The actin-activated Mg\(^{2+}\)-ATPase and in vitro motility activities of the three Acanthamoeba myosin I isozymes depend upon phosphorylation of their single heavy chains by myosin I heavy chain kinase. Previously, the kinase had been shown to be activated by autophosphorylation, which is enhanced by acidic phospholipids, or simply by binding to purified plasma membranes in the absence of significant autophosphorylation. In this paper, we show that the rate of phosphorylation of myosin I by unphosphorylated kinase is ~20-fold faster when both the myosin I and the kinase are bound to acidic phospholipid vesicles than when both are soluble. This activation is not due to an increase in the local concentrations of vesicle-bound kinase and myosin I. Thus, acidic phospholipids, like membranes, can activate myosin I heavy chain kinase in the absence of significant autophosphorylation, i.e. membrane proteins are not required. Kinetic studies show that both binding of kinase to phospholipid vesicles and autophosphorylation of kinase in the absence of phospholipid increase the \( V_{\text{max}} \) relative to soluble, unphosphorylated kinase with either an increase in the apparent \( K_m \) (when myosin I is the substrate) or no significant change in \( K_m \) (when a synthetic peptide is the substrate). Kinetic data showed that autophosphorylation of phospholipid-bound kinase is both intermolecular and intervesicular, and that phosphorylation of phospholipid-bound myosin I by phospholipid-bound kinase is also intervesicular even when the kinase and myosin are bound to the same vesicles. The relevance of these results to the activation of myosin I heavy chain kinase and phosphorylation of myosin I isozymes in situ are discussed.

Each of the three isoforms of Acanthamoeba myosin I has a single ~110–140-kDa heavy chain with an ~80-kDa N-terminal domain, that is highly similar in sequence to the subfragment 1 domain of conventional myosin II, and a ~50-kDa nonfilamentous C-terminal domain that has no sequence similarity to the C-terminal domain of conventional myosin (1, 2; for reviews, see Refs. 3–5). The N-terminal head domain contains an ATP-binding site (6) and an ATP-sensitive, F-actin-binding site (7, 8), as do all myosins. The C-terminal tail domain contains an ATP-insensitive, F-actin-binding site (8–10) and a membrane (and acidic phospholipid)-binding site (10, 11) which, thus far, appear to be unique to the myosin I family. Actin-activated Mg\(^{2+}\)-ATPase activity is expressed in vitro only when a single serine (myosin IB and IC) or threonine (myosin IA), situated between the ATP- and actin-binding sites in the globular head (6), is phosphorylated (12, 13). The biological importance of phosphorylation of the myosin I heavy chain is evidenced by the observations (14) that ~80% of myosin IA and ~20% of myosin IB and IC are phosphorylated in situ, that the fraction of phosphorylated myosin IC associated with the contractile vacuole in situ varies with the stage of the contractile vacuole cycle (14), and that antibodies that specifically inhibit phosphorylation of myosin IC (the only myosin associated with the contractile vacuole (3)) inhibit contractile vacuole activity of living cells (15).

Acanthamoeba myosin I heavy chain kinase (16) is a single ~97-kDa polypeptide (17) which is ~50-fold activated by autophosphorylation of up to 8–10 sites (18). The rate of kinase autophosphorylation is ~20-fold greater in the presence of acidic phospholipids (18). As determined by immunoelectron microscopy, ~30% of myosin I heavy chain kinase is associated with the plasma membrane in situ (19) and both the kinase and its substrate, myosin I, bind to plasma membranes in vitro (19, 20). The rate of autophosphorylation of plasma membrane-bound kinase is only slightly greater (~2- to 5-fold) than the rate of autophosphorylation of soluble kinase, but the membrane-bound kinase is much more active than expected from its level of phosphorylation (21). Thus, before the present work, there seemed to be two different ways to activate myosin I heavy chain kinase: (i) autophosphorylation, which is stimulated by acidic phospholipids, and (ii) association of kinase with plasma membranes independent of autophosphorylation.

The experiments described in this paper were initiated to determine whether autophosphorylation-independent activation of myosin I heavy chain kinase was, as it appeared to be from the earlier experiments, unique to membranes and, if so, to attempt to identify the membrane components responsible for this activity. Initial experiments showed, however, that activation of kinase by acidic phospholipids and plasma membranes were, contrary to previous indications, qualitatively similar. This had not been observed in the earlier experiments (21) because phospholipid-bound kinase is autophosphorylated much more rapidly than membrane-bound kinase making it experimentally more difficult to show that phospholipid-bound kinase is activated before it is significantly autophosphorylated. With appropriate conditions defined, it has been possible to characterize more fully the phospholipid-enhanced autophosphorylation-dependent activation of kinase, the phospholipid-dependent autophosphorylation-independent activation of kinase, and phosphorylation of phospholipid-bound myosin I by phospholipid-bound phosphorylated and nonphosphorylated kinase. The results of these experiments are reported in the present paper and their potential relevance to myosin I-related activities in vivo is discussed.

MATERIALS AND METHODS

Purification of Myosins I and Myosin I Heavy Chain Kinase—Unphosphorylated myosin I heavy chain kinase and myosin IA, IB, and IC were purified from Acanthamoeba castellanii as described previously.
The abbreviation used is: PS, phosphatidylserine.

RESULTS

Binding of Kinase to Acidic Phospholipid Vesicles—Kinase bound very poorly to vesicles that did not contain PS (Fig. 1A),

![Graph A](image1)

![Graph B](image2)

![Graph C](image3)

Fig. 1. Interactions of unphosphorylated myosin I heavy chain kinase with phospholipid vesicles. A, effect of phosphatidylserine (PS) content on binding: kinase (72 nM) was mixed at room temperature with phospholipid vesicles (0.24 mM total lipid) containing increasing percentages of PS in 20 mM imidazole, pH 7.0, containing 1 mM EGTA, 2 mM MgCl₂, and 1 mg/ml bovine serum albumin. The mixtures were centrifuged at 200,000 × g for 30 min, and the amount of kinase in the pellet was determined by quantitative densitometry of SDS-PAGE gels stained with Coomassie Blue. B, conditions for total binding: kinase (172 nM) was mixed at room temperature with phospholipid vesicles (0.24 mM total lipid) containing 30% PS in 20 mM imidazole, pH 7.0, containing 1 mM EGTA, 2 mM MgCl₂, and 1 mg/ml bovine serum albumin. The mixtures were centrifuged at 200,000 × g for 30 min at 20 °C, and the amount of kinase in the pellet (P) and supernatant (S) was quantified by Coomassie Blue-stained SDS-PAGE gels. To sediment all of the vesicles, as determined by analysis of total phosphorus, it was necessary that the buffer contain 2 mM Mg²⁺. Lanes 1S and 1P, 7.5% PS; lanes 2S and 2P, 15% PS; lanes 3S and 3P, 30% PS; lanes 4S and 4P, 90% PS; lanes 5S and 5P, no PS. C, irreversibility of binding: 100 mM kinase was incubated with phospholipid vesicles (2.5 mM total lipid containing varying percentages of PS) in 20 mM imidazole, pH 7.0, containing 1 mM EGTA, 2 mM MgCl₂, and 1 mg/ml bovine serum albumin. The mixtures were centrifuged at 200,000 × g for 30 min at 20 °C, and the amount of kinase in the pellet (P) and supernatant (S) was quantified by Coomassie Blue-stained SDS-PAGE gels. To sediment all of the vesicles, as determined by analysis of total phosphorus, it was necessary that the buffer contain 2 mM Mg²⁺. Lanes 1S and 1P, 7.5% PS; lanes 2S and 2P, 15% PS; lanes 3S and 3P, 30% PS; lanes 4S and 4P, 90% PS; lanes 5S and 5P, no PS. C, irreversibility of binding: 100 mM kinase was incubated with phospholipid vesicles (2.0 mM total lipid, 30% PS) in a total volume of 250 μl of 20 mM imidazole, pH 7.0, containing 1 mM EGTA, 2 mM MgCl₂, and 1 mg/ml bovine serum albumin, and an aliquot of 50 μl was removed. The remaining vesicles were pelleted, the supernatant was removed, and the pellet was resuspended in 200 μl of fresh buffer from which a 50-μl aliquot was removed. The vesicles in the remaining suspension were pelleted, the supernatant was removed, and the pellet was resuspended in 150 μl of fresh buffer. Identical aliquots (50 μl) of the original suspension, the first and second supernatants, and the resuspended first and second pellets were incubated with [γ-³²P]ATP in autophosphorylation buffer in the presence of 0.2 mM 30% PS vesicles. The entire sample was then subjected to SDS-PAGE, and the gels were stained by Coomassie blue (upper panel) and quantified by densitometry of autoradiograms (lower panel). Lane 1, original suspension; lane 2, first supernatant; lane 3, first resuspended pellet; lane 4, second supernatant; lane 5, second resuspended pellet. Parallel control experiments showed that under these conditions identical, maximal autophosphorylation (9 mol/mol) was obtained for amounts of kinase between 0.17 and 8.5 pmol and that the densitometric values were directly proportional to the amount of radioactivity. Therefore, the densitometric values were a direct measure of the amount of kinase in each sample (see "Results").

![Graph D](image4)

Fig. 2. Autophosphorylation of phospholipid-bound myosin I heavy chain kinase as a function of phosphatidylserine (PS) concentration. Kinase (255 nM) was added to phospholipid vesicles (2.5 mM total lipid) containing 7.5%, 15%, 30%, or 90% PS under conditions in which all of the kinase bound to the vesicles. The suspensions were diluted approximately 9-fold (27 nM kinase) in autophosphorylation buffer and incubated at 30 °C for 0.5 min with [γ-³²P]ATP. The extent of autophosphorylation was quantified by SDS-PAGE and autoradiography. The open circle is a control experiment with soluble kinase in the absence of phospholipid. Phospholipid vesicles containing no PS could not be used as a control because kinase binds very poorly to vesicles in the absence of PS (see Fig. 1).
Under these conditions (Fig. 1), the rate of autophosphorylation of soluble kinase incubated for 1 min at 23°C (filled circles) and of kinase bound to phospholipid vesicles (30% PS) incubated at 10°C for 30 s (open triangles) or 15°C for 20 s (open circles) are plotted as functions of kinase concentration. The rate of autophosphorylation of soluble kinase was constant for at least 2 min and of phospholipid-bound kinase for at least 1 min. For the experiment with phospholipid-bound kinase, kinase (153 nM) was added to phospholipid vesicles (2 mM total lipid, 30% PS) under conditions in which all of the kinase was bound to the vesicles. Aliquots were then diluted to the indicated concentrations of vesicle-bound kinase with either buffer only (open triangles) or buffer containing kinase-free vesicles to maintain the same phospholipid vesicle concentration (0.29 mM total lipid) in all samples. B, the same data are replotted as the log of initial velocity versus the log of kinase concentration. The observed slopes of 2 for both soluble and phospholipid-bound kinase indicate that autophosphorylation is both intermolecular and intervesicular (see text).

and the binding efficiency increased with increased concentration of PS up to 30% which was sufficient for maximal binding under these conditions (Fig. 1A). With a higher ratio of vesicles to kinase, all of the kinase bound to vesicles that contained as little as 7.5% PS (Fig. 1B). The kinase did not dissociate when the vesicles were pelleted and resuspended twice in the same volume of fresh buffer (Fig. 1C). In this experiment, too little protein dissociated from the vesicles to be detected by Coomassie Blue (Fig. 1C, upper panel) so the amounts of free and bound kinase were determined by quantitative autoradiography after labeling all samples equally with 32P (Fig. 1C, lower panel; see legend for methodological details). The first supernatant (lane 2) contained 2.6% of the total kinase in the original suspension (lane 1), the first resuspended pellet (lane 3) contained 92% of which 93% was recovered in the second resuspended pellet (lane 5). No kinase was detected in the second supernatant (lane 4); as little as 1% of the original amount would have been readily detected. Thus, either binding was essentially irreversible under these conditions or the rate of dissociation of kinase was very slow relative to the time of the experiment. In all of the remaining experiments described in this paper, the concentration of phospholipid vesicles was sufficient to bind all of the kinase (and also all of the myosin I, as determined by similar assays).

Autophosphorylation of Phospholipid-bound Kinase—The initial rates of autophosphorylation were higher for phospholipid-bound kinase than for soluble kinase and increased with the proportion of PS in the vesicles (Fig. 2); under these assay conditions, the rate was about 12-fold faster with vesicles containing 7.5% PS than for soluble kinase, about 20-fold faster with vesicles containing 30% PS, and about 24-fold faster with vesicles containing 90% PS (about the same enhancement as previously found for 100% PS vesicles (18)). The final extent of autophosphorylation, however, was essentially the same in the presence and absence of phospholipid, —9 mol/mol, as previously observed (18).

The rates of autophosphorylation of soluble kinase, i.e. in the absence of phospholipid (Fig. 3A, filled circles), and of kinase bound to 30% PS vesicles (Fig. 3A, open triangles) were both directly proportional to the kinase concentration. These results indicate that autophosphorylation of both soluble and phospholipid-bound kinase has a substantial intermolecular component. Under both conditions, a slope of 2 was obtained for the plot of the log of the initial velocity of autophosphorylation versus the log of the kinase concentration (Fig. 3B, filled circles and open triangles) as expected for an intermolecular reaction (27); the slope is 1 for intramolecular reactions (27). Moreover, as the different concentrations of phospholipid-bound kinase...
were obtained by dilution of aliquots of a single batch of vesicles containing bound kinase and, as we have shown, the kinase does not dissociate from the vesicles, autophosphorylation must be intervesicular as well as intermolecular because the kinase concentration within each vesicle would not have been affected by dilution of the vesicles (see "Discussion"). Identical results were obtained when the vesicles with bound kinase were diluted with buffer that contained kinase-free vesicles to maintain a constant concentration of phospholipid (Fig. 3, A and B, open circles).

Phosphorylation of Myosin I by Phospholipid-bound Kinase—The initial rate of phosphorylation of myosin I was substantially greater when both the kinase and myosin I were bound to phospholipid vesicles than when both were in solution in the absence of phospholipid (Fig. 4). Although the initial rate of autophosphorylation of phospholipid-bound kinase was about 85% inhibited by the presence of myosin I (compare the rates in Fig. 4 to the rate for 30% PS vesicles in Fig. 2), autophosphorylation of phospholipid-bound kinase was still about 3–10-fold faster than of soluble kinase (Fig. 4).

It seemed likely that the increase in myosin I phosphorylation in the presence of phospholipids was due entirely to the effect of phospholipid on the kinase because soluble and phospholipid-bound myosin I are equally good substrates for soluble, phosphorylated kinase (21) which does not bind to phospholipids (19). In order to determine if the enhanced rate of phosphorylation of phospholipid-bound myosin I by phospholipid-bound kinase were due simply to the faster rate of autophosphorylation of phospholipid-bound kinase, the activities of phospholipid-bound and soluble kinase were measured as a function of the extent of kinase phosphorylation. These experiments were possible because kinase that is bound to phospholipid vesicles when unphosphorylated remains bound after it is autophosphorylated (19 and data not shown). Parallel experiments were carried out with each of the three myosin isozymes as substrate (Fig. 5). The activity of soluble kinase was approximately 100-fold lower than the activity of bound kinase.

**TABLE I**

Comparison of phosphorylation of phospholipid-bound myosin I B by kinase bound to the same and different phospholipid vesicles.

To bind kinase and myosin to the same vesicles, phospholipid vesicles (2.0 mM, 30% PS) were mixed with 177 nM kinase in 20 mM imidazole, pH 7.0, 0.1 mg/ml bovine serum albumin, 2 mM MgCl₂, and 1 mM EGTA at 4 °C and subsequently diluted to half the concentration and incubated for 3 min at 4 °C with 7.33 μM myosin IB. Under these conditions, all vesicles contain both kinase and myosin. Aliquots of the suspension of vesicles were diluted in phosphorylation medium to the concentrations shown, then incubated at 30 °C for 25 s, and myosin phosphorylation was measured as described under "Materials and Methods." In a parallel experiment, 177 nM kinase was mixed with phospholipid vesicles (2 mM, 30% PS) as above, and 9.02 μM myosin IB was mixed separately with phospholipid vesicles (0.77 mM, 30% PS). The two vesicle suspensions were mixed and diluted into phosphorylation medium, and myosin phosphorylation was determined as described.

| Concentration | Myosin phosphorylation |
|---------------|------------------------|
| Kinase        | Myosin IB              |
| nM | μM | mol/mol | mol/mol |
| 28 | 2.3 | 0.18 | 0.17 |
| 19 | 1.5 | 0.12 | 0.13 |
| 14 | 1.2 | 0.09 | 0.10 |

| Concentration | Myosin phosphorylation |
|---------------|------------------------|
| Kinase        | Myosin IB              |
| nM | μM | mol/mol | mol/mol |
| 28 | 2.3 | 0.18 | 0.17 |
| 19 | 1.5 | 0.12 | 0.13 |
| 14 | 1.2 | 0.09 | 0.10 |

* Same vesicle and mixed vesicles refer to experiments in which the kinase and myosin I were bound to the same vesicles or to different vesicles that were then mixed.

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**Fig. 5.** Relationship between the phosphorylation of myosin I and the level of phosphorylation of soluble (open circles) and phospholipid-bound (filled circles) myosin I heavy chain kinase. Soluble kinase (50 nM) or phospholipid-bound kinase (50 nM kinase, 1.7 mM total lipid, 30% PS) were allowed to autophosphorylate for different periods of time, and aliquots were then diluted to 6.9 nM kinase (for incubation with myosin IA and IB) or 7.3 nM kinase (for incubation with myosin IC) in phosphorylation medium containing 0.64 μM myosin I. The reactions were stopped after 0.5 min, and the extent of autophosphorylation of kinase and phosphorylation of myosin I was determined. A, substrate, myosin IA; B, substrate, myosin IB; C, substrate, myosin IC.
Phospholipid-bound Myosin I Heavy Chain Kinase

To determine if the phosphorylation of phospholipid-bound myosin I by phospholipid-bound kinase was predominantly intravesicular or, like kinase autophosphorylation, intervesicular, the phosphorylation of myosin I by kinase bound to the same and to different phospholipid vesicles was compared at three different vesicle concentrations (Table I). To ensure that all of the vesicles contained bound kinase as well as bound myosin I (see "Discussion"), the kinase/phospholipid ratio in this experiment was about 5 times that used in the experiment described in Fig. 4; by SDS-PAGE analysis, all of the kinase and myosin I was bound to the phospholipid vesicles (data not shown). The rates of myosin I phosphorylation were concentration-dependent and essentially identical irrespective of whether the kinase and myosin were bound to the same phospholipid vesicles or to different phospholipid vesicles (Table I). If significant intravesicular phosphorylation occurred, vesicle dilution would not affect the rate or extent of phosphorylation when the myosin I and kinase were bound to the same vesicles. The fact that kinase autophosphorylation is intervesicular and, therefore, dependent on the vesicle concentration, does not complicate the interpretation of these experiments because the rate of phosphorylation of myosin IB by vesicle-bound kinase is the same for unphosphorylated and phosphorylated kinase (Fig. 5B). Thus, the data in Table I strongly suggest that phosphorylation of myosin I is predominantly intervesicular even when the kinase and myosin are bound to the same vesicles, i.e. independent of the relative kinase and myosin concentrations within the same vesicle. These results are consistent with data obtained previously for the phosphorylation of membrane-bound myosin I by membrane-bound kinase (21).

These results also provide additional evidence that the differences between soluble and phospholipid-bound systems described in Figs. 4 and 5 on were not due to locally high concentrations of kinase and myosin I on the same vesicles. The substrate concentration dependence of the activities of soluble unphosphorylated and highly phosphorylated kinase (9 mol/mol) and phospholipid-bound unphosphorylated kinase were determined with myosin IA, myosin IB, and the synthetic peptide PC9 as substrates (Fig. 6, Table II). Similar results were obtained for myosin IA and myosin IB: the $V_{\text{max}}$ of soluble, maximally phosphorylated kinase was 100–200-fold higher, and the $V_{\text{max}}$ of phospholipid-bound unphosphorylated kinase was 10–20-fold higher than the $V_{\text{max}}$ of soluble, unphosphorylated kinase.

**Fig. 6. Double reciprocal plots of the rates of phosphorylation of myosin I and PC9 by soluble unphosphorylated and phosphorylated myosin I heavy chain kinase and phospholipid-bound unphosphorylated myosin I heavy chain kinase.** Soluble unphosphorylated kinase (filled circles), soluble kinase prephosphorylated to 3.1 mol of phosphate/mol of kinase (open triangles) or unphosphorylated kinase bound to phospholipid vesicles (open circles) were incubated with myosin IA (A) or myosin IB (B) for 0.5 min or PC9 (C) for 1 min at the indicated concentrations, and the extent of phosphorylation of myosin I, PC9, and phospholipid-bound kinase was determined. With myosin IA or IB as substrate, kinase (43 nM) was bound to phospholipid vesicles (2.5 mM total lipid, 30% PS) and diluted to 5 nM kinase in the phosphorylation buffer; soluble unphosphorylated and phosphorylated kinases were also 5 nM. With PC9 as substrate, kinase (69 nM) was added to phospholipid vesicles (2.1 mM total lipid, 30% PS) and diluted to 13.8 nM kinase in the assay mixture; soluble unphosphorylated kinase was 55 nM, and soluble phosphorylated kinase was 6.9 nM. All reactions were linear for the period of incubation. The concentrations of myosin I in the presence and absence of phospholipids cannot be compared directly because, in the presence of phospholipid, none of the myosin was in solution. PC9 does not bind to phospholipid vesicles.
The kinase activity resulting from autophosphorylation of soluble kinase in the absence of phospholipid vesicles is also due entirely to an increase in $V_{\text{max}}$, again with either an increase (for myosin I) or no significant change (for PC9) in $K_m$. The concentration dependence of the rates of autophosphorylation of soluble and vesicle-bound kinase reported in this paper indicates that both reactions are substantially intermolecular.

That, contrary to our original supposition (21), membrane proteins are not required for autophosphorylation-independent activation of kinase by phospholipids, does not mean that there is no regulatory role for membrane proteins in the myosin I cascade. Membrane proteins are probably involved in the specificity of binding of both kinase and the myosin I isoforms to different membranes in situ and in vitro (19, 28, 29). Moreover, phospholipid vesicles have a higher capacity than isolated plasma membranes for both kinase and myosin I, and autophosphorylation of phospholipid-bound kinase is more rapid and more extensive than autophosphorylation of membrane-bound kinase (18, 19, 21). Addition of purified membrane proteins to phospholipid vesicles reduces their binding capacity for myosin I to that of purified plasma membranes.

About 70% of the phospholipid vesicles used in the experiments reported in this paper had diameters between 20 and 70 nm; the other 30% had diameters between 70 and 180 nm. Because of the much greater surface area of the larger vesicles, only ~16% of the kinase and myosin I would have been bound to vesicles of less than 70 nm diameter, on the assumption that both proteins bind to vesicles in proportion to the number of

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**DISCUSSION**

This laboratory had previously shown that myosin I heavy chain kinase is activated in solution by autophosphorylation (18), that the rate of autophosphorylation is greatly enhanced by acidic phospholipids (18) and, to a lesser extent, by isolated plasma membranes (21), and that the ability of the kinase to phosphorylate myosin I is also activated by binding the kinase to purified plasma membranes even in the absence of significant autophosphorylation (21). The data in the present paper show that phosphorylation of myosin I by myosin I heavy chain kinase is also activated, independent of autophosphorylation, when kinase is bound to acidic phospholipids; thus, autophosphorylation-independent activation does not, as previously speculated, require plasma membrane proteins. This activation is the result of an increase in $V_{\text{max}}$ with either an increase in apparent $K_m$ (when myosin I is the substrate) or no significant change in $K_m$ (when PC9 is the substrate). The increase in kinase activity resulting from autophosphorylation of soluble kinase in the absence of phospholipid vesicles is also due entirely to an increase in $V_{\text{max}}$, again with either an increase (for myosin I) or no significant change (for PC9) in $K_m$. The concentration dependence of the rates of autophosphorylation of soluble and vesicle-bound kinase reported in this paper indicates that both reactions are substantially intermolecular.

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**The substrate concentrations were calculated as if the substrates were in solution in the aqueous medium. This is correct in the absence of phospholipids, but in the presence of phospholipid all of the myosin I (but not PC9) and kinase were bound to the phospholipid vesicles. In these experiments, the relevant substrate concentrations would be the density of myosin on the vesicle surface and the vesicle concentration (see "Discussion"). This should not affect comparisons between the substrates under identical conditions, but the concentrations of phospholipid-bound myosins cannot be compared meaningfully to the concentrations of soluble myosins and PC9.**

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**FIG. 4.** Effect of myosin I concentration on the phosphorylation of myosin I and autophosphorylation of myosin I heavy chain kinase. Kinase (265 nM) bound to phospholipid vesicles (2.1 mM total lipid, 30% PS) was diluted to 27 nM in phosphorylation buffer containing the indicated concentrations of myosin IB and then incubated at 30°C for 45 s, and the extent of autophosphorylation of myosin I and kinase was determined.

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**TABLE II**

| Kinase | $V_{\text{max}}$ | $K_m$ |
|--------|-----------------|-------|
|        | IA  | IB  | PC9 | IA  | IB  | PC9 |
| Soluble |      |     |     |      |     |     |
| Unphosphorylated | 0.03 | 0.06 | 1.5 | 0.3 | 0.2 | 110 |
| Phosphorylated | 3.3 | 7.8 | 17.2 | 2.3 | 5.5 | 64 |
| Phospholipid-bound, unphosphorylated | 0.75 | 0.64 | 11.5 | 0.7 | 0.5 | 64 |

$^a$ The substrate concentrations were calculated as if the substrates were in solution in the aqueous medium. This is correct in the absence of phospholipids, but in the presence of phospholipid all of the myosin I (but not PC9) and kinase were bound to the phospholipid vesicles. In these experiments, the relevant substrate concentrations would be the density of myosin on the vesicle surface and the vesicle concentration (see "Discussion"). This should not affect comparisons between the substrates under identical conditions, but the concentrations of phospholipid-bound myosins cannot be compared meaningfully to the concentrations of soluble myosins and PC9.
phospholipid molecules on the vesicle surface. At the concentrations of kinase and phospholipid used in most of the experiments reported in this paper, the ratio of bound kinase molecules to single-bilayer vesicles with diameters of 20 nm, 40 nm, 60 nm, 80 nm, and 120 nm would have been 1:20, 1:15, 1:2, 1:1, and 4:1, respectively, calculated on the basis of 1650 phospholipid molecules on the surface of a 20-nm diameter vesicle (30) (the kinase to vesicle ratio would be substantially greater for multi-bilayer vesicles). At the apparent $K_m$, for vesicle-bound myosin I, there would have been about 100 times more vesicle-bound myosin I than vesicle-bound kinase (0.6 $\mu$M myosin I and 5 nM kinase, Fig. 6 and Table I). In those experiments, about 84% of the phospholipid-bound kinase and myosin I would have been associated with vesicles that contained 1 to 4 kinase molecules and 100 to 400 myosin I molecules. Therefore, it was theoretically possible for both intermolecular kinase autophosphorylation and myosin phosphorylation by kinase to have occurred mostly within the same vesicle. However, autophosphorylation of vesicle-bound kinase decreased upon dilution of the vesicles as expected if autophosphorylation occurred between kinase molecules on different vesicles, i.e. kinase autophosphorylation was intervesicular as well as intermolecular. Similarly, the observation that myosin I was phosphorylated at the same rate when kinase and myosin were bound to different vesicles as when kinase and myosin were bound to the same vesicles strongly indicates that myosin phosphorylation was also predominantly intervesicular (in that experiment, by calculation, 98% of the kinase was bound to vesicles containing 1 to 20 kinase molecules and vesicles with bound myosin I contained no less than 20 myosin molecules). Since both reactions are predominantly, if not exclusively, intervesicular, the enhancement of autophosphorylation of kinase and kinase-catalyzed phosphorylation of myosin I when the proteins are bound to phospholipid vesicles cannot be due to an increase in their local concentrations on the vesicles.

The observations that both kinase autophosphorylation and myosin phosphorylation are activated by binding of kinase to phospholipids and that the two reactions are competitive suggest that both reactions are regulated by the same mechanism. Previous experiments established that the N-terminal (7-kDa) region of the kinase is required for binding of kinase to membranes and acidic phospholipid vesicles (22), and that an ~54-kDa peptide fragment derived from the C-terminal region of the kinase is more active catalytically than the native enzyme (for both kinase autophosphorylation and myosin I phosphorylation). Also, the C-terminal kinase fragment neither binds to nor is activated by either plasma membranes or acidic phospholipid vesicles (31). Thus, it seems likely that the catalytic domain in the C-terminal half of the native kinase is either in a low activity conformation or inaccessible to substrate (either myosin I or another kinase molecule) unless the kinase is either sufficiently phosphorylated or its N-terminal region is bound to membranes or acidic phospholipid vesicles. This is consistent with our earlier data which showed that phosphorylation and binding to phospholipid vesicles produced similar changes in the accessibility of the catalytic domain of the kinase to trypsin (33). The very much higher $K_m$ and $I_{so}$ for PC9 than for the native substrates also suggests that the interactions between the kinase and its substrates involve more than just the primary sequence around the phosphorylation site (the sequence of PC9 is the same as the phosphorylation site of myosin IC and similar to the sequences of the phosphorylation sites of myosins IA and IB). A similar suggestion has been made recently for smooth muscle myosin light chain kinase (32). These, and many other aspects of the regulation of myosin I heavy chain kinase activity remain unclear. Particularly intriguing questions concern the localization and role of each of the multiple autophosphorylation sites. It is of some interest to compare the data in this paper to the situation in situ. About 70% of the myosin I kinase of Acanthamoeba plasma membranes (19). From the kinetic data for soluble kinase presented in this paper, the activity of the cytoplasmic kinase would be expected to vary with the extent to which it is phosphorylated which, presumably, would be a function of the relative rates of its autophosphorylation and dephosphorylation. Approximately 90% of the total myosin IA is cytoplasmic (29), at an estimated concentration of ~0.1 $\mu$M if it were uniformly distributed. However, myosin IA appears to be localized to the subplasma membrane, actin-rich cortex, the local concentration of myosin IA might be closer to the $K_m$ of 2 $\mu$M reported in this paper for soluble phosphorylated kinase. Cytoplasmic myosin IA is mostly phosphorylated (14), which indicates that, in situ, the rate of phosphorylation of cytoplasmic myosin IA by myosin I heavy chain kinase exceeds the rate of dephosphorylation of myosin I by a cytoplasmic phosphatase.

About 30% of myosin I heavy chain kinase and 40% of myosin IB and IC are associated with the Acanthamoeba plasma membrane in situ (29). From the data in this and a previous paper, the membrane-associated kinase would be expected to be in a relatively activated state, whether phosphorylated or not. There are about 100 molecules of myosin IB and myosin IC per $\mu$m² of plasma membrane (29), assuming uniform distribution within the membrane. This is similar to but less than the estimated concentration of myosin molecules on the surface of the phospholipid vesicles (500 molecules/$\mu$m², at the apparent $K_m$ of 0.5 $\mu$M for phospholipid-bound unphosphorylated kinase. However, if membrane-associated kinase in situ behaves similarly to phospholipid-bound kinase in vitro, membrane-associated myosin I would be a poor substrate for membrane-associated kinase, because phosphorylation of phospholipid-bound myosin by phospholipid-bound kinase is largely intervesicular. By strict analogy, kinase would not be able to phosphorylate myosin I within the same plasma membrane. Therefore, the present results leave uncertain the mechanism by which ~20% of plasma membrane-associated myosin I remains phosphorylated at steady state in situ (14). One possibility is that membrane-associated myosin I is phosphorylated in situ by cytoplasmic myosin I heavy chain kinase, which is enriched in the subplasma membrane cortex (19), and accounts for about 70% of the total kinase in the cell (19). This is consistent with our earlier results which showed that even though soluble autophosphorylated kinase does not bind to membranes in vitro (19), it can phosphorylate membrane-bound myosin I (21). Alternatively, membrane-associated kinase and myosin I may interact differently in situ than when bound to phospholipids in vitro. Also, there may be specific situations in which two membrane surfaces are sufficiently closely apposed to allow phosphorylation of membrane-bound myosin by membrane-bound kinase, e.g. during endocytosis.

Many questions remain. For example, is myosin I heavy chain kinase activated in situ by both of the mechanisms that operate in vitro: association with membranes and autophosphorylation? What are the concentrations in situ of cytoplasmic and membrane-bound phosphokinase? These questions might be answered by immunoelectron microscopy utilizing antibodies specific for phosphokinase, as described elsewhere for quantifying phosphorysiton I in several cellular compartments (14), but such experiments require information on the sequences and roles of the multiple autophosphorylation sites that is not yet available.
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Zhen Yuan Wang, Hanna Brzeska, Ivan C. Baines and Edward D. Korn

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