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Association of a cellular myosin II with anionic phospholipids and the neuronal plasma membrane

(brain myosin/lipid binding/myosin immunolocalization/neuroblastoma cells)

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ABSTRACT **Myosin II has been observed in close proximity to the neuronal plasma membrane, suggesting the possibility that at least one isoform of neuronal myosin II may be capable of direct association. Here, we demonstrate that a significant fraction (>30%, saturable around 90%) of brain myosin II, but not myosins from skeletal or cardiac muscle, can bind to lipid vesicles composed of the anionic phospholipid L-α-phosphatidyl-L-serine but not with vesicles made from the neutral phospholipid L-α-phosphatidylcholine. Binding to lipid vesicles made from L-α-phosphatidyl-L-serine is enhanced in the presence of millimolar amounts of free calcium. ATPase activity remains unimpaired after vesicle association. Myosin II was also shown to remain in tight association with purified plasma membranes, even after depletion of actin. The above observations suggest that mechanisms involving membrane-bound myosin II are required to facilitate metazoan cell motility.**

In vertebrate neurons, both myosin I and myosin II are found at the leading edge of growth cones, suggesting that both molecular motors may play a role in neurite outgrowth (1). This is in contrast to the picture derived from *Acanthamoeba* and *Dictyostelium* amebae, where molecular genetic (2, 3) and immunological (4–6) approaches suggest that myosin II, the conventional double-headed isoform of myosin, does not play a major role in ameboid motility but is implicated in cytokinesis (2–4) and cell surface mobility (7). Within amebae, single-headed myosin I is found at the tips of pseudopodia (4, 6) and is thought to facilitate locomotion through its ability to associate directly with phospholipids within the plasma membrane (8, 9). Presumably, if a myosin II molecule were also capable of membrane association it would be able to act in a way similar to mechanisms already postulated for myosin I (9, 10).

Consistent with this hypothesis, we demonstrate here that a major fraction of brain myosin II can bind directly to anionic lipid vesicles made from L-α-phosphatidyl-L-serine (PS) but not to vesicles made from the neutral phospholipid L-α-phosphatidylcholine (PC). Such vesicle-binding properties are similar to those of myosin I from amebae (8, 9). This characteristic property is an attribute of brain myosin II and is not exhibited by sarcomeric myosin II preparations. Close association of myosin II with the neuronal plasma membrane has also been visualized at both the light microscopic and ultrastructural levels (1), and the specificity of this association is further demonstrated here on stripped plasma membrane preparations. Possible roles of membrane-bound myosin II in growth cone motility are discussed.

MATERIALS AND METHODS

Cell Culture and Immunofluorescence. Indirect immunocytochemical techniques were performed on cell monolayers obtained from dissociated rat embryonic day 16 (E16) neuronal tissue cultured in defined medium, as detailed elsewhere (1).

Pre-embedment Immunoperoxidase Staining and Electron Microscopy of Cells. Cells were fixed in 4% paraformaldehyde/10 mM sodium acetate/0.1% glutaraldehyde, pH 7.0, prior to immunoperoxidase staining. After rinsing with phosphate-buffered saline, cells were osmicated by using 0.5% OsO_4 in 0.1 M sodium phosphate buffer, pH 7.0. After dehydration with ethanol, cell monolayers were infiltrated with a graded series of Epon diluted in absolute ethanol, culminating with a final infiltration of pure Epon that was allowed to polymerize overnight at 55°C. Silver–gold thin sections (70–100 nm) were collected within 4–5 μm from the surface of the block, mounted onto 200-mesh Formvar-coated copper grids, and examined on a JEOL 100S electron microscope. To facilitate visualization of the horseradish peroxidase (HRP) reaction product from unlabeled material, counterstaining with heavy metals was not performed.

Plasma Membrane Preparation. Plasma membranes were prepared from Neuro-2A cells grown in culture (1), according to published procedures (8, 11). Batches consisting of 5×10^9 cells were disrupted in 0.35 M sucrose/10 mM 2-{[tris(hydroxymethyl)methyl]amino}ethanesulfonic acid (Tes), pH 7.4, containing an inhibitor cocktail (added immediately before use, comprising 0.1 mM phenylmethylsulfonyl fluoride, 3.0 mM NaN_3, soybean trypsin inhibitor at 10 mg/liter, pepstatin A at 2.5 mg/liter, leupeptin at 2.5 mg/liter, aprotinin at 2.5 mg/liter, benzoyl-L-arginine methyl ester at 10 mg/liter, tosyl-L-arginine methyl ester at 10 mg/liter); unbroken cells and nuclei were removed by a 5-min centrifugation at 250 × *g*. A crude plasma membrane fraction was pelleted from the supernatant at 800 × *g* for 10 min, resuspended in 3 ml of 0.25 M sucrose/10 mM Tes, pH 6.9, then repelleted, resuspended, and fractionated by density gradient centrifugation at 48,000 × *g* for 40 min in 10 ml of 0.25 M sucrose/18% Percoll (Pharmacia)/10 mM Tes plus inhibitor cocktail. The plasma membrane fraction at the top of each gradient was diluted by the addition of 10 ml of 10 mM Tes, pH 6.9, then pelleted at 12,000 × *g* for 15 min. The density gradient centrifugation was repeated and the purified plasma membranes were resuspended in 10 mM Tes, pH 6.9.

Myosin Preparation. The preparation of bovine brain myosin II was based on published procedures (12, 13). Up to three fresh brains were homogenized in 20 mM imidazole·hydrochloride, pH 7.0/2 mM EGTA/2 mM $MgCl_2$. An inhibitor cocktail (above) was included at all stages in the preparation. Upon centrifugation, pellets were resuspended in 30

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Abbreviations: PS, L-α-phosphatidyl-L-serine; PC, L-α-phosphatidylcholine; HRP, horseradish peroxidase; Tes, 2-{[tris(hydroxymethyl)methyl]amino}ethanesulfonic acid; DRG, dorsal root ganglion; BSA, bovine serum albumin.

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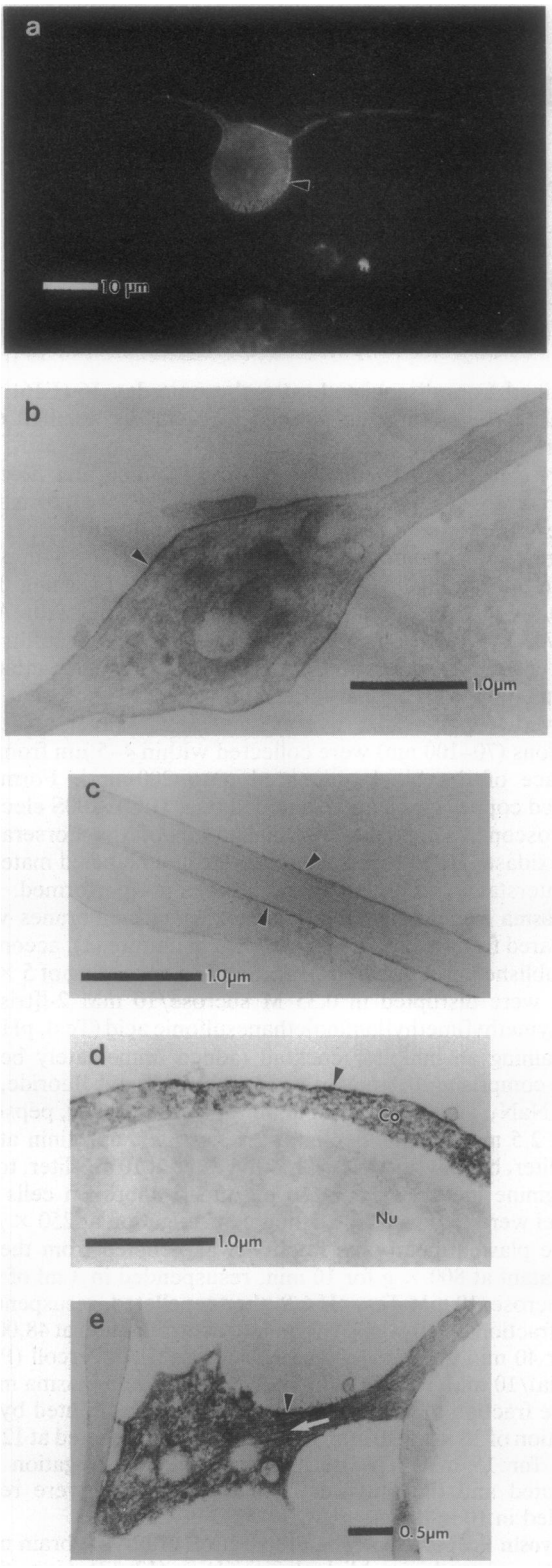

FIG. 1. Localization of myosin II adjacent to the plasma membrane within primary cultures of DRG neurons. Arrowheads denote the presence of myosin II in regions immediately adjacent to the inner surface of the plasma membrane. (*a*) Immunofluorescent microscopic image of DRG cell body and associated processes. Primary antibody is rabbit anti-neuroblastoma myosin II IgG; secondary antibody is a fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG. (*b–e*) Immunoelectron microscopic images. Ultrastructural observations were made using preembedment labeling procedures. Primary antibody is rabbit anti-neuroblastoma myosin II IgG; secondary antibody is HRP-conjugated goat anti-rabbit IgG. The HRP reaction product is visualized as the dark stain superimposed on the lighter background (osmium) staining. (*b*) DRG cell body and proximal region of the neuritic process. (*c*) Shaft of neuritic process. (*d*) Peripheral section of DRG cell body. Note myosin presence within the cell cortex (Co) as compared with the sparsity of staining within the nucleus (Nu). (*e*) Growth cone at tip of DRG process. Note that anti-myosin staining is found throughout the growth cone and also seems to be associated with microtubules entering this region (white arrow), as shown previously (1). Similar results were obtained with mouse Neuro-2A cells.

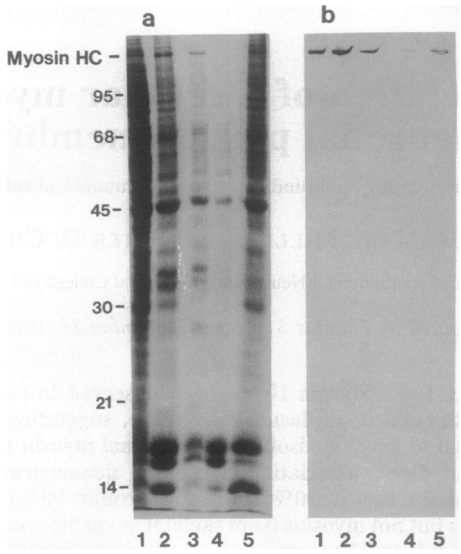

FIG. 2. Association of myosin II with the membrane fraction isolated from Neuro-2A cells. (*a*) Coomassie-blue stained 10% acrylamide/SDS gel. (*b*) Immunoblot of *a*, obtained with rabbit anti-neuroblastoma myosin II IgG as primary antibody and HRP-conjugated goat anti-rabbit IgG as secondary antibody, color being developed by standard procedures (1, 17–19). Numbers on left are molecular weight $\times 10^{-3}$ of standards. Loadings were 15 μg in lane 1 and 10 μg (10 μl) in lane 2; lanes 3–5 were loaded with volumes equal to the volume used in lane 2, so as to allow for direct comparison of the preparative steps. Lanes: 1, whole-cell homogenate; 2, plasma membrane fraction, isolated by standard procedures; and 3, supernatant remaining (30-min spin at 120,000 × *g* at 4°C), after plasma membrane fraction was treated with 1.0 M KCl/2.0 mM MgATP. The pellet remaining was subsequently exposed to 0.6 M KI/30 μM sodium thiosulfate/10 mM Tes (no additional ATP), then incubated for 2 hr at 4°C in an attempt to deplete membrane-associated actin even further. The supernatant and pellet obtained from centrifugation (150,000 × *g* for 30 min) following this treatment are seen in lanes 4 and 5, respectively, each pellet being resuspended in 10 mM Tes, pH 6.9, in an equal volume as compared with the earlier fractions. The final concentration of the plasma membrane preparation was typically around 0.35 mg/ml. HC, heavy chain.

mM Tris·HCl, pH 8.0/1.2 M KCl/2 mM EGTA/4 mM EDTA. After 30 min of stirring on ice, ATP was added to 2 mM, the pH being maintained at 7.5 for 30 min. After centrifugation, the supernatant was subjected to ammonium sulfate fractionation, the material precipitating between 35% and 65% saturation with ammonium sulfate being resuspended and dialyzed overnight against 30 mM NaCl/1 mM EDTA/0.1 mM EGTA/20 mM imidazole, pH 7.0. $MgCl_2$ was added to this material to 2 mM and the pH was adjusted to 6.5. The pellet formed after centrifugation (40,000 × *g* for 30 min) was resuspended in 20 mM imidazole·hydrochloride, pH 7.0/0.6 M KCl/1.0 mM $MgCl_2$/5.0 mM ATP prior to loading onto a Sepharose CL-4B column (Pharmacia). The elution buffer was 10 mM imidazole·hydrochloride, pH 7.0/0.6 M KCl/0.1 mM $MgCl_2$/0.5 mM ATP.

Binding Studies. Aliquots (0.1 mg) of purified myosin II were incubated at room temperature for 30 min with 1.0 ml of buffer containing 1.0 mg of vesicles of either PS or PC.

Vesicles were prepared according to published procedures (14). The buffers used in the experiments reported here were 0.3 M KCl/10 mM imidazole/2.0 mM EGTA/2 mM $MgCl_2$/60 mM potassium glutamate/1.0 mM ATP, at pH 6.4, 7.0, or 7.6, adjusted with HCl. For the pelleting experiments, 0.5 ml of material was subjected to centrifugation at 128,000 × *g* for 20 min. Pellets were resuspended in the same volume of buffer as the supernatants. Alternatively, material was loaded onto a Sepharose CL-4B column (18.5 ml), preequilibrated in the above buffer, and 0.35-ml fractions were collected and an aliquot of this material (0.1 ml) was subjected to lipid analysis by a colorimetric assay (15). The remainder of the material within each aliquot was concentrated in a Savant SpeedVac prior to electrophoretic separation and examination for myosin II on 10% acrylamide/SDS gels.

Other. Standard procedures were employed for SDS/acrylamide gel electrophoresis (16) and for protein blotting (17), the protein being visualized through use of a sensitive indirect immunoperoxidase protocol (18, 19).

RESULTS AND DISCUSSION

Using an affinity-purified antibody, previously shown to recognize neuronal isoforms (1), we have demonstrated a close association of myosin II with the plasma membrane of cultured primary dorsal root ganglion (DRG) neurons, both by immunofluorescence (Fig. 1*a*) and at the ultrastructural level by immunoelectron microscopy (Fig. 1 *b–e*). Similar images have been observed with neuroblastoma cells (data not shown). This association is seen both within the cell body (Fig. 1 *a*, *b*, and *d*), along the length of the neuritic shaft (Fig. 1*c*), and at the growing tip (Fig. 1*e*). This localization demonstrates a close association of myosin II with the plasma membrane, but it does not tell us whether the association is direct or indirect—by binding to cortical actin filaments, for example.

This association of myosin II with the plasma membrane was also demonstrated biochemically. Bulk membranes were prepared from neuroblastoma cells and subjected to SDS/acrylamide gel electrophoresis and immunoblotting, using anti-neuroblastoma myosin II as primary antibody. Myosin II was found to be a significant component of the membrane preparation (Fig. 2). In an attempt to see if this myosin could be stripped away easily from the membrane surface, purified membranes were subjected to sequential extractions with KCl and KI. Despite depletion of actin and unbound and actin-bound myosin II by the sequential salt extractions (Fig.

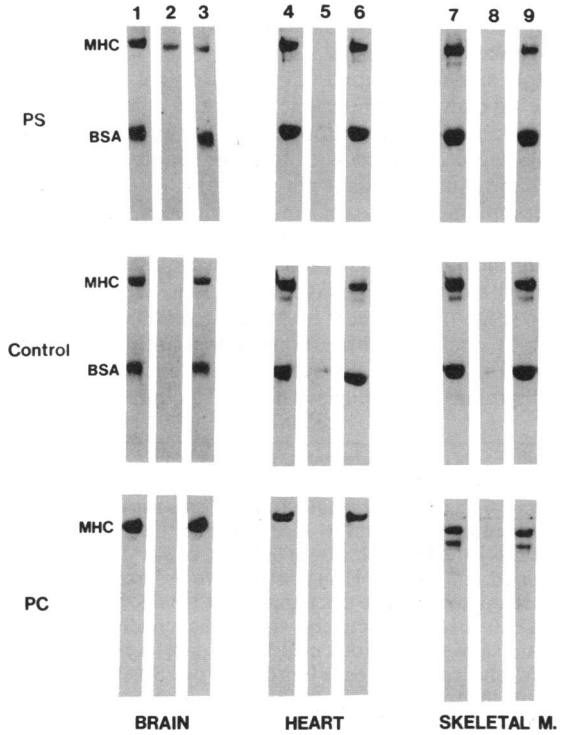

FIG. 3. Interaction of myosin II, prepared from bovine brain, heart, or skeletal muscle, with phospholipid vesicles composed of PS, an anionic phospholipid, or PC, a neutral phospholipid, as demonstrated through a pelleting experiment. Aliquots (0.1 mg) of purified myosin II, prepared from bovine brain (lanes 1–3), heart (lanes 4–6), or skeletal muscle (lanes 7–9), were incubated at room temperature for 30 min with 1.0 ml of buffer containing 1.0 mg of vesicles of either PS (*Top*) or PC (*Bottom*) or in the absence of lipid (*Middle*). After pelleting, aliquots were removed and prepared for gel analysis. From left to right, in each group of three lanes, we see the starting material, the pellet, and the supernatant, respectively. Under the conditions used in these experiments, 50–75% of all lipids are found in the pellet fraction. In some experiments bovine serum albumin (BSA) was included as a volume marker at a concentration of 0.05 mg/ml. MHC, myosin heavy chain.

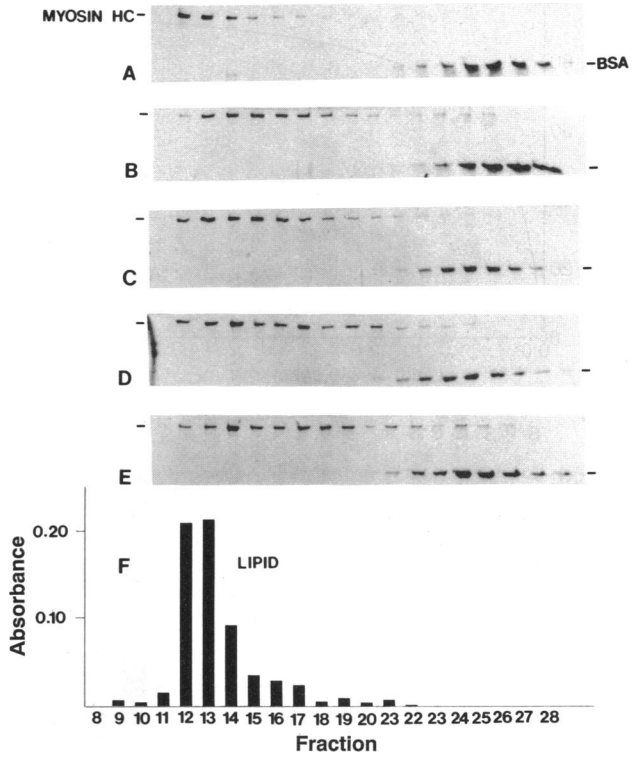

FIG. 4. Interaction of myosin II prepared from bovine brain, heart, or skeletal muscle, with phospholipid vesicles composed of PS or PC, as demonstrated by gel filtration. Aliquots (0.1 mg) of purified myosin II, prepared from bovine brain (*A*, *B*), heart (*D*), or skeletal muscle (*E*), were incubated at room temperature for 30 min in 0.5 ml of buffer containing 1.0 mg of PS (*A*, *D*, and *E*) or were incubated in the absence of lipid (*B*). An additional sample of bovine brain myosin II was incubated for 30 min with 1.0 mg of PC (*C*). Material was then loaded onto a Sepharose CL-4B column (18.5 ml) and 0.35-ml fractions were collected prior to 10% acrylamide/SDS gel electrophoresis and lipid analysis (15). BSA was included as a volume marker at a concentration of 0.1 mg/ml. A representative histogram for lipid elution from this column (bovine brain myosin II + PS experiment) is seen below the column profiles (*F*); the *x*-axis is proportioned so as to fit exactly under the appropriate gel lanes. The first lane depicted in the gel profiles is of fraction 11 of the elution profile; the first lipid fraction depicted is that of fraction 8. No protein or lipid eluted in fractions 1–7. Note that whereas brain myosin II elutes with the lipid when incubated with PS, in all other cases the elution of myosin II trails behind the elution of lipid.

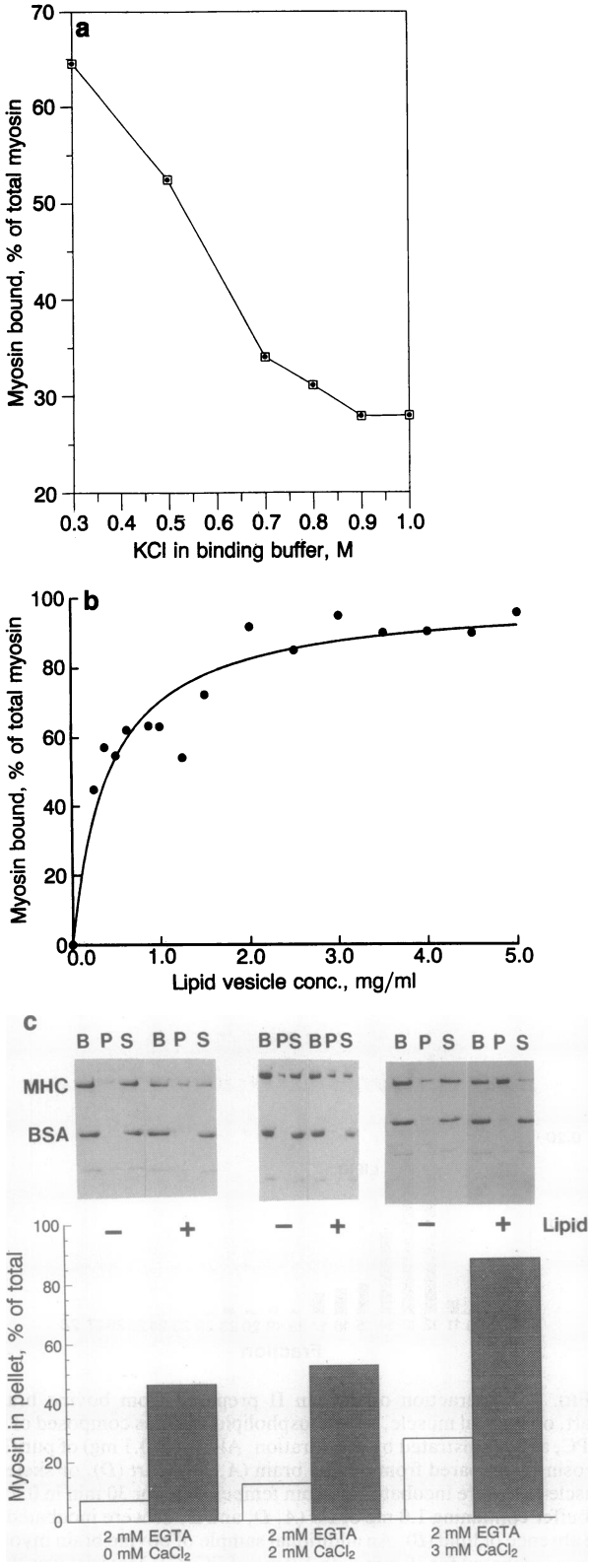

FIG. 5. (*a*) Ionic strength dependence of binding of bovine brain myosin II to anionic vesicles composed of PS. Myosin aliquots (0.1 mg at 0.1 mg/ml) were incubated in binding buffer (10 mM imidazole·hydrochloride, pH 7.0/60 mM potassium glutamate/2 mM EGTA/2 mM $MgCl_2$/1 mM ATP) which had been made up to various ionic strengths with KCl. Binding was quantified by gel densitometry. Points shown are the average of two separate determinations. (*b*) Saturation curve of the binding of bovine brain myosin II to various concentrations of PS vesicles. Binding was carried out under standard conditions ([KCl] = 0.3 M), except that the phospholipid concentration was varied between aliquots. The curve was fitted by use of a nonlinear regression program. (*c*) Effect of free calcium level on the binding of brain myosin II to vesicles made from PS. The binding of brain myosin to vesicles made from PS was assayed by pelleting, as described in the legend to Fig. 3, except that the level of free calcium was varied by inclusion of 0, 2 mM, or 3 mM $CaCl_2$ in a buffer containing 2 mM EGTA. Using an iterative program (20), we found the concentrations of free calcium to be $<1 \times 10^{-9}$ M, 2.38×10^{-5} M, and 1.0 mM, respectively, under the conditions used. The histogram shows results obtained from a single experiment in the presence (shaded) or absence (unshaded) of PS vesicles; the *y*-axis represents the fraction of the total myosin present within the pellet after centrifugation. Data were obtained from densitometry of the corresponding 10% acrylamide/SDS gels, shown above the appropriate bar of the histogram. Each triplet of lanes corresponds to material before centrifugation (B), the pellet (P), and the supernatant (S), after centrifugation. The presence or absence (control) of phospholipid is indicated. MHC, myosin heavy chain. BSA was included in experiments as a nonpelleting solution marker. When results from three such experiments were pooled and quantitated, the percentage (±SD) of myosin found in the pellet was as follows: In the absence of lipid, 18.4 ± 10.0; 14.5 ± 3.9; and 15.3 ± 8.0; in the presence of lipid: 52.9 ± 23.3; 50.1 ± 22.9; and 75.4 ± 20.6 (values in the presence of 2.0 mM EGTA plus 0, 2.0, or 3.0 mM $CaCl_2$, respectively). Application of Student's *t* test to each pair of data indicated that the amount of myosin pelleting with PS was significantly greater in the presence of 1 mM free calcium compared with that pelleting either in the absence of calcium or in the presence of micromolar levels of free calcium ($P < 0.05$).

2, lanes 3 and 4), some myosin II remained associated with the membrane fraction (Fig. 2, lane 5). Only upon addition of 0.05% saponin (or, less effective, 1% Triton X-100) to the extraction buffer was most myosin II effectively stripped from the membrane (data not shown). Two interpretations of the detergent-stripping experiments are possible: detergent may permeablize a subset of inside-out vesicles in which myosin was trapped, or, solubilization of appropriately oriented membrane-bound myosin may have occurred. We favor the latter interpretation for the following reasons. If myosin were trapped within a vesicle, this phenomenon would be observed irrespective of the type of myosin II used; this is not the case (see Fig. 3). Under the conditions of our ATPase assay, it would be difficult to explain full retention of activity (see Table 1) and, further, a pronounced dependence of myosin binding on ionic strength (see Fig. 5*a*) would not be anticipated.

To assess whether brain myosin II could bind directly to phospholipids, centrifugation and gel filtration assays originally employed to examine the lipid binding properties of myosin I (9) were adapted for use with myosin II. In the centrifugation assay, aliquots of purified myosin II prepared from bovine brain, heart, or skeletal muscle were incubated with either the anionic phospholipid PS (Fig. 3, top row of gels) or the neutral phospholipid PC (Fig. 3, bottom row) or were left in the absence of lipid (Fig. 3, middle row). A significant fraction of brain myosin II (30–90%) was capable of pelleting after incubation with PS (Fig. 3 *Top*, lane 2). This did not occur when PS was absent (Fig. 3 *Middle*, lane 2) or was replaced by PC (Fig. 3 *Bottom*, lane 2). Irrespective of the type of phospholipid vesicle used, cardiac or skeletal muscle myosin II did not pellet with the lipids (Fig. 3, lanes 5 and 8) under the conditions used. Results similar to those with PS were obtained with another anionic phospholipid, phosphatidylinositol (data not shown).

In complementary experiments, myosin II preparations from brain and skeletal or cardiac muscles, incubated with lipid vesicles made from PS or PC, were assessed for phospholipid binding by passage through a Sepharose CL-4B column (Fig. 4). In agreement with the results of Fig. 3, brain myosin II coelutes with anionic phospholipids in the void volume (Fig. 4 *A* and *F*) but does not coelute with neutral phospholipids, remaining within the included volume (Fig.

Table 1. K^+,EDTA-ATPase of bovine brain myosin II remains unchanged after binding to lipid vesicles composed of PS

Measurement of bovine brain myosin II ATPase activity	KCl conc., M	K^+,EDTA-ATPase, nmol/min per mg
Prior to incubation with	0.6	340
anionic lipid vesicles	0.3	407
When associated with	0.6	323
anionic lipid vesicles	0.3	401

Bovine brain myosin II (0.1 mg) was incubated with lipid vesicles composed of PS as described in the legend to Fig. 3. After separation of unbound myosin II from myosin II bound to lipid vesicles, by centrifugation at 108,000 × *g* for 20 min, duplicate samples were assayed for K^+,EDTA-ATPase activity in 0.6 M KCl/2.0 mM EGTA/1.0 mM EDTA/1.0 mM dithiothreitol/1.0 mM [γ-^{32}P]ATP/50 mM Tris·HCl, pH 8.0 or in the same buffer but with 0.3 M KCl. K^+,EDTA-ATPase is fully retained after phospholipid binding. In a separate experiment, lipid vesicles made from PS were incubated with 1.0 mM [γ-^{32}P]ATP for 30 min at room temperature in 0.3 M KCl/10 mM imidazole·hydrochloride/2.0 mM EGTA/2.0 mM $MgCl_2$/60 mM potassium glutamate, pH 6.4. This material was then loaded onto a column of Sepharose CL-4B equilibrated in the same buffer; eluted material was assayed for the presence of lipids and radioactivity. No radioactivity was found in the eluted lipid fraction.

4*C*). No coelution with lipid occurred when cardiac or skeletal muscle myosin II replaced brain myosin; these myosins eluted in the same position (Fig. 4 *D* and *E*) as that of unbound myosin in the absence of lipid (Fig. 4*B*).

The binding assay illustrated in Fig. 3 was also used to determine the salt dependence and saturability of myosin II binding to phospholipid vesicles. We found a pronounced dependence on ionic strength of myosin II binding to PS liposomes (Fig. 5*a*); maximum binding was observed at 0.3 M KCl. When the binding assay was performed at a fixed myosin concentration while the concentration of PS vesicles was varied, up to 90% of the myosin was found to pellet at saturation (Fig. 5*b*). While this argues that at least 90% of the myosin II recognized by our antibody was capable of specific association with anionic phospholipid vesicles, we cannot say whether this was due to action of one or more distinct isoforms. The results of rebinding experiments are also consistent with these results (data not shown). Association of myosin II with PS vesicles was enhanced by the addition of millimolar amounts of free calcium as compared with association in the presence of micromolar amounts of free calcium or in the total absence of calcium (Fig. 5*c*). Nevertheless, the majority of the binding was insensitive to calcium.

It is possible that the isozymic forms of brain myosin II involved in phospholipid binding correspond to those possessing the unique 72-residue tailpiece described earlier for an isoform of rat brain myosin II (21). This nonhelical region contains a hydrophobic core flanked by hydrophilic sequences and may serve to anchor this isoform in the plasma membrane (22). When brain myosin II bound to vesicles made from PS and separated from unbound myosin by centrifugation was assayed for K^+,EDTA-ATPase activity, no decline in specific activity was detected (Table 1). These results show that the active site of brain myosin II is unaffected either by association with lipid vesicles made from PS or by the experimental protocol. Because control experiments have demonstrated that ATP cannot cross the lipid bilayer in vesicles made from PS (Table 1), the ATPase results cannot be due to myosin molecules with their heads oriented on the inside of the lipid vesicles. Therefore, the complete retention of K^+,EDTA-ATPase suggests not only that all heads remain intact but also that they are located on the outside of the vesicles, an orientation consistent with the possibility that the tailpiece is responsible for anchoring brain myosin II molecules to the vesicle surface. Lipid binding studies performed on the papain digestion products of brain myosin II under low-salt conditions (12), after their subsequent purification into head and rod fragments, resulted in an inability of either fragment to bind to PS vesicles (data not shown). This implies that a region critical for binding was impaired by the digestion.

This demonstrated ability for one or more isoforms of brain myosin II to bind to anionic phospholipids may account for the observed localization of myosin II adjacent to the plasma membrane within neurons, *in situ* (1) or in culture (Fig. 1), and its tenacious association with isolated membrane fractions (Fig. 2). Such an interaction immediately suggests a plausible mechanism for the role of myosin II in the directional motility of growth cones. Myosin II, anchored to the plasma membrane, will possess cycling heads (crossbridges) capable of moving actin filaments in directions parallel to the plasma membrane. Structures attached to these actin filaments, such as an interlinked network of membrane-anchored actin attachment sites, will also have force exerted on them by this process, bringing about their relative movement as well as the movement of structures to which they are attached. This arrangement confers the ability to move segments of the plasma membrane relative to the location of the anchored myosin, the exact speed and direction of movement depending on the orientation of the cortical actin filaments (23).

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