Published in final edited form as:

Biochem Biophys Res Commun. 2007 June 22; 358(1): 140–144.

Differential regulation of protein phosphatase-1, by neurabin

S. Andrew Bullock^{1,2}, Jimcy Platholi^{1,2}, Ada Gjyzezi¹, Paul M. Heerdt^{1,2}, H.Y. Lim Tung^{1,*}, and Hugh C. Hemmings Jr.^{1,2}

¹ Institute for Neuronal Cell Signaling, Department of Anesthesiology, Weill Medical College of Cornell University, New York, NY 10021, USA

² Department of Pharmacology, Weill Medical College of Cornell University, New York, NY 10021, USA

Abstract

Neurabin is a brain-specific actin and protein phosphatase-1 (PP-1) binding protein that inhibits the purified catalytic subunit of protein phosphatase-1 (PP-1_C). However, endogenous PP-1 exists primarily as multimeric complexes of PP-1_C bound to various regulatory proteins that determine its activity, substrate specificity, subcellular localization and function. The major form of endogenous PP-1 in brain is protein phosphatase-1_I (PP-1_I), a Mg²⁺/ATP-dependent form of PP-1 that consists of PP-1_C, the inhibitor-2 regulatory subunit, an activating protein kinase and other unidentified proteins. We have identified four PP-1_I holoenzyme fractions (PP-1_{IA}, PP-1_{IB}, PP-1_{IC} and PP-1_{ID}) in freshly harvested pig brain separable by poly-L-lysine chromatography. Purified recombinant neurabin (amino acid residues 1-485) inhibited PP-1_{IB} (IC₅₀=1.1 μM), PP-1_{IC} (IC₅₀=0.1 μM) and PP-1_{ID} (IC₅₀=0.2 μM), but activated PP-1_{IA} by up to 3-fold (EC₅₀=40 nM). The PP-1_{IA} activation domain was localized to neurabin¹⁻²¹⁰. Our results indicate a novel mechanism of PP-1 regulation by neurabin as both an inhibitor and an activator of distinct forms of PP-1_I in brain.

Keywords

protein phosphatase-1; neurabin; PP-1 inhibitor; actin binding protein

Introduction

Neurabin is a brain-specific actin and protein phosphatase-1 binding protein found predominantly in dendritic spines, but also in dendrites, axons, axon terminals and glia [1-3]. Previous studies on the regulation of protein phosphatase-1 (PP-1) by neurabin showed that it is a potent and specific inhibitor of the catalytic subunit of PP-1 (PP-1_C) [1,4]. PP-1_C interacts with various proteins, such as targeting subunits, inhibitory subunits and substrates [5]. Most, but not all, of these interactions are mediated by the PP-1_C binding motif (R/K)X(V/I)XF [5]. Targeting subunits determine the subcellular localization and therefore the specialized functions of the various forms of PP-1 [5,6]. The predominant cytosolic form of PP-1 in pig brain is protein phosphatase-1_I (PP-1_I) which consists of the catalytic subunit PP-1_C, the inhibitor-2 regulatory subunit (I-2), and other regulatory subunits, including an unidentified activating protein kinase [7-10].

*Correspondence to: H.Y. Lim Tung, Laboratory of Neurobiochemistry, Institute for Neuronal Cell Signaling, Department of Anesthesiology, Weill Medical College of Cornell University, 525 East 68th Street/Box 50, New York, NY 10021, USA, Tel: 212-746-2769, Fax: 212-746-8316, E mail: hyltung@incsofweillcornell.org

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Neurabin interacts with both PP-1_C and I-2 [11]; it binds PP-1_{C γ 1} with higher affinity than PP-1_{C α} or PP-1_{C β} [12,13]. It has been suggested that PP-1_C bridges the association of I-2 with neurabin, and that neurabin acts as a targeting protein that directs PP-1_C and I-2 to polymerized actin in the control of cell morphology [14]. However, the PP-1_C and I-2 complex (PP-1_I) is inactive in its basal state and requires phosphorylation by an activating kinase for activation. Whether neurabin regulates the activity of native brain PP-1_I is unknown since previous studies were performed with isolated PP-1_C or immunoprecipitates in the absence of kinase activation. We determined the effects of recombinant neurabin on the activity of endogenous PP-1_I complexes purified from fresh pig brain. These studies indicate that neurabin has differential effects on the activity of specific forms of native brain PP-1_I.

Experimental procedures

Reagents

TP, benzamidine, DEAE-Sepharose, poly-L-lysine agarose, phenylmethylsulfonyl fluoride (PMSF) and bovine serum albumin (BSA) were from Sigma (Saint Louis, MO). Superdex 200 and [γ -³²P]ATP were from GE Healthcare (Piscataway, NJ).

Protein preparation

³²P-labeled phosphorylase a was prepared by phosphorylation of phosphorylase b by phosphorylase kinase as described [15]. The catalytic subunits of protein phosphatase-1 (PP-1_C) and protein phosphatase-2A (PP-2A_C) were purified to homogeneity from pig brain as described [15]. Human DARPP-32 was cloned from a human cDNA library, overexpressed and purified from transfected *E. coli*. (Invitrogen, CA). Phospho-Thr³⁴-DARRP-32 was prepared by phosphorylation of DARPP-32 with PKA as described [16].

Preparation of neurabin

Full-length neurabin forms high molecular weight oligomers that cannot be purified (data not shown). Soluble C-terminal fragments of rat neurabin (neurabin¹⁻⁴⁸⁵, neurabin¹⁻²¹⁰, neurabin¹⁻¹⁰², neurabin¹⁰³⁻²¹⁰, neurabin⁵⁰⁻¹⁵⁰ and neurabin⁵⁰⁻²¹⁰) were obtained by amplifying the rat cDNA [2] by PCR with the appropriate primers. The cDNA for each fragment with a polyhistidine (6 His) or glutathione-S-transferase (GST) tag was subcloned into the expression vector pTrcHis2-TOPO and used to transform BL21 *E. coli* as recommended by the manufacturer (Invitrogen, CA). Fragments were purified by chromatography on glutathione-agarose or Ni²⁺NTA-agarose (GE Healthcare, Piscataway, NJ). Neurabin¹⁻⁴⁸⁵ was further purified chromatography on Superdex 200 and Mono Q FPLC.

Assay of PP-1_I

Protein phosphatase-1_I was assayed by its ability to dephosphorylate ³²P-labeled phosphorylase a following preincubation with ATP and Mg²⁺. Briefly, the assay consisted of 0.01 ml of PP-1_I in 50 mM imidazole-Cl pH 7.2, 0.2 mM EGTA, 0.1% (v/v) 2-mercaptoethanol, 1 mg/ml BSA (Enzyme Diluent Buffer); 0.01 ml of Reaction Buffer (50 mM imidazole-Cl pH 7.2, 0.2 mM EGTA, 0.1% (v/v) 2-mercaptoethanol, 1 mg/ml BSA, 0.375 mM ATP and 3.75 mM MgCl₂ (Enzyme Assay Buffer); and 0.01 ml of 3 mg/ml ³²P-labeled phosphorylase a in Enzyme Diluent Buffer plus 75 mM caffeine. Protein phosphatase-1_I complex was preincubated with ATP and Mg²⁺ in Enzyme Assay Buffer for 5 min at 30°C prior to initiation of the reaction with ³²P-labeled phosphorylase a. After 10 min at 30°C, the reaction was terminated with 0.2 ml of 25% (w/v) trichloroacetic acid. The suspension was centrifuged at 10,000xg for 5 min and 0.2 ml of the supernatant containing the released [³²P]phosphate was analyzed for Cerenkov radiation in a liquid scintillation counter. One unit of phosphatase activity catalyzes the dephosphorylation of 1 nmol of substrate per min at 30°C.

Purification of PP-1_I

A 50 kg pig was anesthetized with an intramuscular injection of xylazine/ketamine in accordance with a protocol approved by the IACUC of Weill Medical College of Cornell University. Cardiac arrest was induced by intravenous injection of cold saturated KCl. The brain (~100g) was removed within 5 min of cardiac arrest by extraction through the maxilla, chilled on ice, cut into 1 cm pieces, and homogenized in 5 volumes of 50 mM imidazole-Cl pH 7.3, 2 mM EDTA, 2 mM EGTA, 0.1% (v/v) 2-mercaptoethanol, 1 mM benzamidine, 0.1 mM PMSF and 5% (v/v) glycerol by 6 × 30 sec pulses at low speed in a blender. All procedures were performed at 4°C. The homogenate was centrifuged at 10,000xg for 30 min. The supernatant (the soluble extract) was collected, diluted 2-fold in 25 mM imidazole-Cl pH 7.3, 0.2 mM EGTA, 0.1% (v/v) 2-mercaptoethanol, 1 mM benzamidine, 0.1 mM PMSF and 10% (v/v) glycerol (Buffer A), and loaded onto a DEAE-Sepharose column (2.5 × 20 cm) equilibrated in Buffer A. The column was washed with 300 ml of Buffer A plus 50 mM NaCl, and then eluted with Buffer A plus 300 mM NaCl. Active fractions of PP-1_I were collected, diluted 10-fold with Buffer A, and loaded onto a poly-L-lysine column (1.5 × 10 cm) equilibrated in Buffer A. The column was washed with 150 ml of Buffer A, then eluted sequentially with Buffer A plus 50 mM NaCl, 100 mM NaCl, 250 mM and 500 mM NaCl. Active fractions from each step were collected, concentrated by vacuum dialysis and separated on a Superdex 200 column (1.5 × 60 cm) equilibrated in Buffer A plus 200 mM NaCl at a flow rate of 0.75 ml/min. Active fractions were collected, concentrated by vacuum dialysis and stored at -20°C in Buffer A plus 50% (v/v) glycerol.

Miscellaneous methods

Protein concentration was determined by the method of Bradford using BSA as standard [17]. Data were analyzed using GraphPad Prism 4.2 (San Diego, CA) and expressed as mean ±SD. IC₅₀ and EC₅₀ values were determined using a sigmoidal dose-response curve fit program.

Results

Protein phosphatase-1_I was the major form of protein phosphatase-1 in cytosolic extracts of pig brain obtained immediately after cardiac arrest (Figure 1A). The Mg²⁺/ATP dependent phosphorylase phosphatase activity was inhibited by phospho-Thr³⁴-DARPP-32, a specific inhibitor of PP-1 [18]. PP-1_I activity was further purified by DEAE-Sepharose and poly-L-lysine agarose chromatography. Four forms of PP-1_I were identified by differential elution from poly-L-lysine agarose with NaCl, termed PP-1_{IA}, PP-1_{IB}, PP-1_{IC} and PP-1_{ID} (Figure 1B), and were further purified by gel filtration on Superdex 200. GST-neurabin¹⁻⁴⁸⁵ activated PP-1_{IA} with an EC₅₀ of 40 nM, and inhibited PP-1_{IB}, PP-1_{IC} and PP-1_{ID} with IC₅₀ values of 1.1 μM, 0.1 μM and 0.2 μM respectively. The effect of neurabin¹⁻⁴⁸⁵ on PP-1_{IA} was biphasic; reduced activation occurred above ~90 nM (Figure 2A). The effect of GST-neurabin¹⁻⁴⁸⁵ on PP-1_{IA} was specific for PP-1_{IA}; it did not activate purified PP-1_C or PP-2A_C (Figure 2B). Purified GST alone had no effect on these phosphatases (data not shown). PP-1_{IA} had an apparent molecular mass of 250 kDa by gel filtration chromatography (Figure 3), while PP-1_{IB}, PP-1_{IC} and PP-1_{ID} had apparent molecular masses of 250, 115 and 210 kDa, respectively, as determined by gel filtration (data not shown). Thus all four forms of native brain PP-1_I are multiprotein complexes consisting of PP-1_C, I-2 and additional unidentified proteins.

To identify the domain in neurabin responsible for activation of PP-1_{IA}, 6His-N-terminal fragments of neurabin were prepared and tested for their effects on PP-1_{IA} activity. Neurabin¹⁻²¹⁰ activated PP-1_{IA} with an EC₅₀ of ~30 nM and inhibited PP-1_{IB}, PP-1_{IC} and PP-1_{ID} with IC₅₀ values of ~1 μM, ~1 μM and ~30 nM respectively (Figure 4A). Neurabin

fragments consisting of amino acid residues 1-102, 103-210, 50-150 and 50-210 had no effect on PP-1_{IA} activity (data not shown). The effect of GSTneurabin¹⁻²¹⁰ on the activity of PP-1_{IA} required preincubation with Mg²⁺ and ATP. Thus, the effect was specific for Mg²⁺/ATP-dependent PP-1_I (Figure 4B).

Discussion

Protein phosphatase-1 is a highly regulated enzyme with multiple functions determined by complex interactions with a number of regulatory proteins and substrates [5,8,19]. The composition of the endogenous PP-1 complexes present in brain and their specific functions are poorly understood. Only PP-1_I has been characterized in any detail in brain [7,9,20]. Another form of PP-1, termed PP-1_{PN}, has also been identified in brain [21]. PP-1_{PN} is present predominantly in the nucleus and may regulate mRNA splicing [22]. Other forms of PP-1 have been identified in brain but they remain to be purified and characterized [23].

Previous work has shown that N-terminal fragments of neurabin inhibit PP-1_C with IC₅₀ values of 0.2-10 nM [1,14]. Most cellular PP-1_C exists bound to regulatory proteins and substrates rather than as the free catalytic subunit [5,8]. A major form of PP-1 in brain is PP-1_I identified by its association with I-2 and requirement for Mg²⁺/ATP [7,9]. In the present work, we identified four forms of PP-1_I, termed PP-1_{IA}, PP-1_{IB}, PP-1_{IC} and PP-1_{ID}. Neurabin inhibited the major form of PP-1, PP-1_{IC}, which accounted for ~60% of total PP-1_I activity.

Interestingly, neurabin had differential effects on the various forms of native PP-1_I. Recombinant neurabin¹⁻⁴⁸⁵ and neurabin¹⁻²¹⁰ activated PP-1_{IA} but inhibited PP-1_{IB}, PP-1_{IC} and PP-1_{ID} activity. These results indicate that neurabin has two domains of interaction with PP-1_I, an activation domain within amino acid residues 1-210 and an inhibitory domain within amino acid residues 210-485 that contains the canonical KIKF PP-1_C binding motif (1,14). The molecular basis for the interaction between neurabin¹⁻²¹⁰ and PP-1_{IA} that results in activation is currently under investigation. Activation by neurabin¹⁻²¹⁰ is specific for Mg²⁺/ATP-activated PP-1_{IA}, indicating specificity for a single form of PP-1_I. This indicates that activation requires modification of PP-1_C by other components of the PP-1_A holoenzyme.

The likely functions of PP-1_I can be deduced from its binding partners. The finding that neurabin interacts with two essential components of PP-1_I (PP-1_C and I-2) and that neurabin targets PP-1_C and I-2 to polymerized actin suggest that PP-1_I is involved in regulating cell morphology (14). This role is supported by the observation that overexpression of neurabin induces filopodia and dendritic spines in cultured hippocampal neurons [14]. Protein phosphatase-1 has also been implicated in modulating the actions of the D1 dopamine receptor, AMPA- and NMDA-type glutamate receptors, GABA_A receptors, and Ca²⁺, K⁺ and Na⁺ channels [19,24]. Further studies are indicated to determine which of the PP-1_I enzymes identified here interact with these substrates and how neurabin might mediate these interactions to affect channel function.

Acknowledgements

This work was supported by the Department of Anesthesiology, Weill Medical College of Cornell University, by the Phaeikhim Foundation for Biomedical Research, and by NIH grant MH 40899.

References

1. McAvoy T, Allen PB, Obaishi H, Nakanishi H, Takai Y, Greengard P, Hemmings HC Jr. Regulation of neurabin I interaction with protein phosphatase-1 by phosphorylation. *Biochemistry* 1999;38:12943–12949. [PubMed: 10504266]
2. Nakanishi H, Obaishi H, Satoh A, Wada M, Mandai K, Satoh K, Nishioka H, Matsuura Y, Mizoguchi A, Takai Y. Neurabin: a novel neural tissue-specific actin filament-binding protein involved in neurite formation. *J Cell Biol* 1997;139:951–961. [PubMed: 9362513]

3. Muly EC, Allen P, Mazloom M, Aranbayeva Z, Greenfield AT, Greengard P. Subcellular distribution of neurabin immunolabeling in primate prefrontal cortex: comparison with spinophilin. *J Comp Neurol* 2004;469:185–197. [PubMed: 14694533]
4. MacMillan IB, Bass MA, Cheng N, Howard EF, Tamura M, Stract BE, Wadzinski BE, Colbran RJ. Brain actin-associated protein phosphatase-1 holoenzymes containing spinophilin, neurabin, and selected catalytic subunit isoforms. *J Biol Chem* 1999;274:35845–35854. [PubMed: 10585469]
5. Cohen PTW. Protein phosphatase-1-targeted in many directions. *J Cell Sci* 2002;115:241–256. [PubMed: 11839776]
6. Hubbard MJ, Cohen P. On target with a new mechanism for the regulation of protein phosphorylation. *Trends Biochem Sci* 1993;18:172–177. [PubMed: 8392229]
7. Tung HYL, Reed LJ. Purification and characterization of protein phosphatase-II activating kinase from bovine brain cytosolic and particulate fractions. *J Biol Chem* 1989;264:2985–2990. [PubMed: 2914940]
8. Cohen P. The structure and regulation of protein phosphatases. *Annu Rev Biochem* 1989;58:453–508. [PubMed: 2549856]
9. Yang SD, Fung YL. Identification and characterization of an ATP.Mg-dependent protein phosphatase from pig brain. *J Biol Chem* 1985;260:13464–13470. [PubMed: 2414281]
10. Yang SD. Identification of the ATP.Mg-dependent protein phosphatase activator (FA) as a myelin basic protein kinase in brain. *J Biol Chem* 1986;261:11786–11791. [PubMed: 3017947]
11. Terry-Lorenzo RT, Elliot E, Weiser DC, Prickett TD, Brautigan DL, Shenolikar S. Neurabins recruit protein phosphatase-1 and inhibitor-2 to the actin cytoskeleton. *J Biol Chem* 2002;48:46535–46543. [PubMed: 12270929]
12. Terry-Lorenzo RT, Carmody LC, Voltz JW, Connor JH, Li S, Smith FD, Milgram SL, Colbran RJ, Shenolikar S. The neuronal actin-binding proteins, neurabin I and neurabin II, recruit specific isoforms of protein phosphatase-1 catalytic subunits. *J Biol Chem* 2002;277:27716–27724. [PubMed: 12016225]
13. Carmody LC, Bauman P, Bass MA, Mavila N, Depaoli-Roach AA, Colbran RJ. A protein phosphatase-1 γ 1 isoform selectivity determinant in dendritic spine-associated neurabin. *J Biol Chem* 2004;279:21714–21723. [PubMed: 15016827]
14. Oliver CJ, Terry-Lorenzo RT, Elliott E, Christensen-Bloomer WA, Li S, Brautigan DL, Colbran RJ, Shenolikar S. Targeting protein phosphatase-1 (PP1) to the actin cytoskeleton: the neurabin/PP1 complex regulates cell morphology. *Mol Cell Biol* 2002;22:4690–4701. [PubMed: 12052877]
15. Cohen P, Alemany S, Hemmings BA, Resink TJ, Stralfors P, Tung HYL. Protein phosphatase-1 and protein phosphatase-2A from rabbit skeletal muscle. *Methods Enzymol* 1988;159:390–409. [PubMed: 2842604]
16. Hemmings HC Jr, Williams KR, Konigsberg WH, Greengard P. DARPP-32, a dopamine- and adenosine 3':5'-monophosphate-regulated neuronal phosphoprotein. I. Amino acid sequence around the phosphorylated threonine. *J Biol Chem* 1984;259:14486–14490. [PubMed: 6501302]
17. Bradford AA. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976;76:248–254. [PubMed: 942051]
18. Hemmings HC Jr, Greengard P, Tung HYL, Cohen P. DARPP-32, a dopamine-regulated neuronal phosphoprotein, is a potent inhibitor of protein phosphatase-1. *Nature* 1984;310:503–505. [PubMed: 6087160]
19. Greengard P, Allen PB, Nairn AC. Beyond the dopamine receptor: the DARPP-32/protein phosphatase-1 cascade. *Neuron* 1999;23:435–447. [PubMed: 10433257]
20. Paudel AA, Agarwal-Mawal HK. Neuronal Cdc-2-like protein kinase (Cdk5/p25) is associated with protein phosphatase-1 and phosphorylates inhibitor-2. *J Biol Chem* 2001;276:23712–23718. [PubMed: 11320080]
21. Allen PB, Kwon YG, Nairn AC, Greengard P. Isolation and characterization of PNUTS, a putative protein phosphatase-1 nuclear targeting subunit. *J Biol Chem* 1998;273:4089–4095. [PubMed: 9461602]
22. Kim YM, Watanabe T, Allen PB, Kim YM, Lee SJ, Greengard P, Nairn AC, Kwon YG. PNUTS, a protein phosphatase-1 (PP-1) nuclear targeting subunit. Characterization of its PP1- and RNA-binding domains and regulation. *J Biol Chem* 2003;278:13819–13828. [PubMed: 12574161]

23. Colbran RJ, Bass MA, McNeill B, Bollen M, Zhao S, Wadzinski BE, Strack S. Association of brain protein phosphatase-1 with cytoskeletal targeting/regulatory subunits. *J Neurochem* 1997;920–929. [PubMed: 9282913]
24. Greengard P. The neurobiology of slow synaptic transmission. *Science* 2001;294:1024–1038. [PubMed: 11691979]

Fig 1. Cytosolic protein phosphatase activity in pig brain and Identification of four forms of PP-1_I by chromatography on poly-L-lysine agarose

A. Pig brain cytosol extract was prepared and assayed for phosphorylase phosphatase activity in the absence or presence of 1.25 mM Mg^{2+} and 0.125 mM ATP. The contribution of PP-1 activity to the phosphorylase phosphatase activity was determined using 100 nM phospho-Thr³⁴-DARPP-32, a specific inhibitor of PP-1_C. **B.** PP-1_I eluted from DEAE-Sephacel was separated by chromatography on poly-L-lysine agarose column. The column was washed with Buffer A plus 0 NaCl and then eluted sequentially with Buffer A plus 50 mM NaCl, 100 mM, 250 mM NaCl or 500 mM NaCl, as indicated. The eluates were assayed for PP-1_I activity in the presence (●) and absence (■) of Mg^{2+} and ATP. Similar results were obtained in 3 independent experiments.

Fig 2. Effect of neurabin¹⁻⁴⁸⁵ on the activities of PP-1_I, PP-1_C and PP-2_{AC}

A. PP-1_{IA} (●), PP-1_{IB} (■), PP-1_{IC} (▲) and PP-1_{ID} (★) were assayed in the presence of various concentrations of GST-neurabin¹⁻⁴⁸⁵ with 0.005 unit of PP-1_I. **B.** PP-1_{IA} (●), PP-1_C (▲) and PP-2_{AC} (■) were assayed in the presence of various concentrations of neurabin¹⁻⁴⁸⁵ with 0.005 unit PP-1_{IA}, 0.01 unit PP-1_C or 0.01 unit PP-2_{AC}.

Fig 3. Chromatography of PP-1_{IA} on Superdex 200

PP-1_{IA} obtained from the poly-L-lysine agarose column was separated on a Superdex 200 gel filtration column. Protein phosphatase activity was assayed in the absence (■) or presence of (●) 200 nM GST-neurabin¹⁻⁴⁸⁵ in Mg²⁺ and ATP. The arrows indicate the elution positions of the marker proteins thyroglobulin (660 kDa), apoferritin (440 kDa), catalase (200 kDa), bovine serum albumin (68 kDa) and ovalbumin (45 kDa). The calculated molecular mass of PP-1_{IA} is 250 kDa.

Fig 4. Effect of neurabin¹⁻²¹⁰ on the activity of PP-1_I

A. PP-1_{IA} (●), PP-1_{IB} (■), PP-1_I (▲) and PP-1_{ID} (★) purified through the Superdex 200 step were assayed in the presence of various concentrations of 6xHis-neurabin¹⁻²¹⁰ with 0.005 unit of PP-1_{IA}. B. PP-1_{IA} was assayed in the presence of various concentrations of 6xHis-neurabin¹⁻²¹⁰ in the presence (●) or absence (■) of Mg²⁺/ATP. 100% activity is equivalent to 0.005 unit of phosphorylase phosphatase activity with (●) or without (■) preincubation with Mg²⁺/ATP.