Arachidonoyl-diacylglycerol Kinase

SPECIFIC IN VITRO INHIBITION BY POLYPHOSPHINOISITIDES SUGGESTS A MECHANISM FOR REGULATION OF PHOSPHATIDYLINOSITOL BIOSYNTHESIS*

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We previously described the purification of a membrane-bound diacylglycerol kinase highly selective for sn-1-acyl-2-arachidonoyl diacylglycerols (Walsh, J. P., Suen, R., Lemaître, R. N., and Glomset, J. A. (1994) J. Biol. Chem. 269, 21155–21164). This enzyme appears to be responsible for the rapid clearance of the arachidonate-rich pool of diacylglycerols generated during stimulus-induced phosphoinositide turnover. We have now shown that phosphatidylinositol 4,5-bisphosphate is a potent and specific inhibitor of arachidonoyl-diacylglycerol kinase. Kinetic analyses indicated a Ki for phosphatidylinositol 4,5-bisphosphate of 0.04 mol % Phosphatidic acid also was an inhibitor with a Ki of 0.7 mol %. Other phospholipids had only small effects at these concentrations. A series of multiply phosphorylated lipid analogs also inhibited the enzyme, indicating that the head group phosphomonoesters are the primary determinants of the polyphosphoinositide effect. However, these compounds were not as potent as phosphatidylinositol 4,5-bisphosphate, indicating some specificity for the polyphosphoinositide additional to its total charge. Five other diacylglycerol kinases were activated to varying degrees by phosphatidylinositol 4,5-bisphosphate and phosphatidic acid, suggesting that inhibition by acidic lipids may be specific for the arachidonoyl-DAG kinase isoenzyme. Given the presumed role of arachidonoyl-diacylglycerol kinase in the phosphoinositide cycle, this inhibition may represent a mechanism for polyphosphoinositides to regulate their own synthesis.

Diacylglycerol kinases catalyze the ATP-dependent phosphorylation of sn-1,2-diacylglycerol (DAG)1 to phosphatidic acid (PA) (1–3). As such, they are widely regarded as attenuators of the DAG signaling and protein kinase C activation that occur during stimulus-induced PI turnover (4). The recent identification of a specific DAG kinase essential for PI-mediated invertebrate visual transduction is a striking confirmation of the involvement of DAG kinases in the PI cycle (5).

It has recently become evident that DAG kinases are a diverse family of isoenzymes (2). The first of these to be purified and cloned was an 82.6-kDa isoform expressed predominantly in brain and thymus (6, 7). Several homologs of this enzyme also have been cloned, each of which has its own highly specific pattern of expression in cells and tissues (8–12). Additional DAG kinases, which appear distinct from the cloned isoforms described above, also have been reported (13–21). However, detailed enzymologic data on these are not available at this time. Our laboratory has described and purified a membrane-bound DAG kinase highly selective for DAG molecular species containing arachidonate as the sn-2 fatty acyl moiety (21–24). This activity can be distinguished from other DAG kinases by a variety of enzymologic properties in addition to its substrate specificity (21–24). Arachidonoyl-DAG kinase activity varies widely between different tissues, but it is detectable in all cells and tissues we have examined (21–24). Given the marked enrichment of animal cell phosphatidylinositols in arachidonate at the glycerol sn-2 position, the substrate selectivity of arachidonoyl-DAG kinase suggests a special role for this isoenzyme in PI biosynthesis (21–24).

This multiplicity of DAG kinases is reminiscent of the diversity seen in the protein kinase C and PI-specific phospholipase C isoenzyme families. As with the phospholipases C and protein kinases C, this diversity most likely reflects multiple mechanisms of enzyme regulation in the cells expressing the various DAG kinase isoforms (25–28). The detailed mechanisms of DAG kinase regulation are, however, poorly understood. The 82.6-kDa DAG kinase and its homologs are thought to translocate to membranes during cell activation and are activated in vitro by Ca2⁺ and phosphatidylserine (29). Limited evidence for activation of some DAG kinases by reversible protein phosphorylation also has been reported (30–32).

Acidic phospholipids have been shown to modulate the in vitro activities of several membrane enzymes involved in stimulus transduction (33–43). Since levels of these lipids fluctuate during cell stimulation, this modulation is thought to reflect regulation of the enzymes by the in vivo membrane microenvironment. We have now examined the effects of acidic phospholipids on arachidonoyl-DAG kinase in a well characterized, mixed micellar assay system. Phosphatidylinositol 4,5-bisphosphate (PI(4,5)) was shown to be a potent and specific inhibitor of arachidonoyl-DAG kinase. Phosphatidylinositol 4-phosphate (PI(4)) and PA also were inhibitors, but at 18-fold higher concentrations. Five other DAG kinases were examined and found

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1 The abbreviations used are: DAG, sn-1,2-diacylglycerol; PA, 1-α-phosphatidic acid; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP2, phosphatidylinositol 4,5-bisphosphate; OTAC, octadecyltrimethylammonium chloride; ESI-MS, electrospray ionization mass spectrometry; MS/MS, tandem mass spectrometry; TLC, thin-layer chromatography; CMC, critical micelle concentration; 18:0–20:4 PA, sn-1-stearoyl-2-arachidonoylphosphatidic acid; 16:0–18:1 PA, sn-1-palmitoyl-2-docosahexaenoic acid.
to be activated to varying degrees by PIP2 and PA, suggesting that inhibition by acidic lipids may be a specific property of the arachidonoyl-DAG kinase isoform. Given the likely role of arachidonoyl-DAG kinase in PI biosynthesis, these data suggest that inhibition by polyphosphoinositides may represent a mechanism for feedback regulation of this enzyme by PIP2, its presumed final product.

EXPERIMENTAL PROCEDURES

Materials—PIP2, PIP3, sorbitan trioleate, α-myoinositol 1,4,5-trisphosphate, inositol hexaphosphate (phytic acid), and triethanolamine HCl (Sigma). Dodecyl phosphoglycerates from Landauskas (7). Phosphatidic acids were from Avanti. Frozen porcine thymus were from Pel Freeze. Diethylaminoethyl cellulose (DE52) ion-exchange resin was from Whatman. Octadecytrimethylammonium chloride (OTAC) was prepared from the bromide salt (24) by ion exchange over a preparative anion-exchange column of DE52.

Aromatic and terpene hydrocarbons were from Sisco Research Laboratories. Carbohydrates were from Aldrich. All other materials were obtained as described previously (24).

Assay of Diacylglycerol Kinase—The DAG kinase assays employed represent minor modifications of our previously described methods (24). For a standard assay, an appropriate volume of DAG stock solution was evaporated under a stream of nitrogen in a glass test tube. To the DAG droplet were added: 50 µl of 4 x assay buffer, detergent, phospholipid, histone, dithiothreitol, water, and enzyme to a final volume of 180 µl. The reaction was initiated by the addition of 20 µl of [γ-32P]ATP solution. The standard assay contained, in a volume of 200 µl, 7.5 mM Triton X-100, 7.5 mM Triton X-114, 50 mM triethanolamineHCl, pH 7.5, 100 mM NaCl, 1 mM MgCl2, 0.1 mM [γ-32P]ATP, 0.3 mM (20 mol %) DAG, 1 mM EGTA, 1 mM diethanolamine, 1 µg of histone, 0.75 µg of 2.6-dinitrobenzoylhistidine, and 0.75 µg of triethanolamine/nitrophenylphosphate (octylglucoside) was employed instead of Triton. When phospholipids or other amphiphiles were added to the assays, the concentration of detergent was reduced to keep the total micellar concentration of detergent equal to 15 µM in the Triton X-100 and 15 µM in the Triton X-114 assays. For assays containing Triton, the detergent monomer concentrations were negligible, and the decrease in total detergent was simply equal to the amphiphiles added. For assays containing octylglucoside, monomeric detergent was assumed to decrease in proportion with the micellar mol fraction (44). The monomer concentration for pure octylglucoside used in these calculations was assumed to be 25 mM (45). Reactions were allowed to proceed for 10 min at 25°C, and terminated by the addition of 3.0 ml of CHCl3/ethanol (2:1 (v/v)). The residue was dissolved in 100 ml of methanol. Concentrated HCl (5 ml) was added slowly to diphenylchlorophosphate (4.0 ml, 25 mmol) in 20 ml of H2O. Ethanolamine was added slowly to diphenylchlorophosphate (4.0 ml, 25 mmol) in 20 ml of H2O. The reaction was stirred for an additional hour, after which, the methanol was evaporated. The product was precipitated at −20°C from H2O/ethanol (1:1 (v/v)) and washed at −20°C with petroleum ether/diethyl ether (2:1 (v/v)). The residue was dissolved in 80 ml of CHCl3 and extracted with 80 ml of H2O (41 (v/v)) followed by 60 ml of H2O. Ethanolamine was added to the CHCl3, and the mixture was evaporated to 20 ml of CHCl3. The residue was re-dissolved in 20 ml of CHCl3, evaporated, and the product was crystallized from petroleum ether/diethyl ether (1:1 (v/v)). The final yield was 4.7 g. The product was homogeneous by TLC on silica gel (Rf 0.08, benzene/ethylether/acetic acid, 25:25:1 (v/v)). ESI-MS: m/z 361 (100) MH+, m/z 378 (93) MHN2+, M/S/M (MH+); m/z 361 (49) MH+, m/z 343 (100) MH+-H2O, m/z 325 (5) MH2+-H2O, m/z 285 (5) C19H10O2Cl, m/z 137 (88) pentaoctenyltrihexadecylpentaoctate acid H+, m/z 119 (97) pentaerythritol H+-H2O, m/z 101 (99) pentaerythritol H+-H2O. Some additional product could be obtained by concentrating the mother liquor, but it was contaminated with a small amount of faster migrating material presumed to be the dihexadecyl ether.

To prepare the trisphosphorylated product, mono(200 MHz in Me2SO-d6) and the mixture was evaporated at 80°C. The catalyst was removed by filtration, and the solution was concentrated to about 15 ml by evaporation.

Diacylglycerol Kinases—Arachidonoyl-DAG kinase was purified to apparent homogeneity from bovine testis as described previously (24). Protein concentrations of the arachidonoyl-DAG kinase used in this work were too low to be reliably assayed, and activities are therefore expressed as pmol/min/µl of the original pooled fractions (24). Testis cytosol DAG kinase, and cytosolic DAG kinases from 3T3 cells were also prepared as described previously (24). The 3T3 cell DAG kinases, referred to in this work as Thymus DAG kinase A and 3T3 cell DAG kinases, were identical to the type I and II enzymes described by Stathopoulos et al. (15). Porcine thymus cytosol DAG kinases were prepared according to the procedure of Sakane et al. through the DEAE cellulose step (34). The activity eluting at 100 mM NaCl, referred to in this work as thymus DAG kinase A, is the 82.6-kDa brain enzyme subsequently cloned by the procedure of Sakane et al. through the DEAE cellulose step (34). The activity eluting at 200 mM NaCl is referred to as thymus DAG kinase B.

Organic Syntheses—Procedures for preparation of the acidic phospholipid analogs used in this work are described below. Electrospray ionization mass spectrometry (ESI-MS), including tandem mass spectrometry (MS/MS), of the products was performed as described by Kerwin et al. (48) except that acidic compounds were injected in 5 mM methanolic HCl. Neutral compounds were injected in 5 mM methanolic ammonium acetate. Characteristic ions are expressed as m/z (relative intensity). Some structures were additionally confirmed by 1H NMR at 200 MHz in Me2SO-d6. 20% deuterium chloride (20:1 (v/v)). Chemical shifts are reported as δ (parts/million) relative to tetramethylsilane. Oxalate-impregnated plates for TLC of multiply phosphorylated compounds were prepared by co-solvented by stirring. The reaction was terminated. The mixture was dissolved with 50 ml of Dowex AG 50W-X8. Evaporation of the benzene yielded 134 g of clear, syrupy product (theoretical 136 g). To prepare the hexadecyl ether, acetylated pentaerythritol (20 g, 76 mmol) was partially acetylated with acetic acid (89 ml, 1.55 mol) by refluxing under a Dean-Stark trap in 150 ml of benzene containing 2 g of toluenesulfonic acid. After 24 h, 28 ml (1.55 mol) of water had collected in the trap, and the reaction was terminated. The mixture was dried with 50 ml of Dowex AG 50W-X8. Evaporation of the benzene yielded 134 g of clear, syrupy product (theoretical 136 g). To prepare the hexadecyl ether, acetylated pentaerythritol (20 g, 76 mmol) was used to prepare mono-o-hexadecylpentaerythritol trisphosphate, pentae-
This material was dissolved in 100 ml of H2O. The pH was adjusted to 11.0 by addition of 10 % NaOH, and the product was precipitated at 0°C by the addition of 100 ml of methanol. The precipitate was collected by filtration and washed successively with 50% ice-cold aqueous methanol, absolute ethanol 3 times, ethyl acetate 2 times, and diethyl ether 3 times. Yield was 3.86 g (95%). The final product migrated as a single spot on TLC (Rf 0.14, benzene/diethyl ether/acetic acid, 25:25:1 (v/v/v)).

To prepare 2-hexadecylglycerol-1,3-bisphosphate, 2-hexadecylglycerol was first prepared from 1-bromohexadecane and 1,3-benzylidene glycerol. The 2-hexadecylglycerol was then phosphorylated with excess phosphoryl triimidazole as described previously (51). The measured CMCs of the multiply phosphorylated lipid analogs were determined by raising the temperature to 55°C, which is above the cloud point of the Triton X-100/Triton X-114 mixture (53). The upper aqueous phase was removed and replaced with an equal volume of detergent-free buffer, and the mixture was cooled to 25°C to allow reformation of a single phase (53). Partitioning of the phosphorylated amphiphiles was then assessed by assaying the two solutions for Triton (e275 = 1.49 mm−1 cm−1, isopropanol/H2O, 1:1 (v/v)) and organic phosphorus (46).

**RESULTS**

Inhibition of Arachidonoyl-DAG Kinase by Acidic Phospholipids—The effects of 18:0–20:4 PA, 16:0–18:1 PA, and PIP2 on arachidonoyl-DAG kinase activity are shown in Fig. 2. The two PA species gave identical inhibition curves, indicating that the presence of an sn-2 arachidonoyl group is not required for this effect. The inhibition curve with PIP2 was identical to that of PA (data not shown). Phosphatidylinositol 4,5-bisphosphate was an 18-fold more potent inhibitor than PA (Fig. 2). We have previously shown that other phospholipids have negligible effects on arachidonoyl-DAG kinase at these concentrations (24). Phosphatidylinositol, in particular, caused minimal inhibition at less than 5 mol % (24).

Addition of 20 mol % phosphatidylcholine had no effect on the PIP2 inhibition. The addition of up to 50 μM Ca2+ or 5 mM Mg2+ also was without effect. In the presence of 20 mol % octadecyltrimethylammonium chloride, 50% inhibition occurred at 1.4 mol % PIP2 and 75% inhibition at 2.2 mol %. Presumably, the decreased inhibitory potency of PIP2 in OTAC reflects complexation by the quaternary amine. However, it does demonstrate that PIP2 is capable of inhibiting the enzyme even in micelles bearing a net positive charge. Examination of surface dilution behavior by varying the micelle concentration while holding the surface concentrations of PIP2 and 18:0–20:4 DAG constant demonstrated that activity in the presence of 0.04 or 0.10 mol % PIP2 was dependent on the PIP2 surface concentration and independent of micelle concentration (data not shown). This is consistent with our previous observation that several other phospholipids do not alter the surface

2 Octadecyltrimethylammonium chloride is also an activator of DAG kinases (24). The stimulation of arachidonoyl-DAG kinase by OTAC in this assay system is 2.2-fold both at 10 and 20 mol %. Assuming each PIP2 binds five octadecyltrimethylammonium ions, binding of the quaternary amine cannot account for the effect of PIP2.

**Fig. 1. Structures of multiply phosphorylated lipid analogs used in this work.** Structures of phospholipid analogs used in this work are shown together with phosphatidic acid and phosphatidylinositol 4,5-bisphosphate.
dilution behavior of arachidonoyl-DAG kinase (24). Inositol polyphosphates, including D-
myo-inositol 1,4,5-trisphosphate and inositol hexaphosphate had no effect on arachidonoyl-DAG kinase activity at 1.0 mM, nor did they reverse the inhibition by PIP2 (data not shown).

Inhibition of Arachidonoyl-DAG Kinase by Other Multiply Phosphorylated Amphiphiles—The foregoing observations suggest that arachidonoyl-DAG kinase inhibition by PIP2 is highly specific for this phospholipid. Moreover, the inability of PI to inhibit at these concentrations and the much weaker inhibition by PIP suggest that the phosphomonoesters are major structural determinants of this effect. None of the naturally occurring lipids examined, however, is as highly charged as PIP2, and it was possible that addition of any highly charged amphiphile to the assay would cause similar inhibition. To test this, amphiphilic compounds bearing one, two, or three phosphate monoesters in their headgroups (Fig. 1) were prepared and examined with arachidonoyl-DAG kinase. As shown in Fig. 3, increasing the number of phosphates in the headgroup did increase DAG kinase inhibition. However, these analogs were not as potent as PA and PIP2. Concentrations of the mono- and bisphosphorylated analogs required for 50% inhibition were 3- and 7-fold higher, respectively, than the concentrations of PA and PIP2 required for this level of inhibition. Even the trisphosphorylated analog was less than half as potent a DAG kinase inhibitor as PIP2. These results strongly indicate that the inhibition is specific in some way for the structure of PIP2 and not just a function of its total charge.

The bis- and trisphosphorylated amphiphiles were freely soluble in water, behaving as detergents. It was important to show under the assay conditions used that these compounds incorporate completely into Triton micelles, as failure to do so would render the calculated mol fractions invalid. The CMC in 100 mM NaCl of monohexadecyl-pentaerythritol trisphosphate was 34 μM, and for 2-hexadecylglycerol bisphosphate it was 16 μM. The CMC of PIP2 under these conditions was less than 2 μM. Assuming ideal partitioning between the aqueous and micellar pseudophases (44), greater than 99% of these amphiphiles must be in the micelles. Partitioning of these compounds into Triton micelles was also estimated by cloud point separation as described under “Experimental Procedures.” These results indicated that greater than 98% of the bis- and trisphosphorylated analogs and approximately 80% of the do-decyl phosphate were in the micelles (data not shown). The inability of these compounds to inhibit arachidonoyl-DAG kinase as effectively as PIP2 cannot, therefore, be due to a failure to partition into the micelles.

Mechanism of PIP2 Inhibition—Double-reciprocal plots of arachidonoyl-DAG kinase on DAG and ATP. Double-reciprocal plots of the 18:0–20:4 DAG dependence at four different MgATP concentrations are shown. The inset shows the slopes of the primary plots as a function of 1/[MgATP]. The MgATP concentrations used were, clockwise from top: ○, 70 μM; ●, 100 μM; □, 150 μM; ▲, 200 μM.

3 Due largely to charge separation, ionic detergents incorporate into mixed micelles with nonionic detergents to a much greater extent than predicted by ideal mixing (54). The 100 mM NaCl should attenuate this effect somewhat. Nevertheless, these calculations probably underesti-

4 The Triton phase partitioning was performed at 55 °C. At this temperature, the partitioning of the ionic amphiphiles into mixed micelles is expected to be less than at 25 °C (55). Therefore, these measurements probably underestimate the extent of incorporation.
Arachidonoyl-DAG Kinase Regulation by PIP2

Fig. 5. Kinetics of arachidonoyl-DAG kinase inhibition by PIP2. Panel A shows double-reciprocal plots of the 18:0–20:4 DAG dependence at four different PIP2 concentrations. Panel B shows effects of the same concentrations of PIP2 on double-reciprocal plots of the MgATP dependence. The insets show the slopes of the primary plots as a function of PIP2. To calculate the true $K_i$ for PIP2, the horizontal intercept of the secondary plot for 18:0–20:4 DAG was corrected for the concentration of MgATP used in the assays (100 μM). No correction of the MgATP intercept is required. The PIP2 concentrations used were as follows: ●, 0.06 mol %; ○, 0.04 mol %; ●, 0.02 mol %; ○, no PIP2.

MgATP are shown in Fig. 4. The intersection of all the plots on the 1/DAG axis suggests a random order equilibrium mechanism, with $K_m = K_s = 153$ μM for MgATP and $K_m = K_s = 3.3$ mol % for 18:0–20:4 DAG. Random order of addition is the only type of equilibrium mechanism that can cause this type of kinetic behavior (56). A nonequilibrium, steady state mechanism is excluded by our previous observation that octadecyltrimethylammonium bromide is a partially noncompetitive activator of arachidonoyl-DAG kinase (24). Given these kinetics, the activator must be accelerating some rate-limiting step after binding of both substrates (56). In its absence, binding of substrates must thus be in a near equilibrium state, excluding any type of nonequilibrium mechanism for the kinetics in Fig. 4. Double-reciprocal plots of the effects of PIP2 on the 18:0–20:4 DAG dependence are shown in Fig. 5A. Inhibition was strictly noncompetitive with a $K_i$ of 0.04 mol %. The noncompetitive kinetics with respect to DAG are consistent with the absence of selectivity for an sn-2 arachidonoyl group in PA (Fig. 2). Strictly competitive kinetics were observed when the PIP2 inhibition was examined with respect to MgATP, again with a $K_i$ of 0.04 mol % (Fig. 5B). Inhibition by PA was also strictly competitive with MgATP but with a $K_i$ of 0.7 mol % indicating that the mechanism is the same as that of PIP2 (Fig. 6). This result also implies that octadecyltrimethylammonium bromide cannot be activating the enzyme by complexation of PA (generated by the reaction) and consequent reversal of PA mediated inhibition. Since inhibition by PA is competitive with MgATP, reversal of any such inhibition should decrease the apparent $K_m$ for this substrate. However, we have shown previously that activation of arachidonoyl-DAG kinase by octadecyltrimethylammonium cation is due to an increase in $V_m$ and that the apparent $K_m$ for MgATP is unchanged (24). Such a mechanism is also excluded by the observation that chlorpromazine, another cationic amphilie, readily reversed PA inhibition but did not activate DAG kinase in the absence of added PA (data not shown).

Effects of Acidic Phospholipids on Other DAG Kinase Isoforms—Diacylglycerol kinases are now known to be a large family of isoenzymes (2). It was thus of interest to examine whether inhibition by PIP2 or PA is seen with other DAG kinases. The octylglucoside assay was used for these experiments because other DAG kinases tend to have low activity in Triton (24). As shown in Fig. 7A, five cytosolic DAG kinase isoforms were activated, in some cases markedly, by phosphatic acid. As shown in Fig. 7B, PIP2 also activated these five DAG kinases. Only the arachidonoyl-DAG kinase was inhibited by PIP2 or PA. The same general effects were seen in Triton (Table I), although, as expected, the activities were lower than in octylglucoside. This apparent absolute specificity for the arachidonoyl-DAG kinase isoform is strong additional evidence that the inhibition reflects a specific interaction of PIP2 with the enzyme and is not an artifact of the assay method employed. Expressed as a mole fraction, PIP2 was a less potent arachidonoyl-DAG kinase inhibitor in octylglucoside than in Triton. The behavior of PIP2 in octylglucoside as a substrate for phospholipase C-γ1 is also very different from its behavior in Triton (34). With the phospholipase C kinetic effects of enzyme tyrosine phosphorylation could be observed only in a Triton-based assay and not in octylglucoside (34). Given the many differences between these two detergents, the significance of these observations is unknown. With all five of the other DAG kinase isoforms, the maximal activities observed with octylglucoside/PA were similar to those obtained in the deoxycholate assay normally used for these enzymes (6, 24), raising the possibility that PA and PIP2 may regulate the activities of these enzymes in vivo.
specifically inhibited by PIP2. The inhibition of arachidonoyl-DAG kinase by PIP2 in Triton X-100 is expressed as a function of PIP2 surface concentration, was identical to that in the Triton mixture (data not shown).

Inhibition of arachidonoyl-DAG kinase by PIP2 in Triton X-100, expressed as a percentage of the maximal activity with that isoform in octylglucoside/PA as described in the legend to Fig. 4.

**Fig. 7. Effects of acidic phospholipids on DAG kinase isoenzymes.** Panel A shows the effect of PA on the activities of arachidonoyl-DAG kinase and five other DAG kinase isoforms. Assays were performed in the octylglucoside assay described under "Experimental Procedures" in the presence of the indicated surface concentrations of PA. Phosphatidic acid concentrations greater than 20 mol % could not be tested because of insolubility in the assay mixture above this concentration. Panel B shows the effect of PIP2 on these same DAG kinases. In these assays, the octylglucoside contained 10 mol % phosphatidylserine in addition to the indicated concentrations of PIP2. The basal activities are slightly lower with the arachidonoyl-DAG kinase and slightly higher with the other isoforms because of the phosphatidylserine. Assays at 1.0 and 3.0 mol % PIP2 in octylglucoside without phosphatidylserine showed the same pattern of inhibition and activation. To facilitate comparison, activities in both panels are expressed as percentages of the maximal activity observed with that isoform in the octylglucoside/PA assay. The DAG kinase isoforms used, maximal activities, and molar fractions of PA at which these activities were observed are as follows: \(\bullet\), arachidonoyl-DAG kinase, 33 pmol/min/mg, 0.0 mol %; \(\circ\), 3T3 DAG kinase A, 1.9 nmol/min/mg, 1.0 mol %; \(\Delta\), 3T3 DAG kinase B, 1.0 nmol/min/mg, 20 mol %; \(\oplus\), thymus DAG kinase A, 25 nmol/min/mg, 20 mol %; \(\ominus\), thymus DAG kinase B, 12.3 nmol/min/mg, 20 mol %; \(\Box\), testis cytosol DAG kinase, 8.2 nmol/min/mg, 20 mol %.

**DISCUSSION**

We have shown arachidonoyl-DAG kinase to be potently and specifically inhibited by PIP2. The \(K_i\) for PIP2 inhibition of the enzyme was 0.04 mol %. Assuming a Triton aggregation number of 140, this corresponds to one PIP2 molecule/20 micelles in the assay mixture.\(^5\) As arachidonoyl-DAG kinase inhibition was dependent on the PIP2 surface concentration and independent of micelle concentration, the mechanism must involve intramolecular binding of PIP2 to the enzyme. Rapid equilibration of the solubilized lipids and enzyme between micelles presumably permits this low level of PIP2 to effectively inhibit the enzyme (24, 59). The inability of o-myoinositol 1,4,5-trisphosphate and inositol hexaphosphate to inhibit arachidonoyl-DAG kinase is further evidence that PIP2 must incorporate into the micelle before it can inhibit the enzyme. The observation that PI does not inhibit arachidonoyl-DAG kinase below 5 mol % points to the phosphomonoesters as major determinants of the PIP2 effect. This conclusion is also supported by the potent inhibition seen with the multiply phosphorylated analogs. These analogs were, however, clearly less potent than PIP2 itself, indicating that some structural feature of PIP2 not related to its total charge is also important. The competitive inhibition kinetics with respect to MgATP raise the possibility that the PIP2 phosphomonoester binding sites overlap the binding site for trisphosphate moiety of the MgATP, although an allosteric mechanism cannot be excluded. The observations that five other DAG kinase isoforms are not inhibited by PIP2 or PA are strong additional evidence that this is a specific property of arachidonoyl-DAG kinase and not a nonspecific effect of the mixed micellar assay.

Acidic lipids, including polyphosphoinositides and phosphatidic acid, have been shown to activate several membrane associated enzymes of stimulus transduction, including PI-specific phospholipase C-\(\gamma\) (33), several protein kinase C isoforms (35–37), phospholipase D (38), ADP-ribosylation factor 1 (39), and an ADP-ribosylation factor GTPase-activating protein (40). Phosphatidylinositol-4-phosphate 5-kinase appears to be inhibited by PIP2 and activated by PA (41, 42). Casein kinase I also appears to be inhibited by PIP2 (43). In some of these cases, as with the DAG kinases, these effects were shown to occur only with certain isoforms of these enzymes (37, 42). The surface concentration of PIP2 in Triton required for half-maximal DAG kinase inhibition, 0.04 mol %, compares well with the apparent \(K_m\) of phospholipase C-\(\gamma\) for PIP2 in Triton, which is 0.03–0.03 mol %, depending on the activation state of the enzyme and presence or absence of a PA cofactor (33, 34). Also, activation of protein kinase C by PIP2 in Triton occurs at 0.08–0.2 mol % (35, 36). In other studies, the use of very different assay methods renders comparisons with the present work difficult. Taken together, however, these results do suggest that signaling cascades may be regulated by the lipid microenvironment of subcellular membranes in which they are located. Inasmuch as PIP2 and PA undergo rapid flux during cell stimulation (60–65), this hypothesis seems not unreasonable.

The stimulation of some other DAG kinases by PIP2 and PA, while raising the possibility that these enzymes may be activated by PA or polyphosphoinositides, does not support a role for these isoforms in regulating the level of PIP2. Stimulation of these isoforms by phosphatides may reflect a mechanism for their recruitment to specific intracellular membranes during cell stimulation (66, 67). However, detailed studies of this phenomenon have yet to be performed.

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\(^5\) The aggregation number of Triton X-100 in water is 140 (57). The aggregation number of the Triton 100/114 mixture used in this work is unknown, but probably slightly greater than that of Triton X-100 (58).
The marked enrichment of animal cell phosphatidylinositols in arachidonate strongly suggests a role for arachidonoyl-DAG kinase in PI synthesis (21, 25). Given this, the observed inhibition may represent a mechanism for PIP2 to regulate its own synthesis. Work from several groups has shown that the arachidonate-enriched DAG pool that arises during stimulus-induced PI turnover is rapidly phosphorylated by a diacylglycerol kinase and that much of this PA is ultimately converted back to PI (21, 60–65). Other DAG species are phosphorylated much more slowly (60–65). As other DAG kinases do not exhibit fatty acyl selectivity, these results strongly suggest arachidonoyl-DAG kinase is the isoform responsible for this DAG phosphorylation. Phosphorylation of arachidonoyl-DAG under these conditions coincides with a transient drop in the cellular level of PIP2 (63, 68–70). Regulation of arachidonoyl-DAG kinase by PIP2 feedback is thus entirely consistent with available in vivo data.

The present work demonstrates PIP2 to be a potent and specific in vitro inhibitor of arachidonoyl-DAG kinase. To what extent the mixed micellar system reflects the in vivo regulation of this enzyme remains to be determined. Arachidonoyl-DAG kinase is an integral membrane protein and is likely to be highly localized in the cells in which it is found. The intramembranous mechanism of PIP2 inhibition in vitro suggests an intramembranous in vivo mechanism. Any regulation of this enzyme by PIP2 would then require the phosphoinositide to be present in the same membrane compartment as the DAG kinase.

The subcellular localization of the PI cycle, while likely to be of critical importance to its regulation, is understood to a limited extent (71, 72). Future studies of in vivo DAG kinase regulation will have to address these issues of compartmentation and multiple enzyme isoforms. Cloning of arachidonoyl-DAG kinase, which is in progress in our laboratory, will facilitate new approaches to these questions. The interaction with PIP2 will be amenable to site-directed mutagenesis, and the subcellular location of the enzyme will be open to immunocytochemical approaches. Such studies promise a better understanding of PI-mediated stimulus transduction in animal cells.

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