Legionella micdadei Protein Kinase Catalyzes Phosphorylation of Tubulin and Phosphatidylinositol

ASISH K. SAHA,¹ JOHN N. DOWLING,^{2*} NISHIT K. MUKHOPADHYAY,^{1†} AND ROBERT H. GLEW¹

Department of Microbiology, Biochemistry and Molecular Biology,¹ and Department of Medicine,²
University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261

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Legionella micdadei, a pathogen which enters into host phagocyte phagolysosomal structures, contains at least two protein kinases. We have purified to homogeneity the predominant, nucleotide-independent protein kinase and examined its ability to catalyze the transfer of phosphate from ATP to acceptors in human neutrophils. The *L. micdadei* protein kinase catalyzed the phosphorylation of proteins of 11.5, 14, 19, 23, 28, 34, and 38 kilodaltons (kDa) present in a Triton X-100 extract of neutrophil membranes and of 11.5, 13.5, 25, and 38 kDa in the neutrophil cytosol. Tubulin was a good substrate for the *L. micdadei* protein kinase in vitro. The bacterial kinase also catalyzed the phosphorylation of phosphatidylinositol (PI) at about half the rate at which histones were phosphorylated; phosphatidylinositol-4-phosphate (PIP) was not phosphorylated by the kinase. The PI kinase activity of the *L. micdadei* enzyme was optimum at pH 7.0, and the divalent cation requirement was satisfied best by Mg²⁺ and Ca²⁺. The maximum rate of PI phosphorylation was obtained with 0.6 mM PI; in the presence of MgCl₂ (10 mM), the *K_m* for PI was 0.9 mM and the *K_m* for ATP was 1.5 mM. The detergents octyl- β -D-glucoside (10 to 20 mM) and Triton X-100 (0.5%) stimulated kinase activity twofold when PI was the phosphate acceptor; however, only octyl glucoside stimulated histone kinase activity. Various membrane phospholipids inhibited PI kinase activity. The most potent phospholipid inhibitor was the product of the PI kinase reaction, PIP, which at a 0.6 mM concentration inhibited both PI and tubulin phosphorylation by 80%. The inhibition of kinase activity by PIP when histone served as the acceptor was noncompetitive in character. The *L. micdadei* kinase also phosphorylated PI in intact, [³H]inositol-labeled neutrophils. The PI kinase and histone kinase activities of the *L. micdadei* kinase copurified and cofocused (pI, 5.8) when subjected to isoelectric focusing, suggesting that the two enzymatic activities reside in a single protein.

Legionella species multiply within the phagolysosomes of host monocytes (11, 37) and macrophages (17, 21) and are ingested, but not killed, by human polymorphonuclear neutrophils (12, 36). There is intense interest in the mechanisms by which the legionellae overcome the antimicrobial properties of host phagocytes. We have found that after the ingestion of *L. micdadei*, the subsequent activation of neutrophils in response to both soluble and particulate stimuli is greatly impaired (G. R. Donowitz, I. Reardon, L. Rubin, D. Focht, and J. Dowling, Abstr. 28th Intersci. Conf. Antimicrob. Agents Chemother., 1988, abstr. 290, p. 160). These data suggested that one or more *Legionella* bacterial cell-associated factors has a profound inhibitory effect on neutrophil activation and, consequently, on subsequent antibacterial functions.

Two "virulence" factors produced by the legionellae are known to inhibit the activation of neutrophils in response to various stimuli, thus mimicking the attenuation of neutrophil activation which follows the ingestion of *L. micdadei*. Friedman and co-workers found that the culture filtrates in which *L. pneumophila* were grown were cytotoxic for Chinese hamster ovary cells (7). Quantities of partially purified cytotoxin that had no effect on neutrophil viability or phagocytosis were found to depress hexose monophosphate shunt activity and oxygen consumption stimulated by phagocytosis and the calcium ionophore A23187 and to inhibit iodination and killing of *Escherichia coli* (8, 18). Treatment of neutrophils with toxin preparations also blocked membrane

depolarization in response to A23187, but not in response to phorbol myristate acetate (18). The second moiety known to affect neutrophil oxidative metabolism dates to our (26) finding that *L. micdadei* bacterial cells contain a phosphatase which blocks superoxide anion production by stimulated neutrophils. When the active phosphatase was purified to homogeneity, it was found that it reduces the amount of the second messengers myo-inositol-1,4,5-trisphosphate (IP₃) and sn-1,2-diacylglycerol, produced following receptor-mediated stimulation by two mechanisms (28). First, the phosphatase dephosphorylates phosphatidylinositol-4,5-bisphosphate (PIP₂) both in vitro and in intact neutrophils. This leaves less PIP₂ available as a substrate for phospholipase C following receptor-mediated stimulation, so that lesser amounts of both IP₃ and diacylglycerol are generated (28). Second, the bacterial phosphatase may directly dephosphorylate some of the IP₃ formed from the hydrolysis of PIP₂ (28).

The demonstration that *L. micdadei* contains a phosphatase which inhibits neutrophil activation (26) led us to speculate that the bacterium may be capable of modulating the function of host phagocytic cells by phosphorylating and dephosphorylating critical proteins. Therefore, we examined the possibility that *L. micdadei* has protein kinase activity in addition to phosphatase activity and found that the bacterium contains at least two protein kinases (27). Since these studies were done with the nonphysiological substrate mixed histones as the phosphate acceptor in the kinase assay, knowledge is lacking as to whether host cell proteins might function as substrates for the *L. micdadei* kinases. We purified the predominant, cyclic nucleotide-independent (27) *L. micdadei* protein kinase to homogeneity and found that it

* Corresponding author.

† Present address: Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA 02115.

TABLE 1. Histone and PI kinase activities^a of fractions during purification of *L. micdadei* kinase

Purification step	Total activity (10 ² U) with substrate:		Total protein (mg)	Sp act (10 ³ U/mg) with substrate:			Purification (fold) with substrate:		Recovery (%) with substrate:	
	Histone	PI		Histone	PI	Ratio histone/PI	Histone	PI	Histone	PI
Bacterial sonicate	285	91	178	0.16	0.05	3.1	1.0	1.0	100	100
High-speed supernatant	272	85	170	0.16	0.05	3.2	1.0	1.0	95	93
QAE-Sephadex	250	72	30	0.83	0.24	3.4	5.2	4.7	87	85
Histone affinity	204	64	7.5	2.72	0.85	3.2	17.0	16.6	71	70
Isoelectric focusing	180	55	0.65	27.7	8.5	3.2	173	166	63	60
Sephadex G-150	155	44	0.12	129	36.6	3.5	806	717	54	48
Hydroxylapatite	138	36	0.09	153	39.4	3.9	970	773	48	39

^a One unit of kinase activity equals 1 pmol of ³²P incorporated into substrate per min.

catalyzes the phosphorylation of both tubulin and phosphatidylinositol (PI) at the expense of ATP. Although the phosphorylation of tubulin in neutrophils treated with the bacterial kinase could not be demonstrated, PI in the plasma membrane of intact neutrophils was a substrate for the exogenously added bacterial enzyme. This resulted in a decreased content of PI and increased content of phosphatidylinositol-4-monophosphate (PIP) in the neutrophil membrane. The phosphorylation of both tubulin and PI could lead to aberrations in neutrophil function which would mimic those seen following the phagocytosis of *L. micdadei*.

MATERIALS AND METHODS

Purification of protein kinases. The initial extraction and purification of protein kinases from *L. micdadei* have been described in detail elsewhere (27). Briefly, the high-speed supernatant from a sonicate of *L. micdadei* bacterial cells was applied to a QAE-Sephadex column. About 70 to 80% of the protein kinase activity appeared in the breakthrough fractions. This cyclic nucleotide-independent protein kinase, designated PK I in our earlier publication (27), was purified to homogeneity by chromatography on a histone affinity column, isoelectric focusing, and sequential chromatography on Sephadex G-150 and hydroxylapatite (Table 1).

Protein determination. Protein concentration was estimated by the method of Bradford (2) with bovine serum albumin serving as the standard.

SDS-PAGE. For sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the purified protein kinase was electrophoresed on SDS-containing gels as described by Laemmli (16).

Protein kinase assays. Protein kinase activity was estimated at pH 7.2 with mixed histones as the acceptor (27). The reaction medium contained the following components in a final volume of 0.1 ml of 50 mM Tris hydrochloride (Tris-HCl) buffer, pH 7.2: 10 mM MgCl₂, 50 mM NaF, 50 mM NaCl, 300 µg of mixed histones, 1 mM (20 µCi) of [γ -³²P]ATP (5 to 10 Ci/mmol; New England Nuclear Corp., Boston, Mass.), and 10 µl of enzyme solution. The blank contained an equal volume of boiled enzyme solution. The reaction mixture was incubated at 37°C for 10 min, and the reaction was terminated by the addition of 10% (wt/vol) trichloroacetic acid. The precipitate was dissolved in water, and a sample was counted by liquid scintillation. One unit of kinase activity corresponds to the transfer of 1 pmol of phosphorus to histone per min.

Neutrophil protein substrates. Neutrophils were prepared from human blood by dextran sedimentation, followed by density gradient centrifugation on Ficoll-Hypaque gradients

and isotonic NH₄Cl lysis (1). Washed neutrophils (5×10^7) were suspended in 50 mM Tris-HCl buffer and broken with the aid of a tight-fitting glass homogenizer. The homogenate was centrifuged at $100,000 \times g$ to produce cytosol and membrane fractions. The pelleted membrane fraction was resuspended in Tris-HCl buffer containing 2 mM EGTA (ethylene glycol tetraacetic acid), 0.1% (vol/vol) Triton X-100, and 40 mM β -mercaptoethanol. This suspension was sonicated three times for 30 s each and then stirred for 1 h at 4°C. Solubilized membrane protein was recovered by centrifugation at $100,000 \times g$ for 1 h, and the supernatant was dialyzed overnight against Tris-HCl buffer to remove EGTA.

Purified *L. micdadei* protein kinase was incubated with 10 µl of neutrophil cytosol (11 µg of protein) or solubilized membranes (11 µg) in the presence of [γ -³²P]ATP. ³²P-labeled proteins were resolved by SDS-PAGE with 5 to 20% polyacrylamide gels. Proteins were stained with Coomassie brilliant blue R-250 (Bio-Rad Laboratories, Richmond, Calif.) and press-dried onto Whatman 3MM paper. Radiolabeled proteins were visualized by autoradiography with the aid of an intensifying screen (Cronex Lighting Plus; E. I. du Pont de Nemours and Co., Boston, Mass.). The autoradiogram film was scanned with the aid of a densitometer (Helena Laboratories, Beaumont, Tex.).

Phosphorylation of tubulin. Purified *L. micdadei* kinase was incubated with purified bovine brain tubulin and [γ -³²P]ATP in the same incubation medium used for histones except that 1 mM MnCl₂ was substituted for 10 mM MgCl₂ and 25 µg of tubulin was substituted for the histones. The bovine brain tubulin, prepared by the GTP-dependent assembly-disassembly method (32), was a gift from Richard B. Vallee, Worcester Foundation for Experimental Biology, Shrewsbury, Mass. Evidence for the complete elimination of microtubule-associated proteins (MAPs) from the preparation was obtained by subjecting the tubulin to SDS-PAGE; even when the gel was overloaded (30 to 50 µg of purified tubulin per lane), only a single 50-kilodalton (kDa) band was observed.

Phosphorylation of PI. Purified *L. micdadei* kinase was incubated at pH 7.2 in the presence of [γ -³²P]ATP and PI (from bovine liver; Avanti Polar Lipids, Inc., Birmingham, Ala.) in 50 mM Tris-HCl which contained the following additions in a final volume of 0.1 ml: 10 mM MgCl₂, 50 mM NaF, and 20 µl of enzyme solution (0.05 to 0.1 µg of protein). The reaction was initiated by the addition of ATP and continued for 30 min at 37°C; it was terminated by the addition of 2 ml of chloroform-methanol-11.6 N HCl (200:100:1, vol/vol/vol). Then, 0.5 ml of 1 N HCl was added, and the radiolabeled inositol-containing phospholipids were ex-

FIG. 1. Phosphorylation of neutrophil proteins by the *L. micdadei* kinase. Neutrophil cytosol (A) and solubilized membrane (B) fractions were incubated for 10 min with [γ - 32 P]ATP and purified *L. micdadei* protein kinase. Phosphoproteins were then analyzed by SDS-PAGE and autoradiography, and the X-ray film was scanned with a densitometer. The number at the top of each peak indicates the approximate molecular mass (in kilodaltons).

tracted as described previously (28). Briefly, after the CHCl_3 phase extract was evaporated under a stream of nitrogen and dissolved in chloroform-methanol (2:1, vol/vol) it was applied to a silica gel G plate (Supelco Inc., Bellefonte, Pa.). The experimental samples and phospholipid standards (Sigma Chemical Co., St. Louis, Mo.) were chromatographed side by side with a solvent system consisting of chloroform-methanol-4 N NH_4OH (9:7:2, vol/vol/vol). Thin-layer chromatography (TLC) was performed in the second direction with a solvent system composed of *n*-propanol-4 N NH_4OH (2:1, vol/vol). The ^{32}P -containing phospholipid spots were localized by autoradiography with Kodak XAR-5 film, scraped from the plate, and counted directly in scintillation solution.

Phosphorylation of neutrophil PI. Neutrophil phospholipids were labeled with *myo*-[2- ^3H]inositol for either 2 or 4 h as described previously (28). The labeled neutrophils were then treated with either 50 U of the purified *L. micdadei* kinase or the same quantity of boiled enzyme. The labeled phosphati-

dylinositides were extracted and quantitated as described above.

RESULTS

Purification of the *L. micdadei* kinase. Relative to the bacterial sonicate, the kinase was purified 970-fold, with a yield of 48% (Table 1). The specific activity of the purified kinase, with mixed histones as the phosphate acceptor, was 1.5×10^5 U/mg of protein. When the purified kinase was electrophoresed in SDS-containing gels, a single protein band was detected with a molecular mass of approximately 55,000 Da.

Neutrophil protein substrates. Neutrophil cytosol or solubilized membrane proteins were incubated for 10 min with 62 U of bacterial protein kinase activity, as determined in the standard histone kinase assay, and [γ - ^{32}P]ATP. The ^{32}P -labeled proteins were resolved by SDS-PAGE and visualized by autoradiography. The bacterial enzyme catalyzed the

FIG. 2. Phosphorylation of tubulin by the *L. micdadei* protein kinase (PK). Purified calf brain tubulin was incubated with purified *L. micdadei* protein kinase in the presence of [γ - 32 P]ATP, and the reaction was terminated by rapid freezing. A portion of each sample was subjected to SDS-PAGE and autoradiography to identify radio-labeled proteins. Lanes 1 to 4 show the autoradiograph under the different incubation conditions specified below each lane.

phosphorylation of 11.5-, 13.5-, 25-, and 38-kDa proteins in the cytosol (Fig. 1A) and 11.5-, 14-, 19-, 23-, 28-, 34-, and 38-kDa proteins present in the extract of neutrophil membranes (Fig. 1B).

Phosphorylation of tubulin. As shown in Fig. 2, when purified *L. micdadei* protein kinase was incubated with tubulin and [γ - 32 P]ATP in buffer supplemented with Mn^{2+} or Mg^{2+} , extensive phosphorylation of tubulin occurred. Phosphorylation in the presence of Mn^{2+} was about 10-fold greater than that obtained with Mg^{2+} . Since the tubulin preparation that was used had been stripped of MAPs, the bacterial protein kinase must have catalyzed the phosphorylation of tubulin itself.

Phosphorylation of PI. When the purified *L. micdadei* kinase was incubated with [γ - 32 P]ATP and PI, we observed a linear, time-dependent incorporation of 32 P into a product which was extractable into $CHCl_3$ - CH_3OH and which co-chromatographed precisely with authentic PIP in two different TLC solvent systems. The assay was linear for at least 40 min, and radioactivity was not detected at the position on the thin-layer chromatogram which corresponded to the PIP_2 standard during this time period (data not shown). The phosphorylation of PI was also linearly proportional to enzyme concentration in the range from 0.025 to 0.15 μ g of protein, and no detectable 32 P-labeled lipid product was formed if the kinase was boiled for 5 min before assay (data not shown). As shown in Fig. 3, when the lipid acceptor

FIG. 3. Effect of PI concentration on kinase activity. PI kinase activity was measured at concentrations of PI ranging from 0.1 to 0.8 μ M in 0.1 ml. Incubations were carried out for 30 min, and reactions were terminated by adding $CHCl_3$ - CH_3OH (2:1, vol/vol). The incorporation of radioactivity into PIP was determined as described in Materials and Methods. Inset: Double-reciprocal plot.

TABLE 2. Effects of various compounds on the phosphorylation of PI by *L. micdadei* kinase

Addition to (+) or deletion from (-) complete medium ^a	PI kinase activity ^b (U)
None	17.8
-NaCl	14.4
-NaF	5.7
-MgCl ₂	5.7
-MgCl ₂ , +CaCl ₂	16.7
-MgCl ₂ , +MnCl ₂	11.8
-MgCl ₂ , +ZnCl ₂	4.2
-PI, +PIP (10 μ M)	<0.25

^a Complete incubation medium contained the following: 50 mM NaCl, 10 mM NaF, 10 mM MgCl₂, PI, [γ - 32 P]ATP (20 μ Ci), and 60 U (histone kinase activity) of *L. micdadei* kinase.

^b One unit equals 1 pmol of 32 P transferred to PI per min.

concentration in the assay medium was varied, the maximum rate of 32 P incorporation into the lipid product was obtained when 50 μ g (0.6 mM) of PI was present. The K_m for PI was 0.9 mM (Fig. 3). Under assay conditions which were determined to be optimal for the *L. micdadei* kinase acting on PI or mixed histones, the rate of phosphorylation of 0.6 mM PI was 30 to 40% of that obtained when histones served as the phosphate acceptor. Higher concentrations of PI were inhibitory (Fig. 3), reflecting either true substrate inhibition, an inhibitory detergent effect of lipid on the kinase, or possibly a change in the aggregation state of PI. When PI was replaced with the same concentration of PIP to determine whether the latter could be phosphorylated by the *Legionella* kinase, no detectable phosphorylation occurred (Table 2). The results of a study of ATP dependency of the lipid kinase reaction indicated that the reaction exhibited typical Michaelis-Menten saturation kinetics (Fig. 4); the K_m of the kinase for ATP was 1.5 mM.

Physicochemical properties of the PI kinase. The effect of hydrogen ion concentration on the activity of the bacterial kinase was investigated between pH 5.0 and 8.0. The pH optimum for the phosphorylation of PI was pH 7.0, close to that observed when histone was used as the substrate, namely, pH 7.2 (27) (data not shown).

The ability of various divalent cations (at 10 mM) to

FIG. 4. Effect of ATP concentration on kinase activity. Increasing concentrations of ATP (0.25 to 4 mM) were incorporated in the standard incubation medium, and PI kinase activity was determined as described in Materials and Methods. Inset: Double-reciprocal plot.

TABLE 3. Effect of detergents on histone kinase and PI kinase activities

Addition	PIP synthesis		Histone kinase activity	
	³² P transferred to PI (pmol/min)	Fold increase	³² P incorporated (pmol/min)	Fold increase
None	15.2	1.0	52	1.0
Octyl glucoside				
1 mM	6.8	0.4	56	1.1
10 mM	32.8	2.2	81	1.6
20 mM	32.6	2.1	91	1.8
Triton X-100 (0.5%)	28.2	1.9	48	0.9
Triton X-100 (0.5%) + octyl glucoside (10 mM)	45.0	3.0	56	1.1

support the *L. micdadei* kinase-catalyzed PI phosphorylation reaction was compared (Table 2); Mg^{2+} was best, followed in order by Ca^{2+} and Mn^{2+} .

Inclusion of 0.25 to 0.5% (vol/vol) Triton X-100 or 10 to 20 mM octyl- β -D-glucoside in the assay medium stimulated PI kinase activity twofold. In contrast, 2% Triton X-100 or Cutscum (0.5%) had no effect on PI kinase activity, and sodium deoxytaurocholate, Tween-20, and SDS detergents were all slightly inhibitory. Concentrations of octyl glucoside that were above the detergent's critical micellar concentration (approximately 6 mM) stimulated PI kinase activity more than twofold (Table 3). The same concentrations of octyl glucoside (10 to 20 mM) also nearly doubled the activity of the kinase when histones were used as the substrate. In contrast, while Triton X-100 enhanced the transfer of phosphate from ATP to PI, it did not stimulate phosphorylation of histones (Table 3). The effects of Triton X-100 and octyl glucoside were additive when PI served as the acceptor in the kinase assay (Table 3), further supporting the concept that the two detergents act by different mechanisms.

At 0.6 mM concentration, the acidic lipids phosphatidylglycerol and phosphatidylserine inhibited the PI kinase activity by 68% and 28%, respectively, and the neutral lipids phosphatidylethanolamine and phosphatidylcholine reduced phosphorylation by 67% and 45%, respectively. PIP (0.6 mM) decreased PI phosphorylation by 80%, whereas at the same concentration PIP_2 was only 50% as inhibitory as PIP (data not shown).

Effect of PIP on protein phosphorylation. As shown in Fig. 5, the incorporation of labeled phosphate into tubulin was markedly inhibited by PIP. When the tubulin concentration in the assay medium was held constant at 10 μ g, as little as 0.2 mM PIP inhibited tubulin phosphorylation by greater than 80% (Fig. 5B). For comparison, the inhibition of tubulin phosphorylation by PI, which presumably is the result of substrate competition between PI and tubulin, is shown in Fig. 5A. The inclusion of 0.2 mM PI in the kinase assay along with 10 μ g of tubulin reduced tubulin phosphorylation by approximately 50%.

The nature of the inhibition of the protein kinase activity by PIP was investigated by using an incubation medium in which histone served as the substrate. Histone rather than PI was used as the ^{32}P acceptor in this experiment because the quality of the data obtained with phosphorylated protein products is better than that of the data that result when the product of the PI phosphorylation reaction, PIP, is isolated by TLC. When histone kinase activity was determined at six

FIG. 5. Inhibition of tubulin phosphorylation by PI and PIP. Bovine brain tubulin (10 μ g) was incubated with the purified *L. micdadei* kinase in the presence of $[\gamma\text{-}^{32}P]\text{ATP}$ at 37°C and various concentrations of PI and PIP. The reaction was terminated by rapid freezing. A portion of each sample was subjected to SDS-PAGE and autoradiography to identify radiolabeled proteins, and the X-ray film was scanned with a densitometer. (A) Lane 1, Tubulin; lane 2, tubulin plus 0.2 mM PI; lane 3, tubulin plus 0.6 mM PI; lane 4, tubulin plus 2.0 mM PI. (B) Lane 1, Tubulin; lane 2, tubulin plus 0.2 mM PIP; lane 3, tubulin plus 0.6 mM PIP; lane 4, tubulin plus 2.0 mM PIP.

different concentrations of mixed histones in the absence and presence of 0.6 mM PIP (Fig. 6B), the extent of inhibition of activity was approximately the same at each concentration, suggesting a noncompetitive type of inhibi-

FIG. 6. Effect of PIP on protein kinase activity at various concentrations of histone. Protein kinase activity was measured at concentrations of mixed histones ranging from 0.06 to 0.8 mM, in both the absence (●) and presence (○) of 0.6 mM PIP. The reaction was carried out for 10 min. Histones were precipitated with 10% trichloroacetic acid, and the precipitable radioactivity was determined as described in Materials and Methods.

TABLE 4. Phosphorylation of PI by *L. micdadei* kinase in [³H]inositol-labeled neutrophils

Phospho- inositide	[³ H]inositol (cpm ± SD) in neutrophils labeled for:			
	2 h		4 h	
	Mock treated	Kinase treated	Mock treated	Kinase treated
PI	1,251 ± 9	1,019 ± 22	2,660 ± 50	2,023 ± 1
PIP	259 ± 88	485 ± 47	596 ± 3	1,030 ± 28
PIP ₂	233 ± 30	196 ± 1	208 ± 24	214 ± 9

tion. This impression was confirmed when the data were put in the form of a double-reciprocal plot (Fig. 6A); the two $1/v$ versus $1/[S]$ plots intersected on the x-axis.

Phosphorylation of neutrophil PI. Neutrophils which had been labeled with *myo*-[2-³H]inositol for 2 or 4 h were treated with the purified PI protein kinase for 4 h, and the labeled inositol lipids were then extracted and separated. As shown in Table 4, although the labeling of PI and PIP of the control neutrophils was increased over twofold by 4 h of labeling, the results of treatment with the bacterial enzyme were the same for the two labeling conditions. After a 4-h incubation with the bacterial kinase, 19 to 24% of the PI had been phosphorylated to PIP, resulting in a 73 to 87% increase in the quantity of labeled PIP in the neutrophil plasma membranes. In contrast, the level of PIP₂ did not change in treated neutrophils.

Evidence that PI and protein kinase activities reside in the same protein. When the enzyme from the histone affinity column was subjected to isoelectric focusing and the fractions were analyzed for kinase activity with histones and PI used separately as acceptors (Fig. 7), we found that both activities focused in fraction 23 (pI, ≈5.8).

When the PI kinase activity of the bacterial enzyme was quantitated in the presence of 0.3 mM mixed histones, the activity was only 45% of that obtained in the absence of histones. Likewise, in the presence of 0.5 mM PI, the histone kinase activity of the enzyme was 46% of the control value with only the histone substrate present (data not shown).

DISCUSSION

These results provide some insights about the novel, cyclic AMP-independent kinase of *L. micdadei* which has

FIG. 7. Isoelectric focusing of *L. micdadei* protein kinase and PI kinase activities. Isoelectric focusing was performed with an LKB 8101 focusing column containing a 110-ml sucrose gradient (0 to 50%, wt/vol) and 2% (vol/vol) ampholytes in the pH range 3 to 10. Fractions (2 ml) were collected, analyzed for conductivity (x), and assayed for PI kinase activity (○) and histone kinase activity (●).

the ability to catalyze, at the expense of ATP, the phosphorylation of both a number of neutrophil proteins and PI. Demonstration of phosphorylation of tubulin by a *Legionella* protein kinase is of particular interest because tubulin is the principal component of microtubules, and phosphorylation of tubulin monomers inhibits assembly into microtubules (34, 35). Ultrastructural studies have revealed that granules, vesicles, and phagosomes form intimate linear associations with, and align along, microtubules (25), indicating that microtubules provide the tracking mechanism by which granules come to associate with phagosomes. The inhibition of microtubule assembly by the bacterial kinase might play a role in the inhibition of phagosome-lysosome fusion engendered by the legionellae in phagocytes (10). Although the purified *Legionella* kinase catalyzed extensive phosphorylation of purified calf brain tubulin, ³²P-labeling of a 50-kDa neutrophil protein consistent with tubulin was not detected when the bacterial kinase and [γ -³²P]ATP were incubated with neutrophil membrane and cytosol fractions. However, in the latter studies there may have been neutrophil proteins that are effective phosphate acceptors for the kinase, but which escaped detection either because their potential phosphorylation sites were already occupied by nonlabeled, endogenous phosphate groups or because they were dephosphorylated by phosphatases present in the cytosolic or membrane extract fractions of the neutrophils. It will be of interest to extract the tubulin from neutrophils and monocytes which have been treated with the *Legionella* kinase and determine its extent of phosphorylation. We will also attempt to determine whether the PI-tubulin kinase phosphorylates MAPs in addition to native tubulin. The phosphorylation of MAPs also inhibits the rate and extent of microtubule assembly (14).

While the *L. micdadei* kinase is similar in size (about 55 to 60 kDa) to the eucaryotic PI kinases of murine T lymphocytes (22), porcine liver microsomes (13), and bovine uteri (23), its kinetic properties appear to be quite different. Whereas the K_m s for ATP of the lymphocyte, liver, and uterine kinases were reported to be 140, 60, and 18 μ M, respectively (13, 22, 23), that of the *Legionella* kinase acting on PI is approximately an order of magnitude higher (1.5 mM, Fig. 3). The bacterial kinase also differs from these eucaryotic PI kinases with respect to divalent cation requirements (13, 22, 23) and the effects of detergents and natural membrane lipids (13, 22).

Although a number of phospholipids inhibited the PI kinase activity of the *L. micdadei* enzyme, the most effective inhibitor among the compounds tested was PIP. PIP also inhibited the bacterial kinase-catalyzed phosphorylation of tubulin to the same extent as it inhibited PI phosphorylation. The inhibition of kinase activity by PIP obtained when histones served as the acceptor was noncompetitive in character, suggesting that PIP does not compete with substrates for the active site of the enzyme. Thus, PIP could be a physiological regulator of the *Legionella* kinase. This hypothesis is strengthened by the observation that the protein kinase activity of a similar cyclic nucleotide-independent tubulin kinase obtained from the promastigotes of *Leishmania donovani* (4, 20), which has no PI kinase activity, was not inhibited by PIP (unpublished observations).

Although protein kinases are widely distributed among eucaryotes and procaryotes, there are few precedents for kinases which phosphorylate both lipid and protein substrates. The insulin receptor kinase is an example of such an enzyme. It is capable of autophosphorylation of one of its own subunits (9) and catalyzes phosphorylation of exoge-

nous protein substrates. Sale et al. (29) demonstrated that the purified insulin receptor phosphorylates PI as well as a variety of proteins. The phosphorylation of PI by highly purified insulin receptor preparations has since been confirmed (30). As is true with the *L. micdadei* kinase, the insulin receptor kinase does not catalyze the transfer of ^{32}P from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ to PIP to form PIP_2 (31) but does catalyze the phosphorylation of tubulin (15).

Purified epidermal growth factor (EGF) receptor also possesses tyrosine kinase and PI kinase activities (31). However, when self-phosphorylated EGF receptor was fractionated by gel filtration chromatography, the peak of PI kinase activity was separated from the comigrating peak of protein-tyrosine kinase activity and from the self-phosphorylated EGF receptor (31). These results indicate that the protein and PI kinase activities which copurify with the EGF receptor reside on different proteins (31). Several pieces of evidence, in addition to fact that the *L. micdadei* kinase has been purified to homogeneity, indicate that a single enzyme catalyzes the phosphorylation of PI and the proteins tubulin and histones. First, the ratio of PI kinase to histone kinase activity was relatively constant throughout the purification scheme (Table 1). Second, when the enzyme from the histone affinity column was subjected to isoelectric focusing, both PI and histone kinase activities focused in the same fraction (Fig. 7). Third, when PI and histone were mixed in the same assay, they acted as competitive inhibitors of each other, with histone inhibiting PI kinase activity and PI inhibiting histone kinase activity. Fourth, PIP inhibited the kinase-catalyzed phosphorylation of both PI and tubulin to the same degree. It remains to be determined whether the *Legionella* enzyme self-phosphorylates and, if so, whether the PI kinase activity can be separated from the protein kinase activity and the self-phosphorylated enzyme.

Regardless of whether the PI and protein kinase activities reside in a single molecule, the PI kinase activity of a *Legionella* enzyme might adversely affect host phagocyte antimicrobial functions by a number of mechanisms. When the PI in the neutrophil plasma membrane is phosphorylated by exogenously added bacterial kinase, the result is a decreased content of PI and increased content of PIP in the cell membrane (Table 4). The lower PI content of the plasma membrane might result in decreased arachidonate formation following neutrophil stimulation, because less PI would be available as a substrate for phospholipase A_2 . The increased PIP content could interfere with the activation sequence mediated by the receptor-activated hydrolysis of PIP_2 by the polyphosphoinositide-specific phosphodiesterase (phospholipase C). Phospholipase C appears to prefer PIP_2 to PIP (5), but an increased PIP concentration could result in less PIP_2 being hydrolyzed following neutrophil stimulation through substrate competition. Whereas phosphorylytic cleavage of PIP_2 generates IP_3 , which is active in Ca^{2+} mobilization, in addition to diacylglycerol, hydrolysis of PIP generates IP_2 , which is not biologically active (24).

The function and significance of the *Legionella* kinase are obscure. Although the enzyme is capable of phosphorylating both PI and tubulin in vitro and PI in the plasma membrane of intact neutrophils, the physiologically relevant substrates remain to be identified. Although unlikely, the possibilities that the *Legionella* kinase modulates bacterial phosphorylation reactions and that the demonstration of neutrophil substrates is entirely fortuitous remain to be rigorously excluded. PI is rare in procaryotic cells; it has been found in only the mycobacteria (33) and myxobacteria (5), and was not detected in *Legionella* spp. (6). Although it was reported

that microtubular structures in certain spirochetes and gliding bacteria were composed of tubulin (19), there is no generally accepted, published evidence that procaryotes contain tubulin. However, it remains to be shown that a tubulinlike (G. M. W. Adams, J. Cell Biol. 97:209A, abstr. 794, 1983) or other bacterial protein is not the natural substrate for the *Legionella* kinase.

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