Regulation of protein phosphatase 2A by ARPP-16 and MAST3 kinase in striatum

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INTRODUCTION

Protein phosphorylation and dephosphorylation are fundamental mechanisms underlying synaptic transmission and plasticity. Phosphatases are involved in multiple excitatory and inhibitory pathways regulating synaptic strength and neuronal excitability. In medium spiny neurons in the striatum, synaptic activity and plasticity are regulated by glutamate input from the cortex and dopamine (DA) input from the substantia nigra. Previous studies of striatal neuron signaling have analyzed the dynamic mechanisms of DA- and Ca^{2+}-dependent pathways, and have highlighted a major role for DARPP-32 and RCS, two proteins phosphorylated by cAMP-dependent protein kinase A (PKA), that in turn regulate two major serine/threonine phosphatases expressed in striatum, PP1 and PP2B, respectively. (Walaas et al. 2011). In our current studies, we have identified a new regulatory pathway in striatum involving protein phosphatase 2A (PP2A) and the striatally-enriched phosphoprotein ARPP-16. Previous studies have identified two phosphorylation sites in ARPP-16, one near the C-terminus phosphorylated by PKA, and a second site, Ser46 (Ser62 in ARPP-19, a spliced isoform) located in a central region, that has not been well characterized yet. Our protein interaction studies have shown that ARPP-16 binds to the A subunit of PP2A. Furthermore we have demonstrated a key regulatory role for the microtubule-associated serine/threonine (MAST) 3 kinase, a serine/threonine kinase recently shown to be enriched in striatum. MAST3 phosphorylates ARPP-16 at Ser46 and regulates its inhibitory activity towards PP2A. ARPP-16 is basally phosphorylated to a high level by MAST3 kinase in striatum, leading to inhibition of PP2A towards selective substrates including Thr75 of DARPP-32. PP2A consists of an active dimer of a catalytic C subunit bound to a scaffolding A subunit. Various accessory B subunits also bind to the A subunit where they influence the substrate specificity and the localization of PP2A heterotrimeric isoforms (Xu et al. 2006; Xing et al. 2006). Moreover, MAST-phosphorylated ARPP-16 exhibits higher specificity for the trimeric forms of PP2A compared to its dimeric form, and the inhibition depends on the co-assembled B subunit. Finally, we have demonstrated in preliminary experiments that PKA plays a fundamental role in the MAST-mediated phosphorylation of ARPP-16 and regulation of PP2A activity. Overall, our model suggests, as for DARPP-32 and RCS, a role for ARPP-16 in the control of protein dephosphorylation and by that of the neuronal activity of striatal neurons. Recent studies in non-neuronal systems have shown that ARPP-19 and the related protein ENSA are phosphorylated by the greatwall kinase during the G2/M phase of the cell cycle, resulting in inhibition of PP2A (Mochida et al. 2010; Gharby-Ayachi et al. 2010).

Our results compliment these recent studies, but show that in striatal neurons this PP2A regulatory system is regulated and utilized in a distinct manner.

METHODS

Cell line culture: HEK 293-T cells were grown on un-coated plates in DMEM (Invitrogen), supplemented with 10% FBS.

GST pull-downs: His-ARPP-16 was immobilized onto 80 µl/sample (of 50/50 slurry) Talon metal affinity resin (Clontech Laboratory Inc, Mountain View, CA). Increasing amounts of purified PP2A-A (0, 20 ng, 40 ng, 200 ng, or 400 ng) were added to the beads and samples were incubated for 1 hr at 4° C with rotation. As a control, 40 ng PP2A-A was added to beads with no His-ARPP-16 immobilized.

Plasmids and Transfection: MAST3-HA, ARPP-16-HA, Balfa-FLAG, B56δ-FLAG and PR72-FLAG constructs were transfected in HEK293 cells with Lipofectamine 2000 (Invitrogen) in Opti-MEM media (Invitrogen) an. Protein expression was assayed at least 24 hr later.

ARPP-16 in vitro phosphorylation: Recombinant ARPP-16 fused to 6xHis tag was expressed in Escherichia coli (BL21) and purified using Ni-NTA Agarose (Qiagen). Purified 6xHis-ARPP-16 (1 µM) has been resuspended in 100 µl of phosphorylation buffer (50 mM Hepses pH 7.4, 10 mM Mg Cl2) in presence of 200 µM ATP (Sigma) or 1 mM thio-γ-ATP (Roche) and incubated at 37°C for different time with immunoprecipitated MAST3 kinase

Immunoprecipitation and Malachite green Phosphatase assay: Lysates of transfected cells were incubated with 50 µl (50% slurry) of anti-FLAG conjugated agarose beads for 2 h at 4 ° C. Immunocomplexes were washed three times in lysis buffer without phosphatase inhibitors and two times in PP2A reaction buffer (50 mM Tris-HCl, pH 7, 100 µM $CaCl_2$). Immunocomplexes for Bα-, B56δ- or PR72- PP2A trimers or purified PP2A-AC dimer (0.01 U/µl, Millipore) were resuspended in 100 µl of PP2A reaction buffer and incubated with or without ARPP-16 or thio-γ-phosphorylated ARPP-16 (50 nM, 100 nM, 1 µM) for 10 min at 37° C in presence of 500 µM Threonine phosphopeptide (K-R-p-T-I-R-R). The phosphatase activity was measured by malachite green assay kit (Millipore)

DARPP-32 in vitro phosphorylation and PP2A activity assay: recombinant purified DARPP-32 (200 ug) was phosphorylated by CDK1 at 30 ° C for 1 hour, in buffer containing 50 mM Tris-HCl, pH 7.1, 150 mM KCl, 10 mM magnesium acetate and 200 µM [γ-^{32}P] ATP. Proteins were precipitated in 100% Trichloroacetic acid (TCA) and after extensive washing the pellet was resuspended and dialyzed in 20 mM Tris-HCl pH 7.6, 5 mM β–mercapto-ethanol. For the PP2A assay, different PP2A heterotrimer were incubated with or without ARPP-16 or thio-γ-phosphorylated ARPP-16 (200 nM) in presence of 75 µg of [^{32}P]-DARPP-32 for 10 minutes at 30 ° C. Free [^{32}P] level was measured by scintilation after precipitation of [^{32}P]-phosphoproteins with cold TCA.

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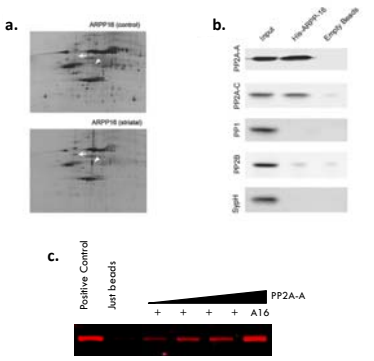
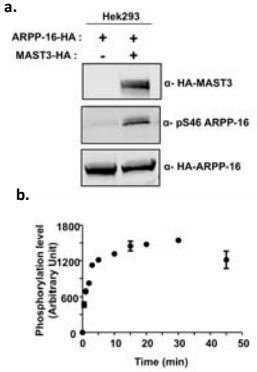
RESULTS

Figure 1: Identification of binding partners of ARPP-16 in rodent striatum, ARPP-16 interacts with the A subunit of PP2A.



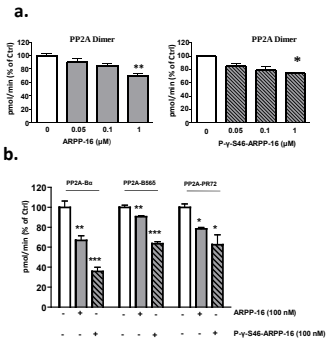
a. Rat brain S2 fractions were incubated with immobilized His-ARPP-16, then eluted proteins separated using DIGE. The striatal sample was labeled with Cy3, while the control sample (non-specific binding of striatal samples to beads with no ARPP-16) was labeled with Cy2. Samples were mixed and analyzed by DIGE. The white arrow indicates a spot corresponding to PP2A-A and the arrowhead indicates a spot corresponding to tubulin. **b.** Striatal lysate fraction S2 was incubated with His-ARRP-16 (100 ug) or beads alone (negative control). Eluted proteins were separated by SDS-PAGE and immunoblotted with antibody against PP2A-A, PP2A-C, PP1, PP2B and Synaptophysin. The lysate input (1%) is showed in lane 1, while lane 3 contains the elution from the negative control. **c.** Increasing amounts of recombinant, SEC-purified PP2A-A were incubated with immobilized His-ARPP-16 and bound protein was analyzed by SDS-PAGE and immunoblotting (lane 1 PP2A input, lane 2 eluat from beads with no His-ARPP-16 that were incubated with PP2A-A).

Figure 2: ARPP-16 is phosphorylated at Ser46 in intact cells and *in vitro* by MAST3 Kinase



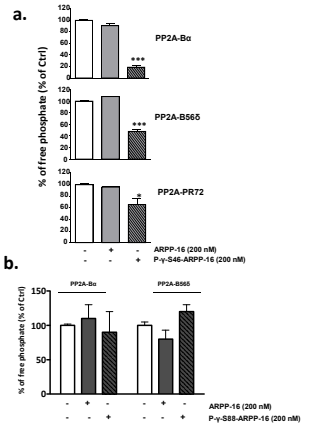
a. HEK293 were transfected with HA-ARPP-16 without or with HA-MAST3 kinase and the phosphorylation at Ser46 of ARPP-16 was analyzed by immunoblotting using phospho-specific antibody. **b.** Recombinant purified ARPP-16 (1µM) was incubated with immunoprecipitated MAST3 Kinase for various times (as indicated), and phosphorylation of Ser46 was measured by immunoblotting. Ser-46 phosphorylation was normalized to total ARPP-16 levels, and to the zero time value in each experiment. Results shown represent the average from three experiments.

Figure 3: P-S46-ARPP-16 selectively inhibits PP2A enzime in is heterotrimeric form.



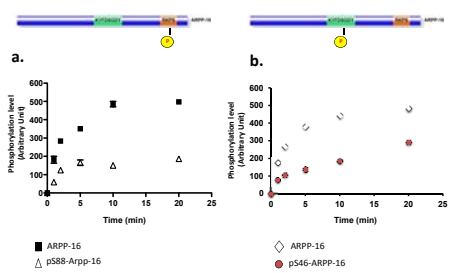
a. Purified PP2A-AC dimer (0.01 U/ µl) was incubated with increasing concentration (0 nM, 50 nM, 100 nM and 1 µM) of recombinant, purified ARPP-16 (upper pannel) or P-γ-Ser46-ARPP-16 (lower panel) for 10 minutes at 37° C with 500 µM phosphopeptide. Phosphate release was detected using a malachite green assay with absorbance at 650 nm. **b.** Recombinant Flag-Bα, Flag-B56δ and Flag-PR72 PP2A trimer over-expressed in HEK293 cells and isolated by immunoprecipitation with anti-Flag Ab, were incubated with 200 nM of ARPP-16 or P-γ-Ser46-ARPP-16 for 10 minutes at 37° C with 500 µM phosphopeptide. Phosphate release was detected using a malachite green assay with absorbance at 650 nm. Results are expressed as percent changes with respect to PP2A alone (white bar). *, $p < 0.05$; **, $p < 0.01$; *** $p < 0.001$, Newman-Keuls multiple comparison test.

Figure 3: P-S46-ARPP-16 but not P-S88-ARPP-16 inhibits PP2A heterotrimers in their ability to dephosphorylate P-T75-DARPP-32 *in vitro*.



a. & **b.** Recombinant Flag-Bα, Flag-B56δ and Flag-PR72 PP2A trimer over-expressed in HEK293 cells and isolated by immunoprecipitation with anti-Flag Ab, were incubated with 200 nM of ARPP-16 or P-γ-Ser46-ARPP-16 or P-γ-Ser88-ARPP-16 (**b**) for 10 minutes at 37° C with P^{32}-P-T75-DARPP32. The ability of Different PP2A trimers to dephosphorylate DARPP32 has been measured by scintillation. Results are expressed as percent changes with respect to PP2A alone (white bar). *, $p < 0.05$; **, $p < 0.01$; *** $p < 0.001$, Newman-Keuls multiple comparison test.

Figure 5: PKA/Ser88-ARPP-16 phosphorylation attenuates MAST3 ability to phosphorylate Ser46-ARPP-16 and viceversa

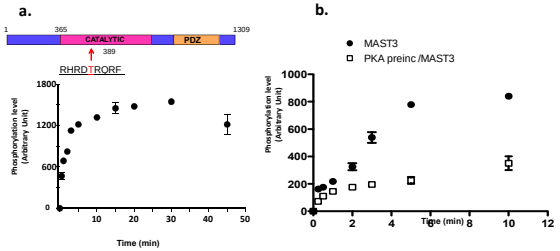


	Vm	Km
ARPP-16	1398±27.21	90.01±5.16
pSer88-ARPP-16	866.9±59.53	158.1±26.35

	Vm	Km
ARPP-16	6999±127.21	1685±51.16
pSer46-ARPP-16	1027±39.8	870.4±47.4

a. Recombinant purified ARPP-16 (1µM) or P-S88-ARPP-16 were incubated with ATP-γ-^{32}P and immunoprecipitated MAST3 Kinase overexpressed in HEK293 cells, for various times (as indicated); the protein were separated by SDS-PAGE and phosphorylation of Ser46 was measured by authoradiography . **b.** Recombinant purified ARPP-16 (1µM) or P-S46-ARPP-16 were incubated with ATP-γ-^{32}P and commercial purified PKA , for various times (as indicated); the protein were separated by SDS-PAGE and phosphorylation of Ser46-ARPP-16 was measured by authoradiography . Results shown represent the average from three experiments

Figure 6: MAST3 is phosphorylated by PKA *in vitro* and this event inhibits MAST3 activity, eg ability to phosphorylate ARPP-16



a. Immunoprecipitated MAST3 Kinase, overexpressed in HEK293 cells, was incubated with ATP-γ-^{32}P and commercial purified PKA for various times (as indicated); the protein were separated by SDS-PAGE and phosphorylation of MAST3 was measured by authoradiography .
b. The activity of phopshorylated MAST3 Kinase was measured as ability to phosphorylate ARPP-16 in comparison with normal MAST3. ARPP-16 (1µM) was incubated with P-MAST3 (previously phosphorylated by PKA) or MAST3 in presence of ATP-γ-^{32}P, for various times (as indicated); the protein were separated by SDS-PAGE and phosphorylation of Ser46-ARPP-16 was measured by authoradiography . Results shown represent the average from three experiments

ACKNOWLEDGMENTS

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CONCLUSIONS

* ARPP-16 negatively regulates PP2A activity and phosphorylation on Ser46 increases PP2A inhibition,

* Three heterotrimers of PP2A are inhibited by p-Ser46-ARPP16, but to varying extent,

* Phospho-ARPP-16 inhibits PP2A ' s ability to dephosphorylate specific substrates in striatum – eg DARPP-32 on Thr75,

* Phosphorylation on Ser88 –ARPP-16 does not affect PP2A activity,

* Phosphorylation of ARPP-16 by PKA or MAST mutually attenuate each others ability to phosphorylate ARPP-16,

* PKA phosphorylates MAST and decreases its activity