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INHIBITION OF PHOSPHATIDYLINOSITOL KINASE IN VASCULAR SMOOTH MUSCLE MEMBRANES BY ADENOSINE AND RELATED COMPOUNDS*

SUSAN R. DOCTROW† and JOHN M LOWENSTEIN‡
Graduate Department of Biochemistry, Brandeis University, Waltham, MA 02254, U.S.A.

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Abstract—Adenosine and 5′-chloro-5′-deoxyadenosine inhibited the phosphorylation of phosphatidylinositol in membranes prepared from aortic smooth muscle. The nucleosides did not affect the breakdown of phosphatidylinositol-4-phosphate. Under certain conditions, the membrane-bound phosphatidylinositol kinase phosphorylated exogenous phosphatidylinositol. The nucleosides inhibited the enzyme competitively with respect to magnesium-ATP and non-competitively with respect to phosphatidylinositol. Adenosine analogs modified in the ribose moiety were inhibitors with potencies comparable to that of adenosine, whereas adenine nucleotides and purine-modified adenosine analogs were much weaker inhibitors. Density gradient fractionation studies showed that phosphatidylinositol kinase is primarily associated with the sarcoplasmic reticulum. Vascular smooth muscle contraction is associated with increased phosphatidylinositol turnover. Inhibition of phosphatidylinositol kinase by intracellular adenosine may, therefore, be a factor involved in regulating vasodilation.

Adenosine is produced by various tissues in response to conditions such as hypoxia or ischemia. In certain tissues it then acts as a vasodilator and causes an increased blood flow [1–3]. Consistent with this physiological action, adenosine decreases the contractile force in strips of isolated vascular smooth muscle [4–6].

α_1-Agonists and other agents that induce contraction of vascular smooth muscle also cause an increased turnover of phosphatidylinositol in the whole tissue [7–10] and in vascular smooth muscle cells in culture [11–13]. Many of these agents act by increasing the concentration of cytosolic calcium [14]. Thus, there is a correlation between PI turnover, calcium mobilization, and contraction in vascular muscle. Enhanced PI§ turnover occurs in many other tissues and cells in response to a variety of agonists that cause a rise in cytosolic calcium [15, 16]. It has been demonstrated in several systems that the most rapid response to agonist binding is the breakdown of the polyphosphoinositides, PIP and PIP_2, to yield inositol triphosphate, inositol diphosphate, and diacylglycerol [17–23]. Inositol triphosphate has been implicated in the release of calcium from intracellular stores [24–26]. It follows that the regulation of PIP and PIP_2 production may be an important means of controlling calcium-dependent processes such as vascular smooth muscle contraction.

We showed previously that when calf aortic muscle membranes are incubated with [γ-^{32}P]ATP, radioactive PIP is produced from endogenous PI [27]. Under our conditions, incorporation of radioactivity into PIP_2, PI, and phosphatidic acid is insignificant. The phosphorylation of PI is inhibited by adenosine and 5′-chloro-5′-deoxyadenosine. In this paper we present studies of the adenosine-sensitive phosphorylation of PI in aortic smooth muscle membranes, with special reference to adenosine analog specificity.

EXPERIMENTAL PROCEDURES

Materials. Adenosine, inosine, 2′,3′-dideoxyadenosine, and N^6-cyclohexyladenosine were from PL Biochemicals, Milwaukee, WI. AOPCP and ACP were from Miles Laboratories, Elkart, IN. N^6-Aminopropyladenosine was synthesized by Dr. Nianci Liang. [^{3}H]Phosphatidylinositol was prepared by Mr. Robert Lutz using the procedure of Takenawa and Nagai [28]. Aristeromycin was a gift from Dr. Robert Abeles, and 5′-chloro-5′-deoxyadenosine was either synthesized by the procedure of Kikugawa and Ichino [29] or was a gift from Dr. John Palmer. AMP, ADP, ATP, cAMP, 2′,3′-AMP (mixed isomers), PIP, and PIP_2 were obtained as sodium salts from the Sigma Chemical Co., St. Louis, MO. 2-Chloroadenosine and 2′,3′-isopropylidene adenosine were from the Sigma Chemical Co. [*R*]N^6-Phenylisopropyladenosine was from Boehringer

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† Present address: Department of Surgical Research, Children's Hospital Medical Center, Boston, MA 02115.
‡ To whom correspondence should be sent.

§ Abbreviations: PI, phosphatidylinositol; AOPCP, adenosine 5′-(α,β-methylene)diphosphonate; ACP, 5′-deoxyadenosine 5′-methylenephosphonate (alternative designation: 6′-deoxyhomoadenosine-6′-phosphonate); TSF, α-toluenesulfonylfluoride; PIP, phosphatidylinositol-4-phosphate; PIP_2, phosphatidylinositol-4,5-diphosphate; NECA, the *N*-ethylamide of adenosine in which the 5′-hydroxymethyl group is replaced by carboxyl; and PIA, N^6-phenylisopropyladenosine.

Mannheim. Bovine liver phosphatidylinositol was from Avanti Biochemicals, Birmingham, AL. Silica gel 60 and cellulose thin-layer plates were from E.M. Reagents, Cincinnati, OH. [γ-^{32}P]ATP, [2-^{3}H]-adenosine, UDP-[U-^{14}C]galactose, [2-^{3}H]inositol, and En^{3}Hance spray were from New England Nuclear, Boston, MA. Triton X-100 was from Bio-Rad and bovine serum albumin from Pentex. Tris base, *p*-toluenesulfonylfluoride, cytochrome *c*, UDP-galactose, *N*-acetylglucosamine, sodium cholate, and dithiothreitol were from the Sigma Chemical Co. Calf aortas were from J. T. Trelegan Inc., Cambridge, MA. All other reagents were from Fisher Scientific, Fair Lawn, NJ.

Vascular smooth muscle fractions. Thirty calf aortas were obtained from the slaughterhouse, where they were rinsed with ice-cold 0.9% NaCl containing 0.1 mM TSF. Vascular smooth muscle was dissected from the aortas, minced, and homogenized as described previously [27]. After removal of large particulate matter, the homogenate was centrifuged at 80,000 *g* for 45 min. The resulting pellet, hereafter called *insoluble material*, was resuspended in 50 mM Tris–HCl buffer, pH 7.4, 0.1 mM TSF (hereafter called Tris–TSF buffer) using a motor-driven Potter–Elvehjem homogenizer.

Membranes. The total insoluble material obtained from 15 g aortic smooth muscle was suspended by homogenization in 20 ml Tris–TSF buffer and layered onto 10 ml of Tris–TSF buffer containing 60% sucrose (w/v) in centrifuge tubes of 36 ml capacity. The tubes were centrifuged in a swinging bucket rotor at 84,000 *g* for 12 hr. The membranes which banded at the interface with the 60% sucrose solution were collected with a Pasteur pipet, diluted 10-fold with Tris–TSF buffer, and centrifuged at 80,000 *g* for 45 min. The resulting pellet, hereafter called *membrane fraction,* was resuspended in Tris–TSF buffer and stored in aliquots at −70°.

Soluble fraction. The 80,000 *g* supernatant fraction was concentrated to a volume of 48 ml in an Amicon pressure concentrator using a PM 10 membrane. The resulting solution was divided into aliquots and stored at −70°.

Density gradient fractionation. The insoluble material obtained from 15 g aortic muscle was suspended in 8 ml Tris–TSF buffer. The suspension was layered onto a linear gradient of 8.5 to 60% (w/v) sucrose in Tris–TSF buffer with a total volume of 26 ml contained in centrifuge tubes of 36-ml capacity. The tubes were centrifuged in a swinging bucket rotor at 84,000 *g* for 12 hr. Fractions of 1.3 ml were collected from the gradient. The fractions were divided into aliquots and stored at −70°.

Assay procedures. Protein was measured by a modified Lowry method which included 2.5% SDS [30], except that the trichloroacetic acid precipitation step was omitted. The Tris–TSF buffer used for membrane preparation and storage did not interfere with this assay. Triton X-100 at a final concentration of about 0.1% interfered with the assay; when it was present, an equal volume of detergent-containing buffer was added to each protein standard and to the blank. Bovine serum albumin was used as standard.

5′-Nucleotidase activity was determined spectrophotometrically by coupling the reaction to adenosine deaminase [31]. Hydrolysis of 2′-AMP and 3′-AMP was insignificant relative to 5′-AMP hydrolysis in all membrane fractions.

Cytochrome *c* oxidase was determined spectrophotometrically. Reaction mixtures contained 30 mM potassium phosphate buffer, pH 7.4, 34 μM reduced cytochrome *c*, and 5–25 μl/ml membrane suspension. Prior to the assays, cytochrome *c* was reduced with 4.1 mM sodium hydrosulfite in 30 mM potassium phosphate buffer. Excess hydrosulfite was then oxidized by vigorous shaking of the solution. Reactions were run at 30° and followed by measuring the change in absorbance at 550 nm. The rate of cytochrome *c* oxidation was calculated using an E_{550} of $19.7 \times 10^3\ M^{-1}\ cm^{-1}$ [32]. Oxidation of cytochrome *c* observed in the absence of membrane protein was subtracted from each value.

UDP-galactose:*N*-acetylglucosamine galactosyl transferase was assayed by the procedure of Fleischer [33]. NADPH–cytochrome *c* reductase was assayed according to Ives *et al.* [34].

PI kinase was assayed in a mixture which contained 50 mM Tris–HCl, pH 7.4, and PI, $MgCl_2$, membrane protein, and [γ-^{32}P]ATP as indicated. Unless otherwise specified, the reaction volume was 150 μl. The reactions were run at 30° and stopped by adding 5 vol. of chloroform/methanol (2/1). The phospholipids were extracted and separated on thin-layer plates coated with silica using solvent II or solvent III as described previously [27]. Solvent III was used routinely because it produces rapid separations that are not affected by detergents. When system II was used for Triton X-100 containing samples, the plates were first developed in chloroform/methanol/glacial acetic acid/water (80/13/8/0.3) [35], air-dried, and then developed in solvent II. The detergent, as well as PI, migrated in the first solvent, while PIP and PIP_2 remained at the origin. The polyphosphoinositides then separated without distortion in solvent II. Lipid spots were located by iodine staining of added standards and by autoradiography, cut from the plastic plates, and placed in scintillation vials containing 2 ml methanol. Beckman ReadySolv MP scintillation fluid (10 ml) was added, and the vials were shaken vigorously. Radioactivity was determined in a Beckman model LS7500 liquid scintillation counter.

Breakdown of ^{32}P-labeled endogenous PIP in the presence and absence of 5′-chloro-5′-deoxyadenosine. Two identical phosphorylation reactions were prepared. Each contained 1.54 mg/ml membrane protein, 50 mM Tris–HCl, pH 7.4, 2 mM $MgCl_2$, 0.1% Triton X-100 and 100 μM [γ-^{32}P]ATP (1200 cpm/pmol) in a total volume of 0.5 ml. Phosphorylation was allowed to proceed at 30° for 4 min. At this time, 50 μl was withdrawn and quenched in chloroform/methanol (2/1) (zero time point for radioactive PIP breakdown). A 0.35-ml aliquot of the remaining reaction was diluted 10-fold with buffer containing 50 mM Tris–HCl, pH 7.4, 2 mM $MgCl_2$, 0.1% Triton X-100, and 100 μM nonradioactive ATP, in the presence and absence of 200 μM 5′-chloro-5′-deoxyadenosine. The temperature was maintained at 30° and 0.5-ml aliquots were withdrawn and quenched with chloroform/methanol

at 2, 4, 6, 8 and 10 min. Phospholipids were separated and analyzed for radioactivity as described above.

5′-N-*Ethylcarboxamidoadenosine (NECA).* This substance was synthesized via the 5′-carboxy 5′-deoxy analog of 2′,3′-isopropylidene adenosine (5′-carboxy IPA) [36]. The latter was converted to the corresponding 5′-*N*-ethylamide by the procedure of Chan and Wong [37] who used $SiCl_4$ to activate carboxyls for amide synthesis. The product was purified by chromatography on a silica gel column using the upper phase of *n*-butanol/acetic acid/water (4/1/5, by vol.) as eluting solvent. The isopropylidene protecting group was removed by hydrolysis in 1 N HCl at 70° for 1 hr. The resulting NECA was purified on an octadecyl silica column by elution with methanol/water (1/1, v/v). Thin-layer chromatography on silica plates using the above solvent showed a single u.v.-absorbing product with an R_f of 0.36. The product contained no detectable 5′-carboxylic acid of adenosine, which would be formed if the amide linkage is hydrolyzed. The 5′-carboxylic acid of adenosine used as a reference compound showed an R_f of 0.11.

RESULTS

Endogenous PI phosphorylation in smooth muscle membranes was examined in the presence of various concentrations of Triton X-100 or sodium cholate (Fig. 1). PIP formation showed a sharp maximum in the presence of 0.1% Triton X-100. Cholate produced only a slight activation at 0.1% and was slightly inhibitory at 0.6%. Triton X-100 (0.1%) was used in all subsequent experiments involving endogenous PI phosphorylation.

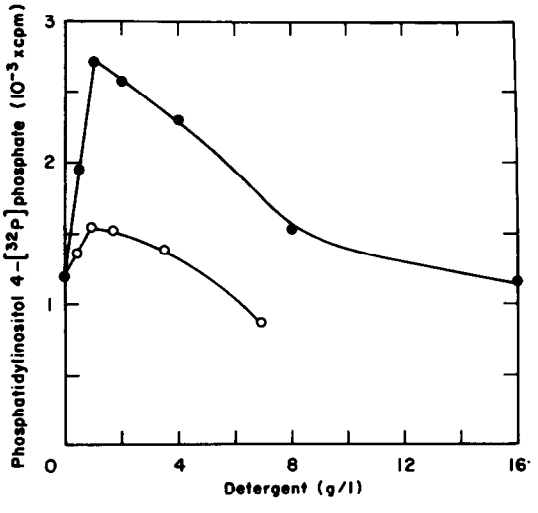

Fig. 1. Effects of detergents on phosphorylation of endogenous phosphatidylinositol in smooth muscle membranes. The reaction mixtures contained 200 μM [γ-^{33}P]ATP (40 cpm/pmol), 2 mM $MgCl_2$, 0.24 mg/ml membrane protein, and either Triton X-100 (●) or sodium cholate (○) in a total volume of 0.5 ml. The reactions were run at 30° for 3 min. After stopping the reactions, detergents were added so that each tube contained 0.63% sodium cholate and 1.6% Triton X-100. Lipids were extracted, separated, and quantitated as described in Experimental Procedures. Points show the mean of two determinations. This experiment was repeated three times with similar results.

Bovine liver PI was added to reaction mixtures to determine whether PI kinase of calf aortic smooth muscle membranes is capable of using exogenous lipid as substrate. A stock suspension of 1 mg bovine liver PI was prepared by sonication of the dried lipid in 1 ml of 0.1% Triton X-100 in 50 mM Tris–HCl buffer, pH 7.4. Exogenous PI was phosphorylated only when the PI was added in this sonicated mixture. When a PI suspension was prepared by sonication in detergent-free 50 mM Tris–HCl buffer, pH 7.4, and added to an incubation mixture that contained 1% Triton X-100, there was no significant increase in PIP production over endogenous values.

To confirm that exogenous PI was actually being phosphorylated, membranes were incubated with [^{3}H]PI and nonradioactive ATP under similar conditions. Lipids were extracted and separated by thin-layer chromatography in solvent system II in the presence of PI, PIP, and PIP_2 standards. After chromatography, the plates were subjected to autoradiography. The resulting autoradiograph showed two radioactive spots which coincided with [^{3}H]PI and [^{3}H]PIP, demonstrating the phosphorylation of the added PI.

5′-Chloro-5′-deoxyadenosine inhibited the rate of PIP formation in calf aortic muscle membranes. In the experiment shown in Fig. 2, the inhibition was about 58 and 83% in the presence of 25 and 100 μM 5′-chloro-5′-deoxyadenosine respectively. Although Triton X-100 was present in this experiment, the inhibition by 5′-chloro-5′-deoxyadenosine also occurs in the absence of detergent [27]. Other experiments revealed that the inhibition of PI phosphorylation was the same in the presence or absence of 0.1% Triton X-100 (results not shown). We next tested whether the adenosine analog was inhibiting PI phosphorylation or accelerating PIP breakdown by a phosphomonoesterase or a phospholipase C. Membranes were phosphorylated with [γ-^{32}P]ATP and then diluted into buffer containing nonradioactive ATP as described in Experimental Procedures. The rates of [^{32}P]PIP disappearance were compared in the presence and absence of 200 μM 5′-chloro-5′-deoxyadenosine in the diluting buffer. Under these conditions, approximately 10% of the labeled PIP disappeared per minute in the presence and absence of nucleoside, i.e. the nucleoside had no effect.

The inhibition of endogenous PI phosphorylation by 5′-chloro-5′-deoxyadenosine was competitive with respect to ATP (Fig. 3). In this experiment, the K_m for ATP was 125 μM, and the K_I for 5′-chloro-5′-deoxyadenosine was 13 μM. In another experiment with a different membrane preparation, the K_m for ATP was 50 μM, and the K_I for 5′-chloro-5′-deoxyadenosine was 20 μM.

The effect of increasing the concentrations of exogenous PI in the presence and absence of 5′-chloro-5′-deoxyadenosine is shown in Fig. 4. The K_m for PI was 140 μM, assuming a molecular weight for PI of about 885 (calculated for the 18:0–20:4 species of PI). As expected, the inhibition by 5′-chloro-5′-deoxyadenosine was non-competitive with respect to PI. Inhibition of PI kinase activity by 5′-chloro-5′-

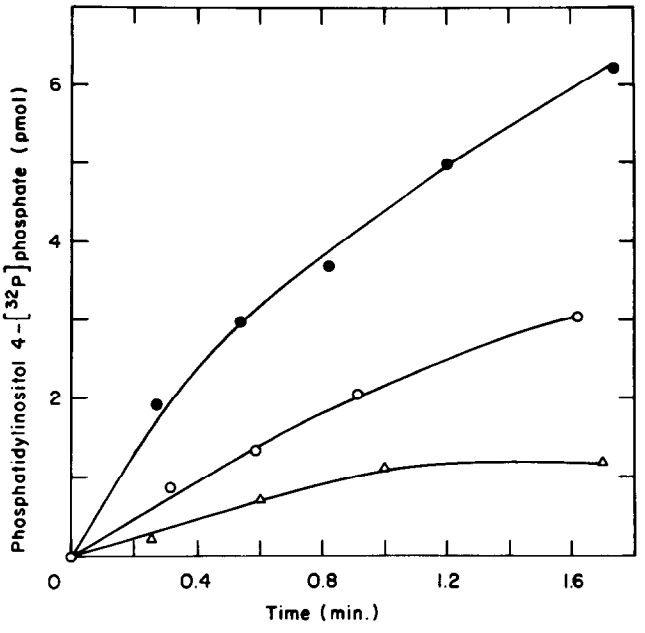

Fig. 2. Effect of 5′-chloro-5′-deoxyadenosine on the rate of endogenous phosphatidylinositol phosphorylation in calf aortic muscle membranes. Reaction mixtures were as described in the legend to Fig. 1, with 0.1% Triton X-100 and 100 μM [γ-^{32}P]ATP. Reactions were started by adding membranes. Aliquots containing 26 μg membrane protein were withdrawn at the times indicated and quenched in 1 ml chloroform/methanol (2/1, v/v). Phospholipids were extracted and separated, and their radioactivity was determined as described in Experimental Procedures. The concentrations of 5′-chloro-5′-deoxyadenosine in the reactions were (●) none; (○) 25 μM; and (△) 100 μM. This experiment was repeated three times with the same results.

deoxyadenosine was also examined at various ATP concentrations in the presence of 0.25 mg/ml bovine liver PI. The results showed that the inhibition is competitive with respect to ATP in a manner similar to the inhibition of endogenous PI phosphorylation shown in Fig. 3.

When the membrane fraction (15 μg protein) was incubated with 100 μM [2-^{3}H]adenosine and 250 μM nonradioactive ATP in a final volume of 0.1 ml for 10 min, under conditions used for the phosphorylation reaction, there was no detectable production of radioactive inosine, adenine, hypoxanthine or AMP as determined by thin-layer chromatography. No detectable production corresponds to less than 3% conversion of adenosine by the membranes.

The effect of adenosine analogs and related compounds on the phosphorylation of exogenous PI by calf aortic membranes was examined (Fig. 5), but similar results were obtained for the phosphorylation of endogenous PI. Fifty percent inhibition occurred at 95 μM adenosine (Fig. 5A). The ribose-modified compounds, 5′-chloro-5′-deoxyadenosine, aristeromycin, and NECA, inhibited PI phosphorylation in the same concentration range. 2′-3′-Dideoxyadenosine was a weaker inhibitor, causing 50% inhibition at 280 μM (Fig. 5A). Two other compounds with potencies similar to that of adenosine were 2′-deoxyadenosine and 2′,3′-isopropylidene adenosine (not shown).

In contrast, compounds in which the purine ring was modified were relatively weak inhibitors of PI phosphorylation (Fig. 5B). In the case of inosine and PIA, 50% inhibition was not attained at the highest concentration tested. N^6-Cyclohexyladenosine and 2-chloroadenosine were also extremely poor inhibitors (not shown).

Adenine nucleotides were weaker inhibitors than adenosine and the uncharged, ribose-modified analogs described above. The compounds tested were AMP, cAMP, and ACP. When AMP was tested, AOPCP, a potent inhibitor of 5′-nucleotidase [38, 39], was added to prevent hydrolysis of AMP to adenosine. In ACP, the 5′-oxygen atom of AMP is replaced by methylene, and the compound is not hydrolyzed by 5′-nucleotidase [39]. AMP and ACP caused less than 50% inhibition at millimolar concentrations. cAMP and ADP caused 50% inhibition at 630 and 340 μM respectively (Fig. 5C).

The insoluble material obtained from a calf aortic smooth muscle homogenate was centrifuged on a linear sucrose density gradient as described in Experimental Procedures. The contents of the tube were collected in 21 fractions of equal size, and each fraction was analyzed for protein, marker enzymes, and PI kinase. The specific activity profiles of these enzymes were compared (not shown).

NADPH–cytochrome *c* reductase, used as marker for sarcoplasmic reticulum, showed the best correlation with PI kinase out of the four marker enzymes measured. In the heavy sarcoplasmic reticulum fractions, the specific activity profiles of these two enzymes were almost identical. In the light sarcoplasmic reticulum fractions, PI kinase was slightly depleted relative to the reductase. PI kinase was

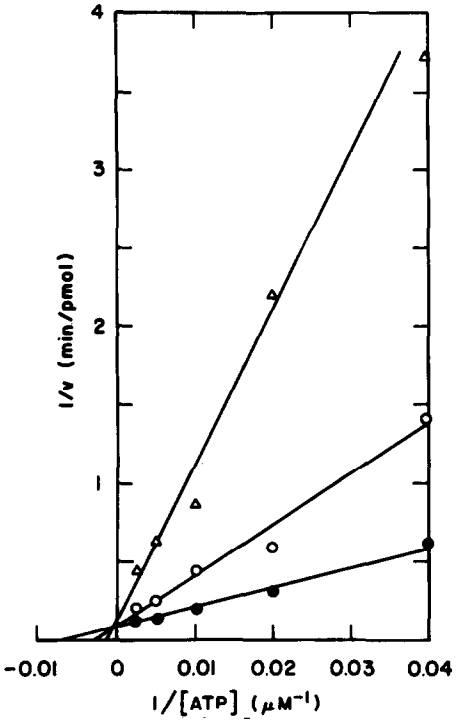

Fig. 3. Inhibition of endogenous phosphatidylinositol phosphorylation by 5′-chloro-5′-deoxyadenosine. The reaction conditions were as described in the legend to Fig. 1, with [γ-^{32}P]ATP, as indicated, and 0.1% Triton X-100. Phospholipids were extracted and analyzed by thin-layer chromatography system II. Five time points spanning 15 sec to 2.0 min were analyzed for each reaction condition and initial rates were calculated from plots of time versus pmol ^{32}P incorporated into PIP. Velocities are expressed in terms of pmol PIP produced per min per 26 μg membrane protein. The concentrations of 5′-chloro-5′-deoxyadenosine were (●) none; (○) 25 μM; and (△) 100 μM.

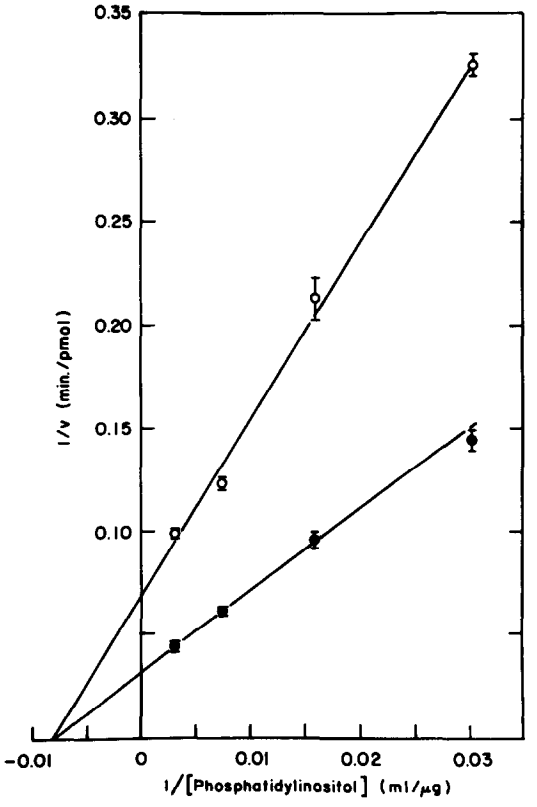

Fig. 4. Effect of exogenous phosphatidylinositol concentration on activity. Reaction mixtures contained 50 mM Tris–HCl, pH 7.4, 2 mM $MgCl_2$, 1% Triton X-100, 0.17 mg/ml membrane protein, 200 μM [γ-^{32}P]ATP (180 dpm/pmol), and bovine liver PI as indicated in a total volume of 150 μM. The stock suspension of bovine liver PI (1 mg/ml) was prepared by sonication of the lipid in 0.1% Triton X-100 for 5 min. The mixture was incubated in the absence of [γ-^{32}P]ATP at 30° for 3 min before the reaction was started by adding [γ-^{32}P]ATP. The reaction was stopped after 3 min. Results show the mean ± SEM for three determinations in the absence (●) and presence (○) of 100 μM 5′-chloro-5′-deoxyadenosine. The rate of phosphorylation of endogenous PI was subtracted from the total rate. This was 0.41 pmol/min in the presence of 5′-chloro-5′-deoxyadenosine and 0.79 pmol/min in its absence.

present in all fractions rich in UDP-galactosyltransferase, used as marker for the Golgi apparatus, but the transferase peak was much sharper than the PI kinase peak. Several of the higher density fractions were poor in Golgi marker but had significant PI kinase enrichment. The PI kinase peak overlapped but did not coincide with the profiles of the mitochondrial and plasma membrane markers. In the soluble fractions, the specific activities of all four marker enzymes and PI kinase were negligible.

DISCUSSION

Various adenosine analogs inhibit PI kinase with specificities that are similar in the presence and absence of exogenous PI. In general, uncharged compounds with modifications in the ribose moiety, but with an intact adenine, inhibited PI kinase about as much as adenosine. Of the adenine nucleotides, AMP and ACP were very weak inhibitors, ADP and cAMP were moderate inhibitors, but none was as potent as adenosine. This may reflect the electrostatic environment at the inhibitor binding site, since AMP and ACP exist as dianions at pH 7.4, cAMP and magnesium-ADP exist as monoanions, and adenosine and the ribose-modified adenosine analogs are uncharged. Adenosine analogs with modified adenine rings were all very weak inhibitors, suggesting that an intact adenine moiety is important for binding of the inhibitor.

Inhibition of PI kinase by 5′-chloro-5′-deoxyadenosine was competitive with respect to ATP, indicating that the nucleoside interferes with ATP binding to the enzyme, presumably by interacting directly with its binding site. The ratio of K_m/K_I varied somewhat in different membrane preparations. This may reflect different degrees of substrate and inhibitor accessibility to PI kinase in different preparations. Despite these variations, the K_I for 5′-chloro-5′-deoxyadenosine was much lower than the K_m for ATP.

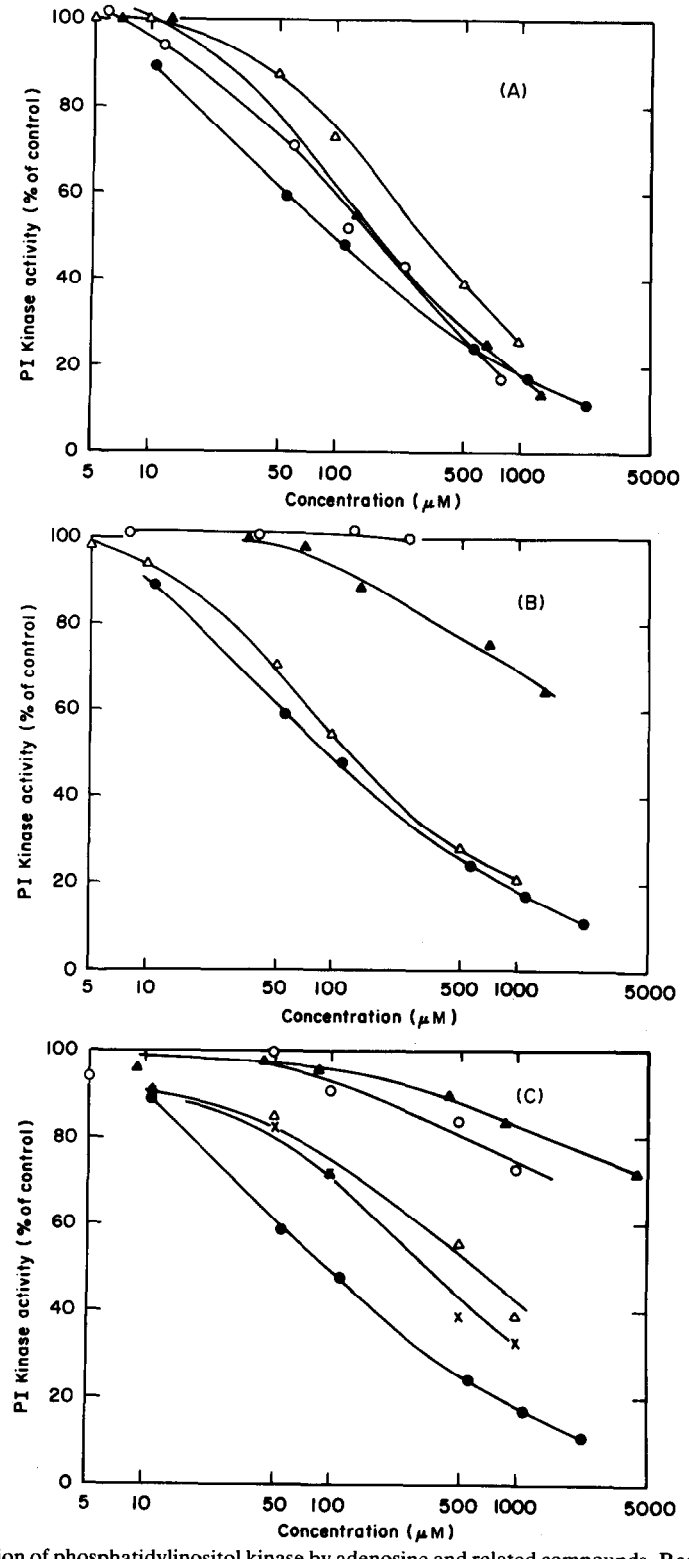

Fig. 5. Inhibition of phosphatidylinositol kinase by adenosine and related compounds. Reaction mixtures contained 50 mM Tris–HCl, pH 7.4, 2 mM $MgCl_2$, 1% Triton X-100, 250 μM [γ-^{32}P]ATP, 0.2 mg/ml bovine liver PI, 0.15 mg/ml membrane protein, and adenosine, or adenosine analogs, or adenine nucleotides at the indicated concentrations. The reactions were run for 2 min. The amount of ^{32}P incorporated into PIP is expressed as percent of a control value determined in the absence of inhibitors. The control activity was about 25 pmol PIP produced per ml reaction mixture per min. When AMP was tested, AOPCP was included at 10% of the AMP concentration in order to inhibit 5′-nucleotidase [39]. Each point represents the mean of two determinations that agreed to within 8%. Each curve was run at least twice. Panel A: (●) adenosine; (○) aristeromycin; (▲) NECA; and (△) 2′,3′-dideoxyadenosine. Panel B: (●) adenosine; (○) N^6-PIA; (▲) inosine; and (△) 5′-chloro-5′-deoxyadenosine. Panel C: (●) adenosine; (○) ACP; (▲) AMP; (△) cAMP; and (×) ADP.

Sucrose density gradient studies show that PI kinase is associated most closely with the sarcoplasmic reticulum. The marker enzyme for sarcoplasmic reticulum, NADPH–cytochrome reductase, exhibited a slightly broader peak than that of PI kinase, with some of the lower density sarcoplasmic reticulum fragments not containing PI kinase. Other fractionation studies of vascular smooth muscle have shown broad peaks of endoplasmic reticulum marker enzymes [34, 40–42]. PI kinase may also be present in the Golgi apparatus, but in our studies the peak due to the Golgi marker, UDP-galactosyltransferase, was much narrower than the peak due to PI kinase. An exclusive localization of PI kinase in Golgi membranes is thus unlikely. Plasma membrane and mitochondria were not enriched with respect to PI kinase. However, the possibility that either organelle contains minor amounts of the enzyme was not eliminated. Our results indicate that, in calf aortic muscle, PI kinase occurs predominantly in intracellular membranes as distinct from plasma membrane. This is in agreement with our previous report [27] that endogenous PI phosphorylation is much greater in microsomal membrane preparations than in plasma membrane-enriched preparations. However, by using exogenous PI in the present study, we eliminated the possibility that our previous observations simply reflected an abundance of endogenous PI in the microsomal preparation. While no previous distribution study of PI kinase in vascular muscle has been reported, the enzyme was found to be localized in the microsomal and Golgi membranes of rat liver [43–45] and kidney cortex [46–48].

In the normoxic perfused rat heart, the total intracellular concentration of adenosine is about 10 μM; it rises to 100 μM during anoxia and to about 1 mM during anoxia in the presence of dipyridamole [49]. In electrically stimulated rat brain, adenosine rises to levels equivalent to an intracellular concentration of 60–100 μM [50]. These values are average levels for whole organs; they may represent a lower limit for adenosine concentrations in vascular muscle cells. It is thus possible that intracellular levels of adenosine in such cells are sufficient to inhibit PI kinase under appropriate conditions.

Hormonal stimuli which act by mobilizing Ca^{2+} are closely associated with an increase in PI turnover [15]. Recent evidence indicates that the polyphosphoinositides PIP and PIP_2 are metabolized most rapidly during agonist stimulation [17–23]. The products formed from PIP_2 by the action of a phospholipase C, namely inositol-1,4,5-triphosphate and diacylglycerol, have both been implicated as transducers of hormonal stimulation [24–26, 51–54]. PI kinase is responsible for replenishing PIP, and hence PIP_2, after they become depleted during hormone action. Regulation of PI kinase activity may thus be a factor in the restoration of the calcium-mobilizing mechanism.

In the case of vascular smooth muscle, PI turnover is enhanced by contractile agents that mobilize Ca^{2+} [7, 9, 10, 55]. An agonist-induced PI effect has also been observed in cerebral microvessels [56] and in atrial muscle [57], both physiological targets of adenosine [58–61]. Agonist-induced polyphosphoinositide breakdown may be involved in the regulation of vascular smooth muscle contraction. One means of regulating the production of diacylglycerol and inositol 1,4,5-triphosphate would be to control the replenishment of the polyphosphoinositides. Thus, inhibition of PI kinase may play a role in adenosine-mediated effects such as relaxation of vascular smooth muscle.

This hypothesis is supported by observations that adenosine decreases cytosolic Ca^{2+} accumulation in both vascular smooth muscle and in atrial muscle [62–64]. In both tissues there is evidence that adenosine acts partly via an intracellular site [6, 64, 65].

The PI kinase we have studied was inhibited by adenosine, but not by AMP, the precursor of adenosine, or by inosine, its inactivation product *in vivo*. It was located intracellularly, where high concentrations of adenosine accumulate during hypoxia. These observations are consistent with the hypothesis that changes in PI kinase activity are involved in adenosine-mediated vasodilation.

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