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A new method to precipitate myosin V from rat brain soluble fraction

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Myosin can be precipitated from soluble fraction under different assay conditions. This paper describes a new method for precipitating myosin V from rat brain soluble fraction. Brains were homogenized in 50 mM imidazole/HCl buffer, pH 8.0, containing 10 mM EDTA/EGTA, 250 mM sucrose, 1 mM DTT and 1 mM benzamidine, centrifuged at 45000×*g* for 40 min and the supernatant was frozen at –20°C. Forty-eight hours later, the supernatant was thawed, centrifuged at 45000×*g* for 40 min and the precipitate was washed in 20 mM imidazole buffer pH 8.0. SDS/PAGE analysis showed four polypeptides in the precipitate: 205, 150, 57 and 43 kDa. The precipitate presented high Mg^{2+}-ATPase activity, which co-purifies with p205. This polypeptide was recognized by a specific myosin V antibody and was proteolised by calpain, generating two stable polypeptides: p130 and p90. The Mg^{2+}-ATPase activity was not stimulated by calcium in both the absence and presence of exogenous calmodulin and the K^+/EDTA-ATPase activity represented 25% of the Mg^{2+}-ATPase activity. In this work, myosin V from rat brain was precipitated by freezing the soluble fraction and was co-purificated with a 45 kDa polypeptide.

Keywords: myosin V, ATPase, F-actin

INTRODUCTION

Myosins constitute a superfamily of ATPases that use energy from ATP to perform movement along the actin cytoskeleton (De La Cruz & Ostap, 2004; Krendel & Mooseker, 2005; O'Connell *et al.*, 2007; Redowicz, 2007). Myosin II, the first identified member of this family of molecular motors, which is responsible for muscle contraction, is present in different tissues of vertebrates and in a wide range of other organisms (Warrick & Spudich, 1987; Ruppel & Spudich, 1996). This myosin consists of two heavy chains of approximately 200 kDa, each one bound to two light chains (Warrick & Spudich, 1987; Ruppel & Spudich, 1996). In addition to myosin II, there are at least another 19 classes of myosins (Krendel & Mooseker, 2005; Foth *et al.*, 2006). Some of them, similarly to myosin II, are composed of two heavy chains, while others contain only one heavy chain (Krendel & Mooseker, 2005; O'Connell *et al.*, 2007; Redowicz, 2007). This family of molecular motors is believed to participate in different cellular motility systems such as cytokinesis (Zang *et al.*, 1997; Uyeda & Nagasaki, 2004; Werner *et al.*, 2007), cell migration (Novak & Titus, 1997), melanosome and secretory vesicle transport (Wu *et al.*, 1997; Rogers *et al.*, 1999; Varadi *et al.*, 2005; Schietroma *et al.*, 2007), vacuolar movement (Hill *et al.*, 1996), cell adhesion (Tuxworth *et al.*, 2001), sensory functions (Gibson *et al.*, 1995; Hasson *et al.*, 1997; Brown & Bridgman, 2004) and cell signaling (Müller *et al.*, 1997; Post *et al.*, 1998).

Myosin V was initially identified in preparations of vertebrate brain actomyosin as a calmodulin-binding 190 kDa polypeptide (Larson *et al.*, 1988; 1990; Espindola *et al.*, 1992). Later, the deduction of its primary structure (Espreafico *et*

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Abbreviations: BCIP, 5-bromo-4-chloro-3-indolyl phosphate; DTT, dithiothreitol; NBT, nitroblue tetrazolium.

al., 1992) revealed that this polypeptide presented homology to the products of the *Saccharomyces cerevisiae* MY02 gene (Johnston *et al.*, 1991) and the mouse *dilute* gene (Mercer *et al.*, 1991). Similarly to myosin II, it consists of two identical heavy chains of approx. 200 kDa, each of which is divided into three domains (Espreafico *et al.*, 1992; Cheney *et al.*, 1993): the motor (or head) domain — located in the N-terminal extremity, contains the ATP and F-actin binding sites; the tail domain — located in the C-terminal, is responsible for the dimerization of the molecule and contains a PEST sequence (a sequence rich in P, E, S and T amino acids) which is associated with targets of calpain-mediated proteolysis (Rechsteiner *et al.*, 1996); and the neck domain — located between the head and tail domains, is the light chain-binding region. The main light chain of myosin V is calmodulin (Larson *et al.*, 1988; Cheney *et al.*, 1993), which belongs to the family of calcium-binding proteins and is one of the main receptors of calcium in the nerve cell. Calcium stimulates the Mg^{2+}-ATPase activity of myosin V (Espindola *et al.*, 1992; Cheney *et al.*, 1993) but inhibits actin movement in the *in vitro* motility assay (Cheney *et al.*, 1993; Nguyen & Higuchi, 2005).

A precipitation stage is common in myosin purification methods. Brain myosin II is precipitated by dialyzing the soluble fraction of brain against low salt-containing buffer (Espindola *et al.*, 1992), while brain myosin V is precipitated selectively by treating the soluble fraction of brain with high salt (Coelho & Larson, 1993). In this work, we obtained a precipitated fraction enriched in myosin V by a new method, which consists in freezing the brain soluble fraction.

MATERIAL AND METHODS

Preparation of actomyosin. The rats were killed by decapitation and their brains immediately extracted, washed in ice-cold saline solution and placed in extraction buffer (50 mM imidazole/HCl pH 8.0, 10 mM EDTA, 10 mM EGTA, 250 mM sucrose, 1 mM DTT and 1 mM benzamidine). About 25 g of rat brain was homogenized in 30 mL of this buffer using a glass homogenizer (Potter) avoiding the formation of foam. This homogenate was then centrifuged at $45\,000 \times g$ for 40 min and the supernatant fraction (S1) was recovered by pouring the contents of the tube into a graduated measuring glass to measure the volume. The S1 fraction was stored in a freezer at –20°C and the P1 fraction discarded. After no less than 48 h, the S1 fraction was thawed in a 27°C bath and centrifuged at $45\,000 \times g$ for 40 min. The P2 fraction was then homogenized in 10 mL of 20 mM imidazole/HCl buffer, pH 8.0, containing 1 mM EDTA and 1 mM DTT and centrifuged at $45\,000 \times g$ for 40 min. The P3 fraction was homogenized in 5 mL of the same buffer as the one used for resuspending P2. The centrifugations were carried out at 4°C and the homogenizations were done in an ice bath.

Determination of ATPase activity. The ATPase activity was determined by quantifying inorganic phosphate (Pi) released by the hydrolysis of ATP, using the colorimetric method of Heinonen and Lahtti (1981). The assays were carried out at 37°C in duplicate, in a final volume of 200 μL and the absorbance reading was done at 355 nm. The Mg^{2+}-ATPase assay was carried out with 25 mM imidazole/HCl pH 7.5, 1 mM DTT, 1 mM EDTA, 60 mM KCl and 4 mM $MgCl_2$, and the K^+/EDTA-ATPase assay with 25 mM imidazole-HCl pH 7.5, 2 mM EDTA, 1 mM DTT and 60 or 600 mM KCl. The reaction was started by addition of ATP and stopped by addition of phosphate determination solution: acetone (P.A.) 50%, ammonium molibdate (10 mM) 25% and sulfuric acid (2.5 M) 25%.

Calpain-mediated proteolysis. Proteolytic digestions were performed using a 1:80 ratio (w/w) of calpain to substrate at 37°C for 10 min in 20 mM imidazole/HCl buffer pH 8.0 containing 1 mM DTT and 2 mM $CaCl_2$. The reaction was interrupted by addition of sample buffer (180 mM Tris/HCl pH 6.8, 6% SDS, 6 mM EDTA, 27.5% glycerol, 3 mM Bromofenol Blue and 29 mM 2-mercaptoetanol) to the test tube, which was immediately heated in boiling water for 2 min.

Analysis of the polypeptide profile and immunoblotting. The polypeptide profile of fractions was analyzed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS/PAGE) in 12% gels, using the method described by Laemmli & Favre (1973) and the plate system employed by Studier (1973). For the antibody assay, the separated polypeptides were electrotransferred to a nitrocellulose membrane according to the method of Towbin *et al.* (1979) and probed with antibody generated against the head domain of myosin V of chicken brain. Immunodetection of specific antigens on Western blots was done using alkaline phosphatase-conjugated secondary antibodies and the color was developed with BCIP and NBT (Harlow & Lane, 1988).

Protein quantification. The protein concentration in the fractions was determined in duplicate according to the Bradford method (Bradford, 1976) using BSA (bovine serum albumin) as the standard. Aliquots of the respective fractions were prediluted to 100 μL with deionized water, after which 3 mL of Bradford reagent was added.

RESULTS

Precipitation of the S1 polypeptides by freezing

Freezing the soluble fraction of rat brain at –20°C for at least 48 h generated a precipitated fraction (P2) which, although it presented various polypeptides, showed four more strongly stained bands in the gel: 205, 150, 57 and 43 kDa (Fig. 1). After washing in 20 mM imidazole/HCl pH 8.0 buffer, containing 1 mM EDTA and 1 mM DTT, the 150 kDa polypeptide and the various polypeptides that presented weaker staining in the gel were recovered in the S3 fraction, while the 205 kDa polypeptide was totally recovered in the precipitated fraction (P3). The 57 kDa polypeptide was also almost completely solubilized in the washing procedure of P2. The 43 kDa polypeptide was divided between the soluble and precipitated fractions (Fig. 1). This figure also shows a polypeptide band migrating at the bromophenol front.

p205 immunoreaction with anti-myosin V

The 205 and 43 kDa polypeptides displayed molecular mass similar, respectively, to the myosin heavy chains (II and V) and actin. To ascertain the presence of myosin V in the P3 fraction, the polypeptides of this fraction were separated by SDS/PAGE and transferred to a nitrocellulose membrane. The membrane was incubated with antibody generated against the head domain of myosin V of chicken brain (Fig. 2). The antibody recognized the polypeptide at around 205 kDa and also marked two smaller polypeptides: one of about 160 kDa and another of about 110 kDa. Although the S1 fraction contained various polypeptides, only the 205 kDa polypeptide was marked by the antibody.

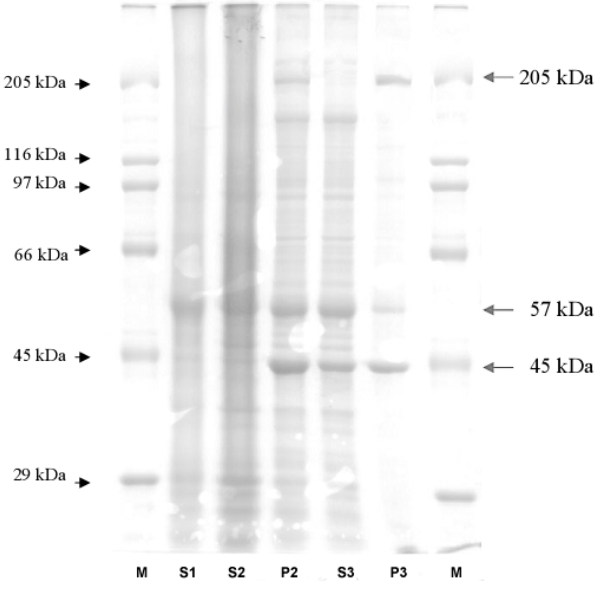

Figure 1. SDS/PAGE of subfractions obtained by freezing of the soluble fraction of rat brain. Approximately 15 μg of S1, 17 μg of S2, 13 μg of P2, 9 μg of S3 and 5 μg of P3 were applied on 12% polyacrylamide gel. The arrows on the right indicate the main polypeptides of fraction P3. (M) Molecular mass standard: myosin, β-galactosidase, phosphorylase *b*, bovine albumin, ovalbumin and carbon anyhdrase. The gel was stained with Coomassie Blue R-250.

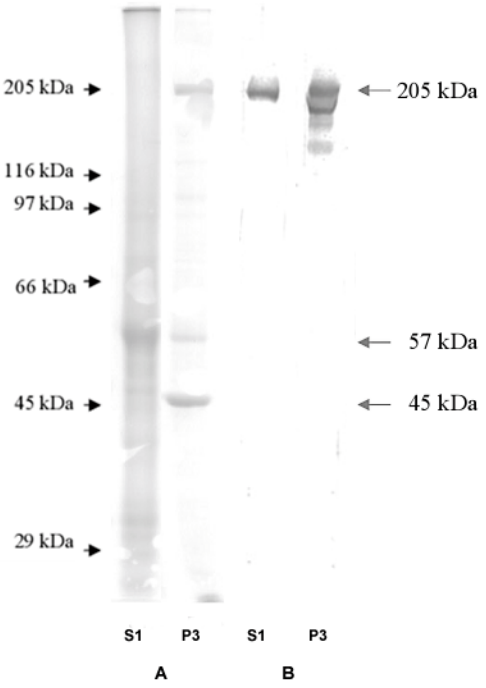

Figure 2. Immunoblot of S1 and P3 using anti-myosin V. Approximately 15 μg of S1 and 5 μg of P3 were applied on 12% polyacrylamide gel. A. Gel stained with Coomassie Blue R-250; B. Immunoblot using anti-myosin V. The arrows on the right indicate the main polypeptides of fraction P3 and the arrows on the left indicate the molecular mass of the standards (see Fig. 1).

Calpain-mediated proteolysis of p205

Brain myosins II and V differ with respect to their sensitivity to calpain, a calcium-dependent cysteine protease. This protease cleaves myosin V, generating stable polypeptides, but does not degrade myosin II. Note, in Fig. 3, that the 205 kDa polypeptide was almost completely degraded when the P3 fraction was incubated with calpain. Note, also, the appearance of two polypeptides: p130 and p90, after incubation of the P3 fraction with calpain. In some preparations, p205 is fully degraded, however, in others a part is still present even after a long time of calpain treatment, suggesting that there is some myosin II in P3.

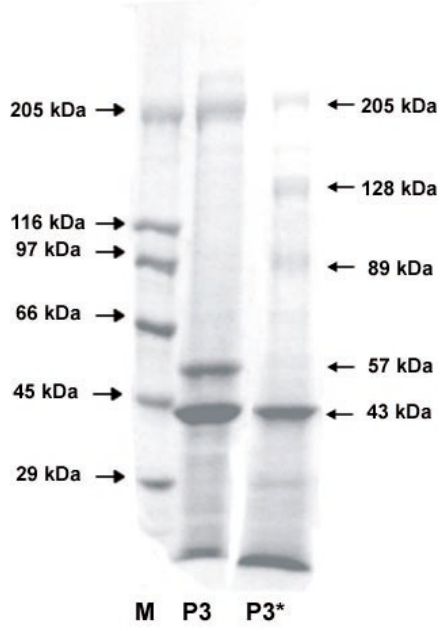

Figure 3. Calpain-mediated proteolysis of p205.
The proteolysis assay was carried out at 37°C for 10 min in reaction medium containing 25 mM imidazole/HCl pH 8.0, 1 mM DTT and 1 mM $CaCl_2$. The proportion of calpain to P3 fraction was 1:80. The reaction was initiated by the addition of calpain and stopped by the addition of sample buffer. For time zero, the sample buffer was added before the calpain. Ten microliters of the assay mixture at time zero (P3) and after 10 min of incubation (P3*), were applied on 12% polyacrylamide gel. The arrows on the right indicate the main polypeptides of fraction P3 (before and after proteolysis). M, indicates the molecular mass standards (see Fig. 1).

ATPase activity of the P3 fraction

Myosins are ATPases that express high Mg^{2+}-ATPase activity only in the presence of F-actin under physiological conditions. They also present activity in the absence of bivalent cations and in the presence of 600 mM of KCl, K^+/EDTA-ATPase activity. The P2 fraction generated by freezing of the soluble fraction of rat brain presented high total Mg^{2+}-ATPase activity and about 50% of this activity was recovered in the P3 fraction (Fig. 4). The specific Mg^{2+}-ATPase activity of fraction P3 was approx. 300 nmol of Pi/mg of protein per min and its K^+/EDTA-ATPase activity was about 72 nmol of Pi/mg of protein/min, which corresponds to about 25% of the Mg^{2+}-ATPase activity (Fig. 5). Calcium at 2 mM, in both the presence and absence of calmodulin, caused a slight inhibition of the Mg^{2+}-ATPase activity of fraction P3 (Fig. 6).

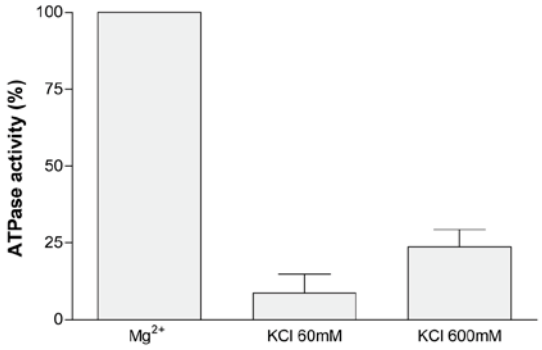

Figure 5. K^+/EDTA-ATPase activity of P3.
Fifteen micrograms of P3 was incubated in reaction medium I (25 mM imidazole/HCl pH 8.0, 1 mM DTT, 1 mM EDTA, 60 mM KCl and 4 mM $MgCl_2$) for determination of the Mg^{2+}-ATPase activity, or in reaction medium II (25 mM imidazole/HCl pH 8.0, 1 mM DTT, 2 mM EDTA containing 60 or 600 mM KCl) for determination of the K^+/EDTA-ATPase activity. The reaction was initiated by the addition of 1 mM ATP and stopped with 2 ml of phosphate determination solution. The bars indicate the standard error for n = 3.

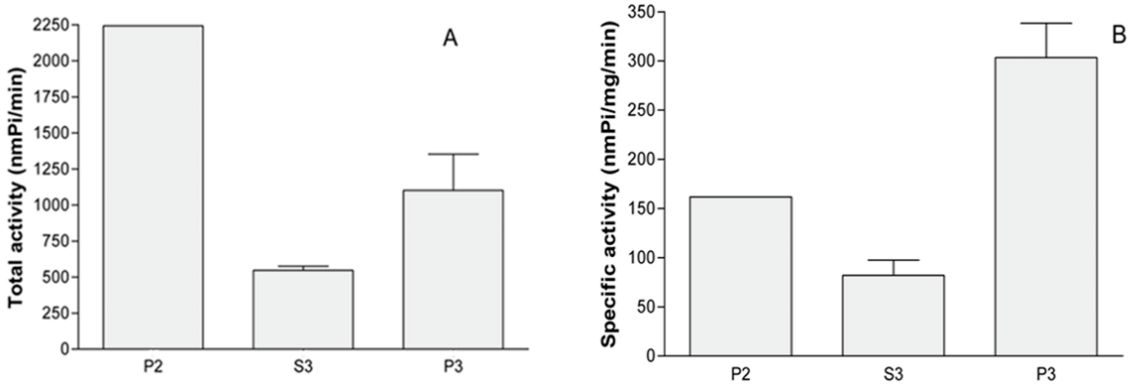

Figure 4. Total and specific Mg^{2+}-ATPase activity of P2, S3 and P3.
Total (A) and specific (B) Mg^{2+}-ATPase activity of P2, S3 and P3. Twenty microliters of these fractions was incubated at 37°C for 10 min in reaction medium containing 25 mM imidazole/HCl pH 8.0, 1 mM DTT, 1 mM EDTA, 60 mM KCl and 4 mM $MgCl_2$. The reaction was initiated by the addition of 1 mM ATP and stopped with 2 ml of phosphate determination solution. The bars indicate the standard error for n = 3.

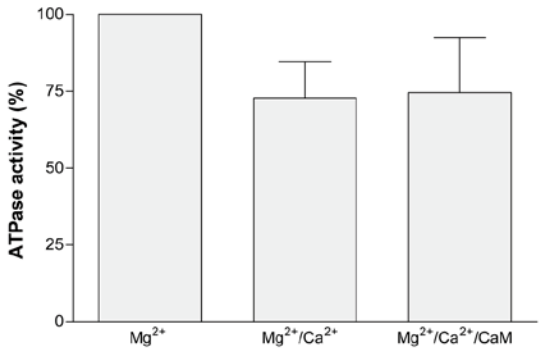

Figure 6. Effect of calcium and calmodulin on the Mg^{2+}-ATPase activity of P3.
Fifteen micrograms of P3 was incubated at 37°C for 20 min in reaction medium I (25 mM imidazole/HCl pH 8.0, 1 mM DTT, 1 mM EDTA, 60 mM KCl and 4 mM $MgCl_2$) containing 2 mM $CaCl_2$ and/or 100 μg/ml calmodulin where indicated. The reaction was initiated by the addition of 1 mM ATP and stopped with 2 ml of phosphate determination solution. The bars indicate the standard error calculated for n = 3. No statistically significant difference was found between the assays and the control ($P>0.05$).

DISCUSSION

The total Mg^{2+}-ATPase activity of the P3 and S3 fractions represented, respectively, 49 and 24% of the Mg^{2+}-ATPase activity of the P2 fraction. Fraction P3 presented an about 3.7-fold higher specific Mg^{2+}-ATPase activity than did fraction S3. Since the 150 kDa polypeptide was not visible in the P3 fraction and the 57 kDa polypeptide appeared predominantly in the S3 fraction, these polypeptides do not seem to be responsible for the Mg^{2+}-ATPase activity observed in fraction P3. The apparently uniform distribution of the 43 kDa polypeptide between fractions P3 and S3 suggests that this polypeptide is also not the Mg^{2+}-ATPase of fraction P3. This polypeptide has molecular mass similar to that of actin. The various other polypeptides of P2 which appeared weakly stained in the gel were also not related to the Mg^{2+}-ATPase activity of P3, since these polypeptides were almost exclusively recovered in the S3 fraction and were not visible in the P3 fraction. On the other hand, the 205-kDa polypeptide was almost exclusively present in fraction P3 and, based on the aforementioned considerations regarding the other polypeptides, it may be the enzyme responsible for the Mg^{2+}-ATPase activity in this fraction. The low molecular mass polypeptide that migrated at the front of the gel was also present only in the P3 fraction and may also be related to that activity.

The 205 kDa polypeptide cross-reacted with an antibody generated against the head domain of myosin V of chicken brain. This antibody reacted only with the 205 kDa polypeptide in fraction S1, indicating that it is truly specific for myosin V, since the S1 fraction possessed a wide variety of polypeptides. The polypeptides of approx. 160 and 110 kDa recognized by the anti-myosin V antibody in the P3 fraction were probably products generated by the proteolysis of p205 during the preparation of the P3 fraction. The heavy chain of brain myosin II also has molecular mass of approx. 205 kDa (Warrick & Spudich, 1987), so marking with anti-myosin V does not exclude the presence of myosin II in the preparation. It is also worth mentioning that, in rat testicle and skeletal muscle, this method of freezing of the soluble fraction causes precipitation of myosin II (Dias & Coelho, 2007). Immunobloting assays using myosin II-specific antibodies showed the presence of myosin II in P3 (not shown). Thus, besides myosin V, the freezing of the brain soluble fraction precipitates conventional myosin, too. The amount of myosin II relative to myosin V varied according with the preparation, but generally p205 was almost fully degraded when P3 was incubated with calpain, a calcium-dependent cysteine protease (Perrin & Huttenlocher, 2002) that cleaves the heavy chain of brain myosin V but does not degrade myosin II (Espindola *et al.*, 1992). Those researchers showed that in an actomyosin preparation from rat brain containing about the same amounts of myosin II and V, 6.7% calpain degraded myosin V totally, but not myosin II. As indicated in Fig. 3, after incubation with 1.25% calpain, most of the P3 205 kDa polypeptide was degraded and two polypeptides appeared: p130 and p90. These polypeptides had molecular mass similar to those generated by *in vitro* calpain-mediated proteolysis of rat brain myosin V (Espíndola *et al.*, 1992; Coelho & Larson, 1993). The immunoblot assay together with this calpain-mediated proteolysis assay strongly suggests that most of the 205 kDa polypeptide of the P3 fraction corresponded to brain myosin V.

The specific Mg^{2+}-ATPase activity of fraction P3 was approx. 300 nmol of Pi/mg per min, which corresponds to about 27% of the Mg^{2+}-ATPase activity of myosin V purified and assayed in the presence of F-actin, calcium and calmodulin (Cheney *et al.*, 1993). In the absence of F-actin, myosin V does not express Mg^{2+}-ATPase activity (Espindola *et al.*, 1992; Cheney *et al.*, 1993). Therefore, the Mg^{2+}-ATPase activity of fraction P3 was extremely high when compared with the Mg^{2+}-ATPase activity of myosin V, presupposing that the 43 kDa polypeptide of this fraction is really actin and, moreover, that this molecule is present at least partially in the form of F-actin. Because the K_m of myosin V for F-actin is extremely low (Nascimento *et al.*, 1996), a small quantity of F-actin in the P3 fraction justifies the high Mg^{2+}-ATPase activity presented by this

fraction. Cheney (1998) suggested that the high ionic strength precipitation of myosin V is due to its interaction with actin filaments and vesicles. Thus, the precipitation of myosin V by freezing of the soluble fraction of rat brain may also be due to its interaction with F-actin.

Unlike the Mg^{2+}-ATPase activity of myosin V, which is stimulated by calcium (Espindola *et al.*, 1992; Cheney *et al.*, 1993; Nascimento *et al.*, 1996), the P3 fraction presented high Mg^{2+}-ATPase activity even in the absence of calcium. Moreover, 2 mM $CaCl_2$ slightly inhibited the Mg^{2+}-ATPase activity of the P3 fraction in both the presence and absence of exogenous calmodulin. It was recently shown that the myosin V molecule can take on two forms: one compact and inactive, which occurs in the absence of calcium, and the other more extended and active, which predominates in the presence of calcium (Krementsov *et al.*, 2004; Wang *et al.*, 2004). This global alteration in the structure of myosin V caused by calcium was postulated as being responsible for the stimulation of myosin V by this cation. Other factors, such as ionic strength and binding of the cargo to the myosin V molecule, may also cause alterations in the myosin V structure and, hence, stimulate its Mg^{2+}-ATPase activity (Krementsov *et al.*, 2004; Wang *et al.*, 2004). Therefore, some component of the P3 fraction, such as a vesicle, may bind to myosin V and stimulate its Mg^{2+}-ATPase activity, even in the absence of calcium. According to this hypothesis, we recently observed that the treatment of fraction P3 with Triton X-100 causes inhibition of the Mg^{2+}-ATPase activity (unpublished).

The ATPase activity in the presence of high K^+ and the absence of bivalent cations, K^+/EDTA-ATPase activity, is a characteristic of myosins, and myosin V also expresses this activity, although its K^+/EDTA-ATPase activity is much lower than that of myosin II (Margossian & Lowey, 1982; Nascimento *et al.*, 1996). The P3 fraction presented a K^+/EDTA-ATPase activity of about 72 nmol of Pi/mg per min, which corresponds to three times that of the control (60 mM KCl) and to approx. 25% of the Mg^{2+}-ATPase activity. It is also worth pointing out that F-actin inhibits the K^+/EDTA-ATPase activity of myosins (De La Cruz *et al.*, 1999) and the presence of F-actin in the P3 fraction might have caused partial inhibition of such activity. The treatment of fraction P3 with Triton X-100 not only inhibited the Mg^{2+}-ATPase activity, as described above, but also stimulated the K^+/EDTA-ATPase activity (not shown). Ongoing experiments in the laboratory seek to clarify the effect of Triton X-100 on the Mg^{2+}- and K^+/EDTA-ATPase activities of P3.

In this paper we showed that, similarly to the treatment of the rat brain soluble fraction with 0.6 M NaCl (Coelho & Larson, 1993), freezing also causes precipitation of myosin V and concomitant coprecipitation of F-actin. If the interaction with F-actin and vesicles really matters to the myosin V precipitation in the brain cytosol treatment with salt as suggested by Cheney (1998) and in the freezing method described in this work, comparative study of the fractions generated using this two methods can help in the identification of possible myosin V targets, e.g., "cargos" trassported by this molecular motor. The high Mg^{2+}-ATPase activity of P3 and its indifference to exogenous calmodulin can be explained by interaction of myosin V with some of this cargo present in this fraction. Therefore the myosin V precipitation method described in this work represents one more tool to study myosin V and can be important in the identification of proteins that interact with this molecular motor and, then, in the identification of the cargos transported by myosin V. While high salt treatment did not precipitate myosin V from cattle brain cytosol (Cheney, 1998), the method described in this work precipitates myosin V from rat brain cytosol, as well as from pig and cattle brain cytosol (Dias *et al.*, manuscript in preparation).

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