

Phosphatidylinositol 4,5-bisphosphate stimulates protein kinase C-mediated phosphorylation of soluble brain proteins

Inhibition by neomycin

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Phosphatidylinositol 4,5-bisphosphate (PIP₂) at 0.1 mol% activated the protein kinase C (PKC)-mediated phosphorylation of 87-, 55- and 47-kDa brain proteins. Neomycin, an aminoglycoside antibiotic that binds PIP₂ with a high affinity, inhibited PIP₂·PKC activity in a concentration-dependent manner. Low concentrations of neomycin (<2 mM) did not affect DG·PKC activity; however, 4 mM neomycin inhibited 50% of this activity. This inhibition of DG-stimulated activity at high neomycin concentration may be the result of its binding to Ca²⁺ and ATP in the assay system. These results suggest that PIP₂ is a physiological activator of PKC, and show that neomycin can be an inhibitor of PIP₂·PKC activity at concentrations where DG·PKC activity is not affected.

Neomycin; Protein kinase C; Phosphatidylinositol 4,5-bisphosphate

1. INTRODUCTION

Neomycin, an ototoxic and nephrotoxic polycationic antibiotic, has been shown to interact with anionic phospholipids such as the polyphosphoinositides [1–3]. It binds phosphatidylinositol 4,5-bisphosphate (PIP₂) preferentially over phosphatidylinositol 4-phosphate (PIP) and lacks affinity for phosphatidylinositol (PI) [3–5]. The strong affinity of neomycin for PIP₂ has provided the basis for its use in purification of PIP₂ [6], and it has generated numerous studies of its role in signal transduction [7–9]. Neomycin appears to dampen the intracellular signal [10,11] by inhibiting the phospholipase C-mediated PIP₂ breakdown that leads to the formation of inositol 1,4,5-triphosphate (IP₃) and 1,2-diacylglycerol (DG). Both these compounds have second messenger function, with IP₃ causing release of intracellular calcium (Ca²⁺) and DG acting synergistically with Ca²⁺ to activate protein kinase C, a calcium/phosphatidylserine (PS)-dependent protein phosphorylating enzyme [12,13]. Recent evidence from this laboratory has demonstrated that PIP₂ in the presence of Ca²⁺ and PS can itself act as an activator of PKC ($K_{PI\text{P}_2} = 0.04$ mol% with histones as a substrate) [14], and that it is a competitive inhibitor of

phorbol ester binding (and, by implication, DG binding) to PKC [15]. This report demonstrates that PIP₂ activates the PKC-mediated phosphorylation of its specific substrates from rat brain: 87 kDa, 55 kDa and 47 kDa proteins and that neomycin inhibits PIP₂·PKC-mediated protein phosphorylation.

2. MATERIALS AND METHODS

All reagents were purchased from Sigma. The purity of lipids was confirmed by thin layer chromatography. [γ -³²P]ATP was bought from NEN. GF/C filters were from Whatman.

2.1. Purification of protein kinase C

The enzyme was purified from rat brains by the method of Woodgett and Hunter [16] with DE52, phenyl-Sepharose 4B and PS-acrylamide column chromatography. The final preparation, a mixture of α -, β - and γ PKC isoenzymes, was concentrated by reverse dialysis against solid polyethylene glycol 8000, dialyzed overnight at 4°C into 20 mM Tris-HCl, pH 7.5, containing 0.1% (v/v) mercaptoethanol, 100 μ M EGTA, and 10% glycerol, and stored at 4°C. The specific activities of DG·PKC and PIP₂·PKC were 3 and 1.3 μ mol/mg per min, respectively, at 37°C in a mixed micellar assay [17] with Triton X-100, 0.3%; PS, 9 mol%; Ca²⁺, 50 μ M; DG, 2 mol% or PIP₂, 0.1 mol%.

2.2. Phosphorylation of brain proteins by PIP₂·PKC and DG·PKC

A calmodulin-depleted soluble fraction was prepared from rat cerebral cortex by DEAE-cellulose chromatography [18]. Briefly, fresh rat cerebral cortex was homogenized in 1 vol. of ice-cold solution A (0.25 M sucrose, 25 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 50 mM mercaptoethanol, 0.3 mM EGTA, and 2 mM phenylmethylsulfonyl fluoride). After centrifugation of the homogenate at 105 000 \times g for 60 min, the supernatant was loaded onto a DEAE-cellulose column equilibrated with solution B (20 mM Tris-HCl, pH 7.5, 50 mM mercaptoethanol) and eluted with 250 mM KCl dissolved

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Abbreviations: DG, diacylglycerol; PIP₂, phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; PS, phosphatidylserine

in solution B. The eluate was used as the source of endogenous PKC and its substrate proteins. The effect of PIP₂ and DG on the phosphorylation of calmodulin-deficient cytosolic proteins was compared under standard PKC assay conditions. Micelles of PS, PS/DG and PS/PIP₂ were prepared by drying the required amount of lipids under a stream of nitrogen in a glass tube followed by solubilization in 3% Triton X-100 by vortexing and incubating for 5 min at 37°C [17]. Aliquots of the eluate containing 100 µg protein were phosphorylated by incorporation of ³²P from [γ -³²P]ATP in the micellar system in presence of 20 mM Tris-HCl, pH 7.5, 50 µM Ca²⁺, 10 mM MgCl₂, and 50 µg/ml leupeptin. The reaction was terminated by the addition of sodium dodecylsulfate stop solution, i.e. 20% (v/v) glycerol/10% (w/v) sodium dodecylsulfate/10% (v/v) 2-mercaptoethanol/0.25 M Tris-HCl, pH 6.7, and heating in a boiling water bath for 2 min. The proteins were separated by SDS-slab gel electrophoresis (with 5–15% linear gradient polyacrylamide gels) and the labelled phosphoproteins were detected by autoradiography. The relative intensity of labelled phosphoproteins was quantified by densitometric tracing of the autoradiogram with a Shimadzu CS-9000 densitometer.

2.3. Effect of neomycin on PIP₂·PKC and DG·PKC activity

PKC activity was assayed in the absence and presence of neomycin (1, 2 and 4 mM) by measuring the incorporation of ³²P from [γ -³²P]ATP into histones III in a total reaction volume of 0.1 ml containing 20 mM Tris-HCl, pH 7.5, 50 µM Ca²⁺, 10 mM MgCl₂, 0.8 mg/ml histone HHS, 50 µg/ml leupeptin, 12.5 µM ATP, 10 µl lipid micelles (PS, PS/DG or PS/PIP₂) and 10 µl PKC (0.1–0.2 µg). The reaction was terminated by adding 1 ml of ice-cold 25% trichloro-

acetic acid (TCA), followed by addition of 1 ml of ice-cold bovine serum albumin (400 µg/ml). The resulting precipitate was washed four times with ice-cold TCA, collected on a 2.5 cm GF/C filter, and counted in 10 ml of hydrofluor. The effect of neomycin on the phosphorylation of calmodulin-deficient soluble cortex proteins was measured in the same manner.

3. RESULTS AND DISCUSSION

Fig. 1 shows the effect of DG and PIP₂ on protein phosphorylation in the calmodulin-depleted soluble fraction of rat brain cerebral cortex. PIP₂ (0.1 mol%) stimulated the phosphorylation of the 87 kDa and 47 kDa (known PKC-substrate proteins) significantly. If DG (2 mol%) was the activator, the phosphorylation for these proteins was stimulated 2-fold greater than it was with PIP₂ (0.1 mol%). These results are in accord with the previous observation [14] that PKC is activated more efficiently by PIP₂ (K_{PIP_2} = 0.04 mol% in Triton/lipid micelles with histones as substrate) than by DG (K_{DG} = 2 mol%) although the maximal velocity of phosphorylation is 2–3 times greater with DG·PKC than with PIP₂·PKC. The role of PKC-mediated phosphorylation of the 87 kDa protein in neurotransmission and neurosecretory processes is well

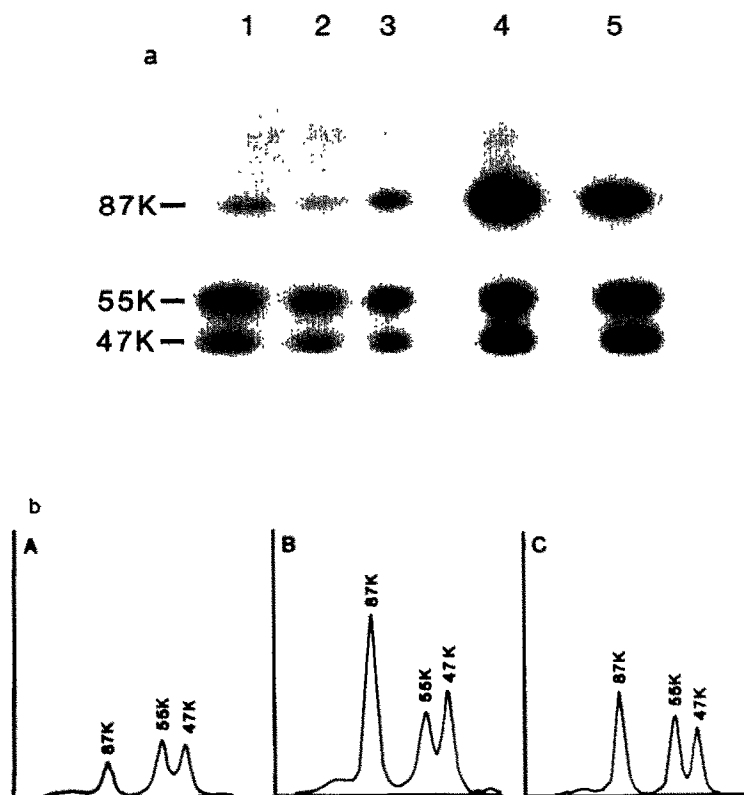


Fig. 1. Autoradiographs showing the stimulation of phosphorylation of 87-, 55- and 47-kDa brain proteins by PIP₂·PKC and DG·PKC (Fig. 1a). The calmodulin-depleted soluble fraction of rat cerebral cortex was prepared by DEAE-cellulose column chromatography [18]. A mixed micellar assay system was used. The additions were: (lane 1) 4 mM EGTA (No Ca²⁺); (lane 2) Ca²⁺; (lane 3) Ca²⁺ + PS; (lane 4) Ca²⁺ + PS + DG; (lane 5) Ca²⁺ + PS + PIP₂. The final concentrations in the reaction mixture were: Ca²⁺ 50 µM; PS 9 mol% or 445 µM; DG 2 mol% or 99 µM; and PIP₂ 0.1 mol% or 4.95 µM. Fig. 1b represents densitometric scans of the above autoradiogram. Panels A, B and C represent lanes 3, 4 and 5, i.e. additions were. (A) Ca²⁺ + PS; (B) Ca²⁺ + PS + DG; and (C) Ca²⁺ + PS + PIP₂.

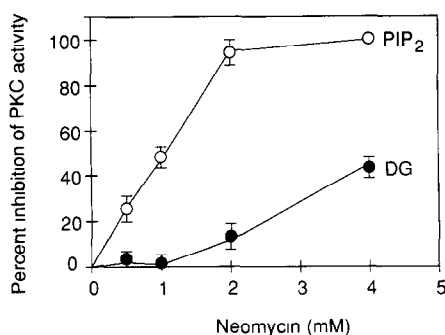


Fig. 2. Effect of neomycin on phosphorylation of histones by PIP₂·PKC and DG·PKC. Neomycin was solubilized in 20 mM Tris-HCl, containing 0.1% mercaptoethanol, and the pH was adjusted to 7.5. The phosphorylation of histones III-S was measured with PS, PS/PIP₂ and PS/DG micelles, as described under section 2. The concentrations of lipids expressed as mol% of TritonX-100 were: PS 9 mol% or 445 μ M; PIP₂ 0.1 mol% or 4.95 μ M; and DG 2 mol% or 99 μ M. PIP₂·PKC or DG·PKC activities were calculated by subtracting the activity obtained for PS/Ca²⁺ from that obtained for PIP₂/PS/Ca²⁺ or DG/PS/Ca²⁺ in the absence and presence of neomycin. 'Percent inhibition' refers to the inhibition of that portion of PKC activity obtained by stimulation with PIP₂ (○) or DG (●). Each point represents the mean \pm SE of 3 different experiments.

documented [19,20]. The 47 kDa protein has been suggested to be related to GAP-43/pp 46/B-50/protein F1, a phosphoprotein implicated in neural and behavioral plasticity of hippocampal long-term potentiation, i.e. memory [21–23]. The phosphorylation of the 55 kDa protein was also stimulated by both DG and PIP₂. Although the identity of the 55 kDa protein is not known, it is possible that it may be related to the microtubule-associated protein tau, which is known to be a substrate for PKC [24]. The tau protein, a family of 4–5 closely related phosphoproteins of 45–62 kDa, plays a multiple role in interaction and regulation of cytoskeletal components.

Since neomycin specifically binds to PIP₂ [3–5], the effect of neomycin on PIP₂-stimulated PKC activity was investigated. Neomycin inhibited PIP₂·PKC activity in a dose-dependent manner in the experiments with histones (Fig. 2) or the calmodulin-depleted soluble fraction of rat cerebral cortex (Fig. 3) as a substrate. Low concentrations of neomycin (<2 mM) did not affect DG·PKC activity, but at a 4 mM concentration neomycin could inhibit this activity by 50%. The inhibition of DG-stimulated PKC activity at a high concentration of neomycin may be the result of its interaction with Ca²⁺ [25] and ATP [26], which are essential cofactors for PKC-mediated protein phosphorylation reactions. These results suggest that neomycin can be an inhibitor of PIP₂·PKC activity, in addition to its other effects on signal transduction, e.g. inhibition of phospholipase C-mediated PIP₂ breakdown [10,11], IP₃-induced Ca²⁺ release [26], Ca²⁺-dependent histamine secretion from GTP- γ S-loaded mast cells [27], and thrombin-stimulated initiation of cell-proliferation [28].

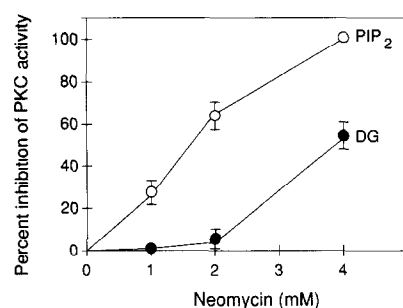


Fig. 3. Effect of neomycin on PIP₂·PKC- and DG·PKC-mediated phosphorylation of calmodulin-depleted soluble proteins from rat cerebral cortex. The calmodulin-deficient cytosol was prepared from rat cerebral cortex by DEAE-cellulose chromatography, and used as a source of PKC and its substrate proteins. Other experimental conditions were the same as described under Fig. 2, with the omission of histone.

Since PIP₂ is an activator of PKC, then room must be made for its new function in the present schemes of inositol lipid cascades. The role of DG, which is generally assumed to be the only receptor-linked PKC activator, will have to be reevaluated. The conventional role of phosphoinositides as the sole source of agonist-induced DG has been challenged by recent reports [29] that stimulus-responsive DG originates mainly from phosphatidylcholine (PC) by the action of PC-specific phospholipase C [30,31] or by a phospholipase-D/phosphatidase-phosphatase pathway [32]. PIP₂ is a normal component of the plasma membrane, with a concentration between 0.1–1 mol% of total lipid. The activation of PKC may start with a rise of intracellular Ca²⁺ (of as yet unknown origin) to a threshold Ca²⁺ level and the subsequent formation of a ternary, active Ca·PIP₂·PKC complex. Further control could be furnished by the inositide shuttle, i.e. the phosphorylation and dephosphorylation of the inositides, since phosphatidylinositol (PI) does not activate PKC [14] or bind to it (unpublished), and phosphatidylinositol 4-phosphate (PIP) is a very weak activator [14], and it translocates the enzyme from cytosol to membrane, and also binds to its activator-receiving region weaker than PIP₂ (unpublished observations). DG might act as a secondary effector of PKC, or may complement PIP₂ in the stimulation of the enzyme during prolonged activation of PKC.

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