOG was added together with 200 μg/ml PS at 300 nM Ca^{2+} (Fig. 2). Under these conditions 300 μM DOG evoked a 43% enhancement of B-50 phosphorylation and perceptible stimulation was observed with about 100 μM of the neutral lipid (Fig. 3). The addition



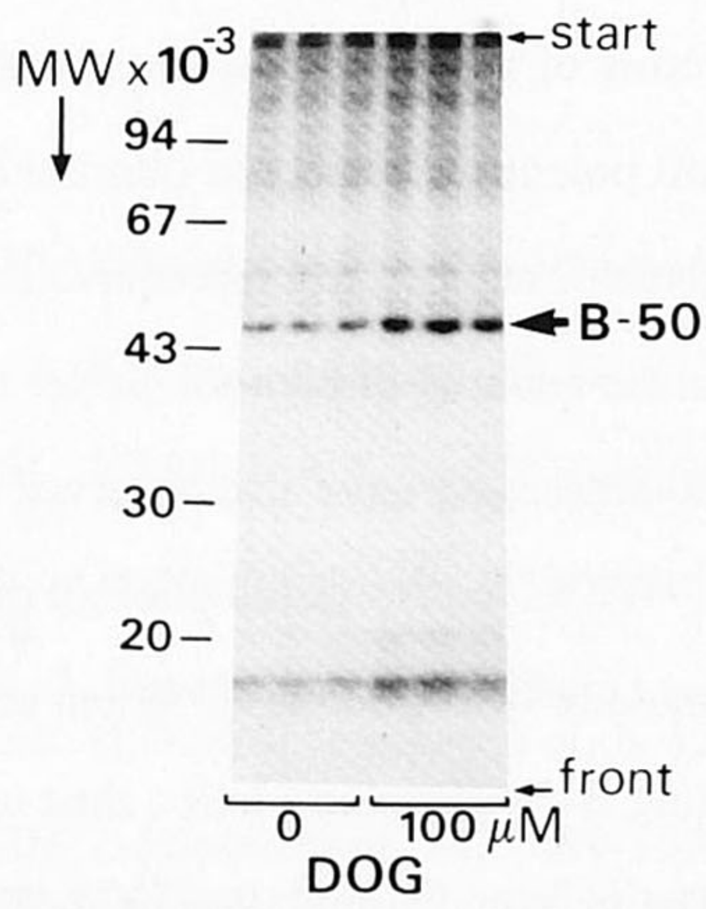


Fig. 1. Stimulation by DOG of B-50 phosphorylation in the presence of SPM and exogenous protein kinase C. SPM were incubated with or without varying amounts of DOG and added protein kinase C (1 μg). Measurement of the extent of B-50 phosphorylation and other experimental details are given in the text.

Fig. 2. Autoradiogram showing the specificity of DOG stimulation of B-50 phosphorylation in SPM. SPM were incubated with 100 μM DOG in the presence of 300 nM Ca^{2+} and 200 mg/ml PS. Labeled proteins were separated by SDS-PAGE prior to autoradiography. Other experimental details are given in the text.

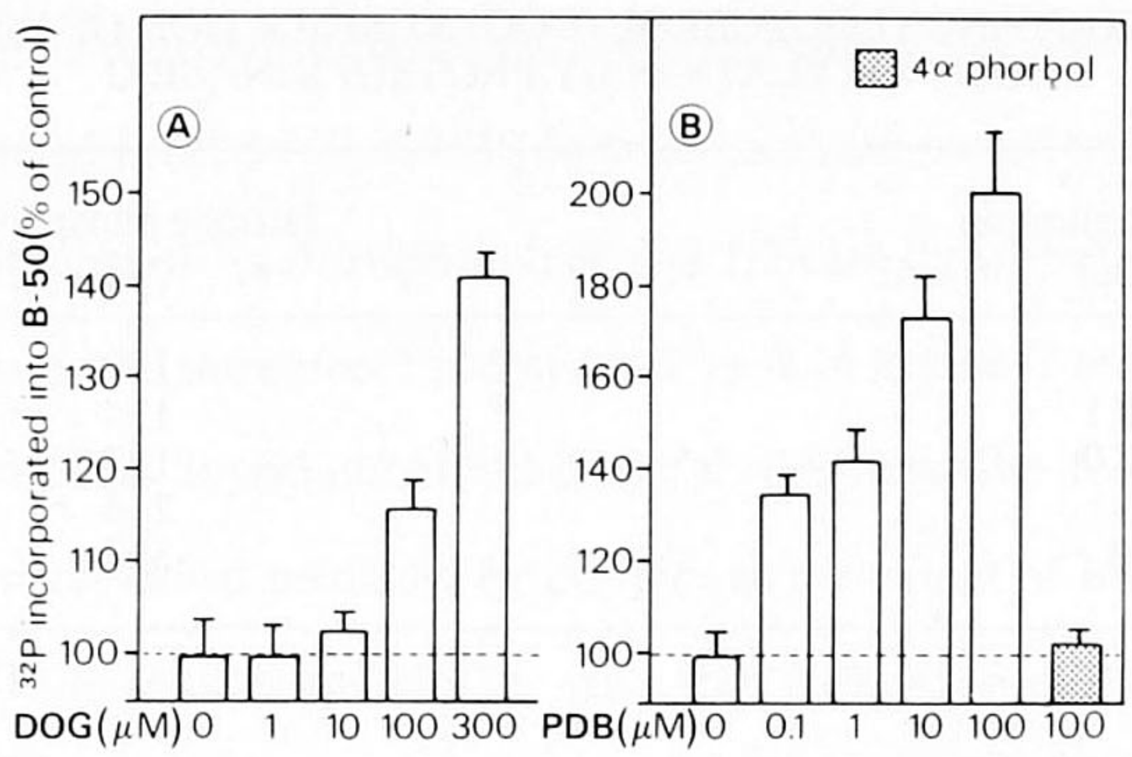


Fig. 3. Effects of DOG, PDB and 4-α-phorbol on endogenous B-50 phosphorylation in SPM. For experimental details, see text.

of PDB also produced a dose-dependent response on B-50 phosphorylation with a significant increase apparent at 100 nM phorbol diester, whereas 4-α-phorbol was without effect (Fig. 3).

DISCUSSION

Our findings describe the first diacylglycerol-activated phosphorylation of a membrane protein in brain and constitutes further evidence that B-50 kinase is a C kinase. Moreover it is noteworthy that the stimulation of B-50 phosphorylation is observed when physiological conditions prevailed, including Ca^{2+} levels which approach those in the resting cell.

1,2-Dioctanoylglycerol is a cell-permeable lipid that among a series of saturated diacylglycerols of varying chain lengths produces maximal stimulation of protein kinase C and mimics most potently a variety of phorbol diester actions on intact cells (17-20). The amount of the diacylglycerol required to exert equivalent effects on the enzyme was approximately 1000 times greater than the quantity of phorbol diester needed (19). In our studies using membrane-bound B-50 and B-50 kinase, we have also observed that PDB is a comparably much more potent activator than DOG. We have also tested phorbol diacetate and found that this compound is a somewhat weaker stimulator of B-50 kinase. When added to SPM, DOG was about 100 times less effective in stimulating B-50 phosphorylation than when it was present together with protein kinase C and histone. The maximal stimulation of B-50 phosphorylation by DOG was reached when protein kinase C was added to SPM. A possible explanation for this may be that only small portions of DOG penetrate the membrane and interact in a proper manner with B-50 kinase. An alternative explanation is that added protein kinase C can reach a population of B-50 molecules in the membrane which are not complexed with the kinase.

Previously, Akers and Routtenberg (21) reported that a 47 M_r brain phosphoprotein, designated F_1, which is associated with membranes and has many similarities to B-50 (22), serves as a substrate for exogenous protein kinase C and undergoes increased phosphorylation in the presence of PS and phorbol 12-myristate 13-acetate. However, their reaction conditions were substantially different from ours and in particular they used a detergent-treated particulate fraction in contrast to the purified native SPM employed in the present investigation.

The demonstration that B-50 kinase can be activated by DOG is consistent with the idea that generation of 1,2-diacylglycerol by stimulated PIP_2 hydrolysis could act indirectly to diminish PIP kinase activity and hence tend to decrease PIP_2 biosynthesis. Short chain diacylglycerols are not produced in brain and an important unsolved problem is the identity of those naturally occuring long chain diacylglycerols capable of activating protein kinase C *in vivo*.

Nonetheless, these results go some way toward providing a rationale for our recent observations that ACTH-induced grooming in the rat is antagonized both by DOG and PDB (23, 24). It is tempting to speculate that these phenomena in the intact animal are brought about by diacylglycerol-induced stimulation of protein kinase C and consequent increased phosphorylation of a synaptic membrane protein such as B-50 that we have shown occurs in SPM *in vitro*.

ACKNOWLEDGMENTS

We gratefully acknowledge the skilled technical assistence of Ms. Marina de Wit and the art work of Ed Kluis.

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