Articles

Phosphatidylinositol 4,5-Bisphosphate Competitively Inhibits Phorbol Ester Binding to Protein Kinase C†

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ABSTRACT: Calcium phospholipid dependent protein kinase C (PKC) is activated by diacylglycerol (DG) and by phorbol esters and is recognized to be the phorbol ester receptor of cells; DG displaces phorbol ester competitively from PKC. A phospholipid, phosphatidylinositol 4,5-bisphosphate (PIP_2), can also activate PKC in the presence of phosphatidylserine (PS) and Ca^{2+} with a K_{PIP_2} of 0.04 mol %. Preliminary experiments have suggested a common binding site for PIP_2 and DG on PKC. Here, we investigate the effect of PIP_2 on phorbol ester binding to PKC in a mixed micellar assay. In the presence of 20 mol % PS, PIP_2 inhibited specific binding of [^{3}H]phorbol 12,13-dibutyrate (PDBu) in a dose-dependent fashion up to 85% at 1 mol %. Inhibition of binding was more pronounced with PIP_2 than with DG. Scatchard analysis indicated that the decrease in binding of PDBu in the presence of PIP_2 is the result of an altered affinity for the phorbol ester rather than of a change in maximal binding. The plot of apparent dissociation constants (K_d') against PIP_2 concentration was linear over a range of 0.01–1 mol % with a K_i of 0.043 mol % and confirmed the competitive nature of inhibition between PDBu and PIP_2. Competition between PIP_2 and phorbol ester could be demonstrated in a liposomal assay system also. These results indicate that PIP_2, DG, and phorbol ester all compete for the same activator-receiving region on the regulatory moiety of protein kinase C, and they lend support to the suggestion that PIP_2 is a primary activator of the enzyme.

Protein kinase C (PKC),[1] a ubiquitous phospholipid/calcium-dependent phosphorylating enzyme first identified by Nishizuka and co-workers, has emerged as one of the centrally important regulators in signal transduction, cell metabolism, and tumor promotion (Nishizuka, 1984, 1986). It is now understood that the enzymatic cleavage of phosphatidylinositol 4,5-bisphosphate (PIP_2) by phospholipase C which results from cell stimulation generates two second messengers: (1) inositol 1,4,5-trisphosphate (IP_3), which mobilizes intracellular Ca^{2+} (Abdel-Latif, 1986; Berridge, 1987); and (2) diacylglycerol (DG), which acts synergistically with Ca^{2+} to activate PKC (Takai et al., 1979; Kishimoto et al., 1980; Mori et al., 1982) after translocating the enzyme from cytosol to plasma membrane (Drust & Martin, 1985; Terbush & Holz, 1986). Interest in PKC increased with the demonstration that this enzyme can also be activated by phorbol esters (Castagna et al., 1982; Yamanishi et al., 1983), a class of tumor promoters that affect cell proliferation, biochemical changes, and intracellular communication (Blumberg, 1980, 1981; Diamond et al., 1980; Enomoto & Yamasaki, 1985). Phorbol ester binding to PKC is also dependent on phospholipids and Ca^{2+} (Ashendel et al., 1983; Kikkawa et al., 1983; Sando & Young, 1983), and phorbol esters, like DG, stimulate PKC by shifting the Ca^{2+} dose–response curve for activation of the enzyme to a lower Ca^{2+} concentration (Castagna et al., 1982). The phorbol ester receptor of cells is now recognized to be largely PKC (Kikkawa et al., 1983; Niedel et al., 1983; Blumberg et al., 1984; Ashendel, 1985), and the fact that DG can competitively inhibit phorbol ester binding (Sharkey et al., 1984; Sharkey & Blumberg, 1985; Ebeling et al., 1985; Hannun & Bell, 1986) is consistent with it being the postulated endogenous phorbol ester analogue (Blumberg et al., 1984).

It has recently been suggested that PIP_2 is more likely than DG to be the primary physiological effector of PKC (Chauhan & Brockerhoff, 1988); PKC was found to be activated much more efficiently by PIP_2 (K_{PIP_2} = 0.04 mol % in Triton/lipid micelles) than by DG (K_{DG} = 2 mol %). The enzyme when activated with PIP_2 had the same requirements as when activated with DG: it needed PS and Ca^{2+}. A common binding site for PIP_2 and DG on PKC was proposed on the basis of the observation that the addition of PIP_2 to an enzyme reaction mixture already maximally activated by DG did not result in a further increase of V_{max}. We report here that PIP_2 inhibits phorbol ester binding (and, by implication, DG binding) to PKC competitively and that the three activators must occupy overlapping, though not completely identical, sites on the regulatory moiety of the kinase.

Experimental Procedures

Materials. Ultrogel ACA 202 was obtained from IBF Biotechnics, phenyl-Sepharose 4B from Pharmacia, DE52 from Whatman, and Triton X-100 from Aldrich. Calf thymus histone type III-S, polyethylene glycol 8000, PDBu, leupeptin, phenylmethanesulfonyl fluoride, bovine serum albumin, PS, PIP_2, and 1,2-*sn*-dioleoylglycerol (DG) were purchased from

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[1] Abbreviations: PDBu, phorbol 12,13-dibutyrate; PKC, protein kinase C; PIP_2, phosphatidylinositol 4,5-bisphosphate; PS, phosphatidylserine, DG, diacylglycerol; PC, phosphatidylcholine; PA, phosphatidic acid.

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Sigma. The purity of lipids was confirmed by thin-layer chromatography (Deshmukh et al., 1981). [γ-^{32}P]ATP (3000 Ci/mmol) and [^{3}H]PDBu (18.8 Ci/mmol) were procured from New England Nuclear, and hydrofluor was from National Diagnostics. Charles River CD male rats were used for the source of protein kinase C.

Purification of Protein Kinase C. The enzyme was partially purified from rat brains essentially by the method of Woodgett and Hunter (1987) with DE52 and phenyl-Sepharose 4B column chromatography. The final preparation was concentrated (1 mg/mL) 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 μM EGTA, and 10% glycerol, and stored at 4 °C. The specific activity of PKC was 30 nmol mg^{-1} min^{-1} at 37 °C in a mixed micellar assay system of 0.3% Triton X-100 (Hannun et al., 1985) containing 9 mol % PS, 2 mol % dioleoylglycerol, and 50 μM Ca^{2+}.

Phorbol Ester Binding. (*a*) *Mixed Micellar Assay.* Triton X-100 mixed micelles containing 20 mol % PS and different concentrations of PIP_2 or DG were prepared by drying the 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 (Hannun et al., 1985). The concentration of lipids is expressed as mole percent Triton X-100. Binding assay was done following the method of Hannun and Bell (1986). Ultrogel ACA 202 columns were prepared by filling 2 mL of gel into silanized Pasteur pipets and then equilibrating the columns with 20 mM Tris-HCl, pH 7.5, 500 μM Ca^{2+}, and 0.015% Triton X-100. The incubation was carried out at room temperature for 5 min in a total volume of 100 μL containing 10 μL of 3% Triton X-100/phospholipid mixed micelles, 500 μM $CaCl_2$, 20 mM Tris-HCl, pH 7.5, [^{3}H]PDBu from 5 to 30 nM, and 5–10 μg of protein sample. Fifty microliters of the incubation mixture was then loaded on the Ultrogel columns, and bound [^{3}H]PDBu and free [^{3}H]PDBu were determined by collecting the eluates in the scintillation vials after washing the columns with 0.9 and 1.8 mL of equilibration buffer, respectively. Hydrofluor (20 mL) was added to the vials, which were vortexed and counted. Nonspecific binding was determined in the presence of excess unlabeled PDBu (10 μM), and specific binding for each sample was calculated as the difference between total and nonspecific binding. To assure equilibration of bound/free phorbol ester, incubations were stopped at 1–10 min. Equilibration was 95% at 1 min and 100% at 2 min (time chosen, 5 min).

(*b*) *Liposomal System.* Displacement of phorbol ester by PIP_2 was also measured by using sonicated liposomes. Small unilamellar liposomes containing PS and PIP_2 were prepared by drying chloroform/methanol solutions of lipids under nitrogen and dispersing the lipid film in 20 mM Tris-HCl, pH 7.5, followed by sonication under argon to clearing (Chauhan et al., 1988). The binding assay was done as described by Sharkey et al. (1984). In brief, 20 mM Tris-HCl, pH 7.5, 1 mM $CaCl_2$, 10 mM $MgCl_2$, 5–10 μg of PKC, [^{3}H]PDBu, 3 mg/mL bovine γ-globulin, 0.3 μmol of PS, and other lipids as indicated, in a total volume of 250 μL in Eppendorf tubes, were incubated for 30 min at 37 °C. After the incubation, tubes were chilled and proteins with lipids precipitated by adding 187 μL of chilled 24% polyethylene glycol (w/v) in 20 mM Tris-HCl, pH 7.5. Samples were then incubated for 15 min at 0 °C to permit precipitation. The tubes were centrifuged for 15 min at 12000 rpm, and 100 μL of the supernatant was counted for free [^{3}H]PDBu. The remaining supernatant was removed carefully, and the tip of the tube containing the pellet was cut and transferred to a scintillation vial and counted in 20 mL of hydrofluor. Nonspecific binding was measured in the presence of 50 μM nonradioactive PDBu and subtracted.

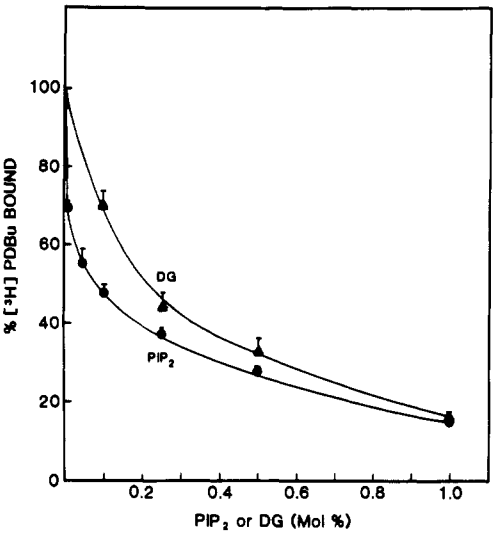

FIGURE 1: Inhibition of specific [^{3}H]PDBu binding by PIP_2 and DG. Binding of [^{3}H]PDBu to PKC was measured with Triton X-100 mixed micelles containing PS at 20 mol % and variable mol % of PIP_2 (●) or DG (▲). The bound [^{3}H]PDBu was separated from unbound [^{3}H]PDBu by chromatography on an Ultrogel ACA 202 column in the presence of 20 mM Tris-HCl, pH 7.5, 500 μM Ca^{2+}, and 0.015% Triton X-100. Nonspecific binding was determined in the presence of excess unlabeled PDBu and subtracted from total binding for each determination to calculate specific binding. The concentration of lipids is expressed as mol % Triton X-100. Each point represents mean ± SE of four different experiments.

Results

Figure 1 compares phorbol ester binding in mixed micellar assays in the presence of either PIP_2 or DG. PIP_2, like DG, has a displacing effect on the specific binding of [^{3}H]PDBu to PKC, with an IC_{50} (PIP_2) of 0.07 mol %. The inhibition by PIP_2 is more pronounced at low concentrations in comparison to DG (IC_{50} = 0.2 mol %). At 1 mol % activator concentration, however, both PIP_2 and DG displaced PDBu to the same extent (85% inhibition of PDBu binding).

A Scatchard plot analysis of binding–inhibition curves between 0.01 and 1 mol % PIP_2 in mixed micellar assay shows that the binding of PIP_2 is competitive with that of phorbol ester (Figure 2). With increasing concentration of PIP_2 there is a marked decrease in the binding affinity for [^{3}H]PDBu but no change in B_{max} (maximal binding). The plot of K_d', the apparent dissociation constant for [^{3}H]PDBu/enzyme in the presence of PIP_2, was a linear function of PIP_2 concentration over a range of apparent dissociation constants of 3–70 nM (Figure 3). Thus, PDBu binding affinity as a function of PIP_2 concentration (I) fits the relationship $K_d' = K_d(1 + I/K_i)$, confirming competition at the binding site. K_d, the dissociation constant for [^{3}H]PDBu/enzyme in the absence of PIP_2, as determined by extrapolation to zero PIP_2 concentration, was 3.0 nM; values of 2.8–7 nM reported by others (Leach et al., 1983; Niedel et al., 1983; Sharkey et al., 1984; Hannun & Bell, 1986; Lee & Bell, 1986) add confidence to our experimental procedures. K_i, the dissociation constant for PIP_2 binding, was 0.043 mol %, as represented by the negative of the horizontal axis intercept.

We reported earlier (Chauhan & Brockerhoff, 1988) that PIP_2 activates protein kinase C in both micellar and liposomal assay. The effect of PIP_2 on phorbol ester binding to PKC was also studied in a liposomal system. The results were similar to those obtained in the micellar assay, and Scatchard

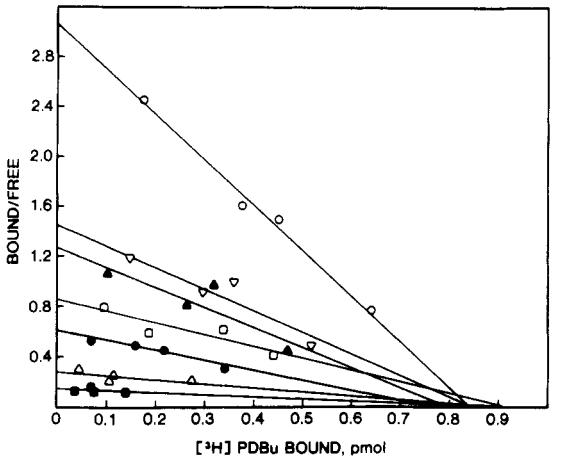

FIGURE 2: Competitive inhibition of [^{3}H]PDBu binding by PIP_2 in mixed micelles. The plot represents a Scatchard analysis of specific binding of [^{3}H]PDBu at variable concentrations of PDBu as a function of PIP_2 concentrations in Triton X-100 mixed micelles containing PS at 20 mol %. [^{3}H]PDBu was incubated for 5 min with PKC, 500 μM Ca^{2+}, 20 mM Tris-HCl, pH 7.5, and 10 μL of 3% Triton X-100/phospholipid mixed micelles in a total reaction volume of 100 μL and then chromatographed on Ultrogel ACA 202. Points are the average of triplicate determinations; lines are determined by linear regression analysis. The concentration of PIP_2 in mol % Triton X-100 was 1 (■), 0.5 (△), 0.25 (●), 0.1 (□), 0.05 (▲), 0.01 (▽), and no PIP_2, i.e., PS alone (○).

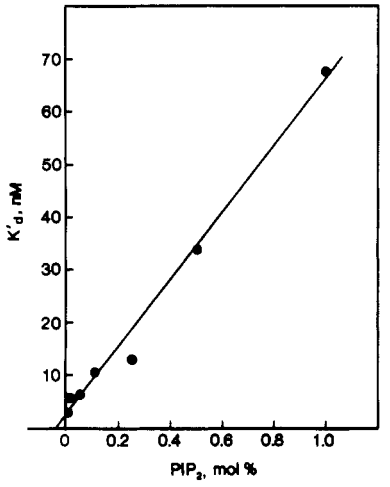

FIGURE 3: Apparent dissociation constants (micellar system) for [^{3}H]PDBu as a function of PIP_2 concentration. K_d', the apparent dissociation constant for [^{3}H]PDBu/enzyme in the presence of PIP_2, was determined from Figure 2 for each PIP_2 concentration (in mol % Triton X-100) by Scatchard analysis. The line is determined by linear regression analysis. K_d, the dissociation constant for [^{3}H]-PDBu/enzyme as represented by the y intercept, is 3.0 nM. K_i, the dissociation constant for PIP_2, represented by the negative of the x intercept, is 0.043 mol %.

plot analysis (Figure 4) confirmed that PIP_2 competitively inhibits phorbol ester binding to PKC in the liposomal system, also.

Discussion

The ability of diacylglycerol to activate protein kinase C was discovered nearly a decade ago (Takai et al., 1979; Kishimoto et al., 1980; Mori et al., 1982) and has since been verified in numerous studies, including our own (Chauhan & Brockerhoff, 1987, 1988). Present consensus holds that DG is, in fact, the natural activator for the enzyme. The finding that the phospholipid PIP_2 is a more potent activator is therefore highly unexpected and in need of vigorous verification. An earlier paper (Chauhan & Brockerhoff, 1988) offered these results: PIP_2 activated PKC with a K_{PIP_2} of 0.04

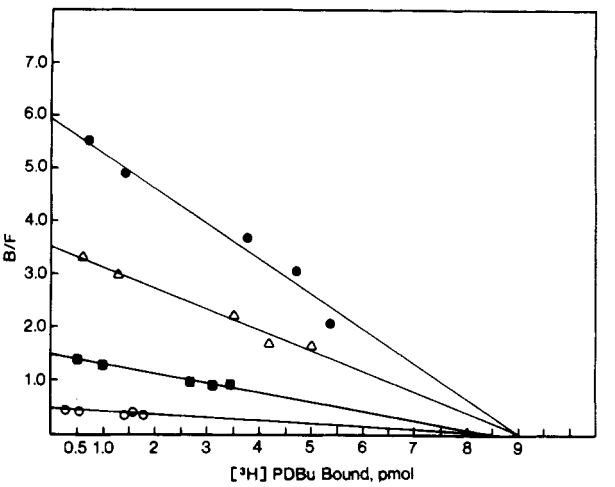

FIGURE 4: Competitive inhibition of [^{3}H]PDBu binding by PIP_2 in a liposomal system (Scatchard plot). The reaction mixture, containing 20 mM Tris-HCl, pH 7.5, 1 mM $CaCl_2$, 10 mM $MgCl_2$, 5–10 μg of PKC, [^{3}H]PDBu, 3 mg/mL bovine γ-globulin, and liposomes containing PS and PIP_2, was incubated for 30 min in a total volume of 0.25 mL. PS, 0.3 μmol/tube; PIP_2, in mol % of total lipid, was 4.76 (○), 1 (■), 0.2 (△), and 0 (●). B/F represents ratio of bound to free [^{3}H]PDBu.

mol % (in Triton X-100 micelles with 9 mol % PS, with histone as the substrate). The efficiency of DG, on the other hand, was much smaller (K_{DG} = 2 mol %) in our assays, though the maximal velocity was 2–3 times larger for DG·PKC than for PIP_2·PKC. With either activator, the systems also required PS and Ca^{2+} for functioning. Phosphatidylinositol and phosphatidylinositol 4-phosphate could not replace PIP_2. That both activators bind to the same region on the kinase and to the same family of PKC isoenzymes was indicated by the fact that, in a system optimized in regard to DG activation (10 mol % DG), further addition of PIP_2 did not cause more activation, while at suboptimal effector concentrations the activation was additive. Finally, there was no synergistic effect between DG and PIP_2; this makes it unlikely that there is more than one effector binding site on the kinase.

Coherent though they are, the available results clearly call for further experimental evidence; therefore, we are here presenting data on the displacement of phorbol ester from protein kinase C by DG and by PIP_2. The experiments with DG (Figure 1) repeat and confirm published reports (Sharkey et al., 1984; Ebeling et al., 1985; Hannun & Bell, 1986) that PKC has an activator-binding site common for DG and phorbol ester. The data for phorbol ester displacement by PIP_2, similarly interpreted, show that this inositol phospholipid occupies the same site on the kinase, with an affinity higher than that of DG.

If PIP_2 is, indeed, as the data suggest, the primary physiological activator for PKC, then room must be made for its new function in the present schemes of inositol lipid metabolism and protein phosphorylation. The role of DG, which has generally been assumed to be the only receptor-linked PKC activator, will certainly have to be reevaluated. There is already mounting evidence that not all the diglyceride in the activated cell is directly linked to the inositol cycle (Bocckino et al., 1985; Exton, 1988). Much, even most, of the cellular DG appearing after agonist stimulation may be derived from phosphatidylcholine (PC) by action of a PC-specific phospholipase C (Besterman et al., 1986; Daniel et al., 1986) or via phospholipase D (Liscovitch et al., 1987; Cabot et al., 1988a,b), rather than from the phosphoinositides. Phosphatidic acid (PA) generated from PC or from DG (by phosphorylation) would probably be located on the extracellular side of

the plasma membrane and might conceivably serve as a vehicle to transport Ca^{2+} into the cell (Serhan et al., 1982; Nayar et al., 1984; Smaal et al., 1987) in the form of Ca(phosphatidate)$_2$ (Chauhan & Brockerhoff, 1984; Chauhan et al., 1988). There is evidence that both PC–phospholipase C and D are regulated by G proteins (Bocckino et al., 1987; Irvine & Exton, 1987).

Though the physiological occurrence of Ca cross-membrane transport has yet to be verified, recent findings—Ca^{2+} appearing before inositol trisphosphate on stimulation of rat pituitary cells (Tashjian et al., 1987), PA preceding DG upon stimulation of hepatocytes with vasopressin (Bocckino et al., 1987), and treatment of cells with PA raising intracellular Ca^{2+} levels (Salmon & Honeyman, 1980; Putney et al., 1980)—suggest that intracellular Ca^{2+} mobilization may be fed from an extracellular pool. The possibility of Ca^{2+} being sequestered in intramembrane Ca cages that open on stimulation (Brockerhoff, 1986a) might also be considered. Calcium so generated might reach a threshold concentration at which it can initiate the activity of both protein kinase C and PIP_2–phospholipase C, which then generates DG to sustain PKC activation. The ability to "translocate" the kinase, i.e., to move it from the cytosol to the plasma membrane by help of DG (Drust & Martin, 1985; Terbush & Holz, 1986) or phorbol esters (Kraft & Anderson, 1983; Tapley & Murray, 1984; Wooton & Wrenn, 1984), appears to be shared by PIP_2, according to experiments with PIP_2-containing liposomes (unpublished observation). Whether such mechanisms are widespread phenomena or restricted to only certain cell types and agonists remains to be explored.

The statement that DG, PIP_2, and phorbol ester all occupy the same activator-binding site on the kinase needs some elaboration. DG is known by its enantiomeric specificity—only the 1,2-*sn*-diacylglycerol is active (Cabot & Jaken, 1984; Ganong et al., 1986; Go et al., 1987; Rando, 1988)—to be linked to the enzyme by three hydrogen bonds, and phorbol ester can also engage in such bonding, with three H-bonds superposable on those formed with DG (Brockerhoff, 1986b). PIP_2 can hydrogen bond with its 1 and 2 ester groups, which likely accept the same protons that the kinase donates to DG; but there is no proton on PIP_2 that would correspond to the *sn*-3-OH of DG or the 9 α-OH of phorbol. Instead, there is a phosphate group in inositol 5-position that—since it is obligatory for activity—must bond to an ionic site on the kinase, conceivably with the involvement of calcium ion. The conformational changes of the regulatory moiety of the kinase induced by either DG or PIP_2 must obviously be different. Such differences may possibly manifest themselves in different substrate specificities for DG·PKC and phorbol·PKC (Shinohara et al., 1985; Kiss & Luo, 1986; Ramsdell et al., 1986) and for PIP_2·PKC (Chauhan & Brockerhoff, 1988). The activator–enzyme bonding interfaces of the three species of activated enzymes cannot be entirely identical, but they must be overlapping to a degree that makes it impossible while one activator is bound to bind another. This is what the kinetic data show.

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Effect of Nucleotides on the Activity of Dinitrogenase Reductase ADP-Ribosyltransferase from *Rhodospirillum rubrum*†

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ABSTRACT: The mechanism by which MgADP stimulates the activity of dinitrogenase reductase ADP-ribosyltransferase (DRAT) has been examined by using dinitrogenase reductases from *Rhodospirillum rubrum*, *Klebsiella pneumoniae*, and *Azotobacter vinelandii* as acceptor substrates. In the presence of 0.2 mM NAD, maximal rates of ADP-ribosylation of all three acceptors were observed at an ADP concentration of 150 μM; in the absence of added ADP, DRAT activity with the dinitrogenase reductases from *R. rubrum* and *K. pneumoniae* was less than 5% of the maximal rate, but the *A. vinelandii* protein was ADP-ribosylated at 40% of the maximal rate. Of eight dinucleotides tested, only ADP, 2′-deoxy-ADP, and ADP-βS served as activators of the DRAT reaction; ADP, 2′-deoxy-ADP, and ADP-βS were also the only dinucleotides found which inhibited acetylene reduction activity by dinitrogenase reductase. The dinucleotide specificities for both DRAT activation and acetylene reduction inhibition were the same for all three dinitrogenase reductases. In the DRAT reaction with the dinitrogenase reductases from *K. pneumoniae* and *A. vinelandii*, the K_m for NAD was 30-fold higher in the absence of ADP than in its presence; the K_m for NAD with the *R. rubrum* acceptor was not measurable. In the presence of saturating ADP, ADP-ribosylation of dinitrogenase reductase from *R. rubrum* was inhibited 63% by 1.5 mM ATP. It is concluded that MgADP stimulates DRAT activity by lowering the K_m for NAD and that MgADP exerts its effect by binding to dinitrogenase reductase. MgATP inhibits DRAT activity by competing with MgADP for binding to dinitrogenase reductase.

Reduction of dinitrogen to ammonium in biological systems is catalyzed by the nitrogenase enzyme complex. The molybdenum–nitrogenase is composed of two oxygen-labile proteins, dinitrogenase reductase and dinitrogenase. Electrons are transferred from dinitrogenase reductase to dinitrogenase with concomitant hydrolysis of MgATP; substrates, which include protons and acetylene as well as dinitrogen, are reduced at the Fe–Mo cofactor site of dinitrogenase [see Orme-Johnson (1985) for a review of nitrogenase]. Dinitrogenase reductase contains two identical subunits of approximately 30 kDa which share a single 4Fe–4S cluster.

MgATP and MgADP bind to dinitrogenase reductase competitively (Walker & Mortenson, 1973; Thorneley & Cornish-Bowden, 1976). Most studies have indicated that dinitrogenase reductase has two nucleotide binding sites per dimer and that MgADP inhibits nitrogenase activity by com-

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