ARPP-16, protein kinase A and MAST3 kinase: a newly identified pathway for regulation of protein phosphatase PP2A in striatal neurons.

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INTRODUCTION

Protein phosphorylation is the most common post-translational modification in neuronal signaling and results from the balanced action of kinases and phosphatases.
We previously identified a family of striatal-enriched phospho-proteins substrates for PKA: DARPP-32, RCS and ARPP-16 (Walaas et al.,1983, Horiuchi et al.,1990)
While the role of DARPP-32 and RCS in the dopamine (DA)-mediated regulation of serine/threonine phosphatases in striatal medium spiny neurons (MSNs) has been well characterized (Walaas et al. 2011) little has been known about the role of ARPP-16.
ARPP-16 is related to two other members of ARPP family, ARPP-19 and ENSA. ARPP-19 and ENSA are ubiquitously distributed and have been identified as phosphatase PP2A inhibitors in mitotic cells. In Xenopus oocytes, phosphorylated by Greatwall kinase (GWT), ARPP-19/ENSA inhibit PP2A during the G2/M phase (Lorca & Castro 2013).
Recently, we found ARPP-16 directly interacts with PP2A in striatum and it is phosphorylated at Ser46 by microtubule-associated serine/threonine kinase 3 (MAST3), a mammalian analogous of GWT, enriched in striatum and still poorly characterized.
Phosphorylation of ARPP-16 at Ser46 by MAST3 converts the protein into an inhibitor of PP2A towards selective substrates including DARPP-32. Moreover, Ser46 of ARPP-16 is phosphorylated to a high basal stoichiometry in striatum, while activation of PKA by cAMP leads to marked dephosphorylation of Ser46 in striatal slices.
In the current study we further investigate the role of the phosphorylation mechanisms in ARPP-16 regulation. We demonstrated, both in vitro and in intact cells, that PKA plays a fundamental role in the MAST-mediated phosphorylation of ARPP-16 and regulation of PP2A. PKA phosphorylation of ARPP-16 at Ser88 negatively acts in an intramolecular fashion on Ser46 phosphorylation, leading to an inhibition of its ability to regulate PP2A. The phosphomimetic S88D-ARPP16 strongly suppresses MAST3 phosphorylation at Ser46 while the single PKA phosphorylation of Ser88 does not have any effect on the phosphatase inhibition. We also find that PKA phosphorylates MAST3 in vitro resulting in kinase inhibition, and that activation of cAMP by forskolin in transfected HEK cells significantly decreases MAST3 activity.
Overall these data suggest that P-Ser46-ARPP-16 acts to basally control PP2A in MSNs, but that DA, acting via PKA, regulates this pathway by inactivating ARPP-16 and/or MAST3 leading to selective potentiation of PP2A signaling. Further experiments are in progress to identify other specific MAST3 substrates in order to characterize and better understand the role of this kinase in the regulation of the synaptic activity in striatum.

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, MAST3T389A/D-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) was resuspended in 100 µl of phosphorylation buffer (50 mM Hepes pH 7.4, 10 mM MgCl2) in the 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
DARPP-32 in vitro phosphorylation and PP2A activity assay: recombinant purified DARPP-32 (200 µg) was phosphorylated by CDK1 at 30° C for 1 h, in buffer containing 50 mM Tris-HCl, pH 7.1, 150 mM KCl, 10 mM magnesium acetate and 200 µM [γ-32P] 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 heterotrimers were incubated with or without ARPP-16 or thio-γ-phosphorylated ARPP-16 (200 nM) in the presence of 75 µg of [32P]-DARPP-32 for 10 min at 30° C. Free [32P] level was measured by scintillation counting after precipitation of [32P]-phosphoproteins with cold TCA. **Cell line treatments:** transfected cells were treated with the cAMP activator forskolin (10 mM) for 30 min then lysed with 25mM Tris-HCl, 150 mM NaCl, 0.1% triton X100 in the presence of protease and phosphatases inhibitor. The levels of the different proteins were analyzed by immunoblotting with specific antibodies after SDS-PAGE. Blots were analyzed and quantified using an Odyssey Infrared Imaging System.
Striatal slice preparation: striatal slices rom 6-8 week old mice or 90-110 g rats were prepared as described with slight procedural modifications (Nishi et al., 1997). The brains were rapidly removed and placed in ice-cold, oxygenated in Krebs-HCO3 buffer (124 mM NaCl, 4 mM KCl, 26 mM NaHCO3, 1.5 mM CaCl2, 1.25 mM KH2PO4, 1.5 mM MgSO4, 10 mM d-glucose, pH 7.4). Coronal slices (350 µm) were prepared using a vibrating blade microtome (VT1000S, Leica Microsystems). Striata were dissected from the slices and then placed in incubation with 2 ml of fresh, oxygenated Krebs-HCO3 buffer and allowed to recover for 30 min with constant oxygenation with 95% O2/5% CO2 at 30° C followed by another 30 min of recovery with adenosine deaminase added to fresh Krebs-HCO3 buffer. Frozen striatal slices from mice or rats were sonicated in 1% SDS containing phosphatase and protease inhibitor cocktails and quantified. Total lysate (30 µg) was loaded on 10-20% gradient gels and immunoblotting was performed

CONCLUSIONS

- **ARPP-16 interacts with phosphatase PP2A by direct binding with the A subunit (Fig. 1),**
- **ARPP-16 is phosphorylated at S46 by MAST3 kinase (Fig. 2),**
- **Three heterotrimers of PP2A are inhibited by p-Ser46-ARPP16, but to varying extents (Fig. 3), phosphorylation on Ser88-ARPP-16 does not affect PP2A activity,**
- **Phosphorylation of ARPP-16 by PKA (on S88) or MAST3 (on S46) mutually attenuate each other's ability to phosphorylate ARPP-16 *in vitro* (Fig. 4),**
- **PKA phosphorylates MAST3 *in vivo* and *in vitro* and decreases its activity *in vitro* (Fig. 5),**
- **ARPP-16 phosphorylation on Ser88 is able, *in vivo*, to regulate in an intra-molecular fashion the phosphorylation on Ser46, at the same time, phosphorylation on Ser46 is just partially able to influence the phosphorylation on Ser88 by PKA (Fig.6),**
- **cAMP plays a key role toward MAST3 in the regulation of phosphorylation on S46-ARPP16 (Fig. 7).**

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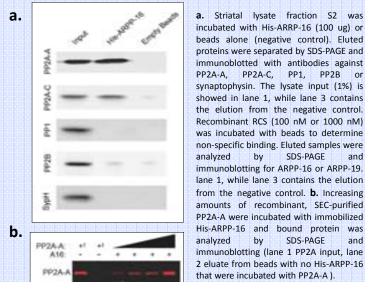
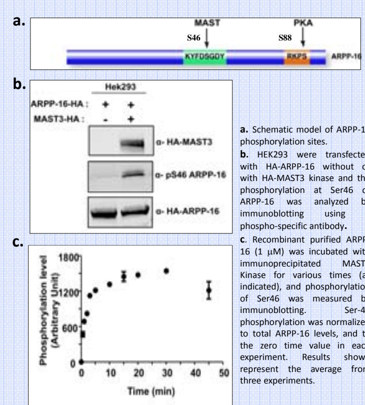
RESULTS

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



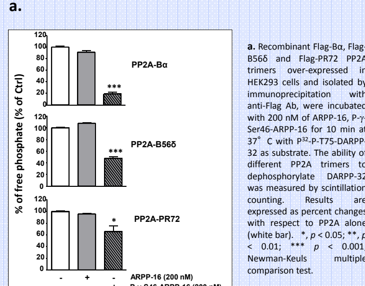
a. Striatal lysate fraction S2 was incubated with His-ARPP-16 (100 ug) or beads alone (negative control). Eluted proteins were separated by SDS-PAGE and immunoblotted with antibodies against PP2A-A, PP2A-C, PP1, PP2B or synaptophysin. The lysate input (1%) is showed in lane 1, while lane 3 contains the elution from the negative control. Recombinant RCS (100 nM or 1000 nM) was incubated with beads to determine non-specific binding. Eluted samples were analyzed by SDS-PAGE and immunoblotting for ARPP-16 or ARPP-19. lane 1, while lane 3 contains the elution from the negative control. **b.** 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 eluate 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



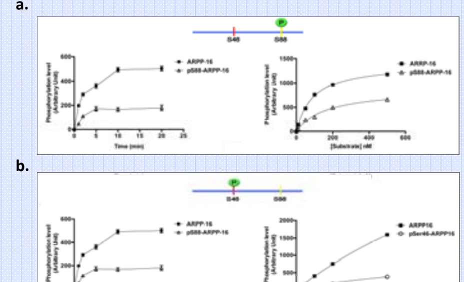
a. Schematic model of ARPP-16 phosphorylation sites.
b. HEK293 were transfected with HA-ARPP-16 without or with HA-MAST3 kinase and the phosphorylation at Ser46 of ARPP-16 was analyzed by immunoblotting using a phospho-specific antibody.
c. Recombinant purified ARPP-16 (1 µM) was incubated with immunoprecipitated 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 inhibits PP2A heterotrimer ability to dephosphorylate P-T75-DARPP-32 *in vitro*.



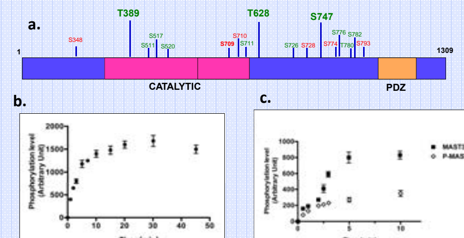
a. Recombinant Flag-Bα, Flag-B56δ and Flag-PR72 PP2A trimers over-expressed in HEK293 cells and isolated by immunoprecipitation with anti-Flag Ab, were incubated with 200 nM of ARPP-16, P-γ-Ser46-ARPP-16 for 10 min at 37° C with P32-P-T75-DARPP-32 as substrate. The ability of different PP2A trimers to dephosphorylate DARPP-32 was measured by scintillation counting. 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 4: PKA/Ser88-ARPP-16 phosphorylation attenuates MAST3 ability to phosphorylate Ser46-ARPP-16 and vice versa.



a. (left panel) Recombinant purified ARPP-16 or P-S88-ARPP-16 (1 µM) were incubated with ATP-γ-^{32}P and immunoprecipitated MAST3 Kinase overexpressed in HEK293 cells, for various times (as indicated); the proteins were separated by SDS-PAGE and phosphorylation of Ser46 was measured by autoradiography. **(right panel)** Increasing concentrations (10-500 nM) of ARPP-16 or P-S88-ARPP16 were incubated with ATP-γ-^{32}P and immunoprecipitated MAST3 Kinase overexpressed in HEK293 cells, for 2 min; proteins were separated by SDS-PAGE and phosphorylation of Ser46 was measured by autoradiography. Phosphorylation by MAST is modulated by previous PKA phosphorylation of ARPP-16 with a decrease in Vmax (from 1398 to 867 ^{32}P incorporation/min).
b. (left panel) Recombinant purified ARPP-16 (1 µM) or P-S46-ARPP-16 were incubated with ATP-γ-^{32}P and commercial purified PKA, for various times (as indicated); the proteins were separated by SDS-PAGE and phosphorylation of Ser46-ARPP-16 was measured by autoradiography. **(right panel)** Increasing concentrations (10-500 nM) of ARPP-16 or P-S46-ARPP16 were incubated with ATP-γ-^{32}P and commercial purified PKA, for 2 min; the proteins were separated by SDS-PAGE and phosphorylation of Ser88 was measured by autoradiography. Phosphorylation by PKA is modulated by previous MAST3 phosphorylation of ARPP-16 with decrease of Vmax (from 6999 to 1027 ^{32}Pincorporation/min).
(**a.** and **b.** left panels) The resulting values for phosphorylation are expressed in arbitrary densitometric units (a.u.) as mean ± SEM of five independent experiments.

Figure 5: MAST3 is phosphorylated by PKA *in vivo* and *in vitro* and this inhibits MAST3 activity.

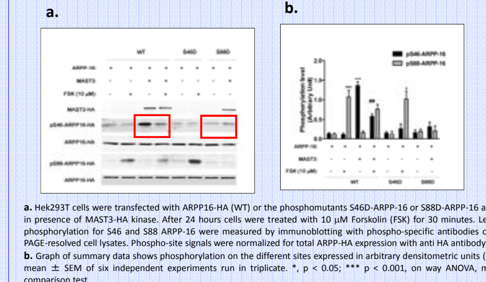


a. Schematic model of MAST3 kinase phosphorylation sites identified by MS analysyi. MAST3-HA was overexpressed in HEK293T cells and after 24 hours cells were treated with forskolin (10 uM for 30 min). MAST3-HA was then isolated by immunoprecipitation, resolved by eletrophoresis and the MAST3 gel band identified by Coomassie staining. The phosphorylated sites on MAST3 sequence were identified by Orbitrap MS/MS analysis. **b.** Immunoprecipitated MAST3 kinase, overexpressed in HEK293T cells, was incubated with ATP-γ-^{32}P and commercial purified PKA for various times (as indicated). The proteins were separated by SDS-PAGE and phosphorylation of MAST3 was measured by autoradiography. **c.** The activity of PKA-phosphorylated MAST3 kinase (P-MAST3) was measured as the ability to phosphorylate ARPP-16 in comparison with unphosphorylated MAST3. Recombinant purified ARPP-16 (100 nM) was incubated with P-MAST3 or MAST3 in the presence of ATP-γ-^{32}P, for various times (as indicated). The proteins were separated by SDS-PAGE and phosphorylation of Ser46-ARPP-16 was measured by autoradiography. **b.** and **c.** Results shown the average from three experiments (error bars show SEM).

d.

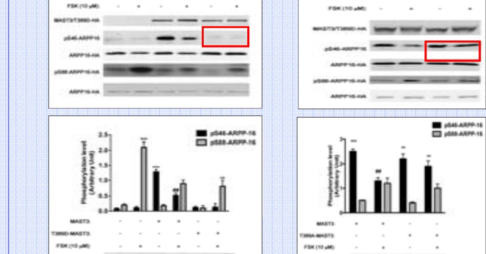
d. To confirm the PKA phosphorylation effect on MAST3 kinase activity we used mutant construct sfor T389-MAST3. The unphosphorylatable T389A-MAST3-HA and the phosphomimetic T389D-MAST3-HA were overexpressed in HEK293T cells. Immunoprecipitated MAST3 mutants were incubated with ARPP-16 (100 nM) in the presence of ATP-γ-^{32}P for different times. T389A-MAST3 was previously incubated for 20 min with PKA before the assay with ARPP-16. The proteins were resolved by SDS-PAGE and phosphorylation of Ser46-ARPP-16 was measured by autoradiography. Results represent the average from three experiments (error bars show SEM).

Figure 6 : Cyclic AMP dependent regulation of ARPP-16 phosphorylation on Ser46 and S88 in intact cell



a. Hek293T cells were transfected with ARPP16-HA (WT) or the phosphomutants S46D-ARPP-16 or S88D-ARPP-16 alone or in presence of MAST3-HA kinase. After 24 hours cells were treated with 10 µM Forskolin (FSK) for 30 minutes. Levels of phosphorylation for S46 and S88 ARPP-16 were measured by immunoblotting with phospho-specific antibodies on SDS-PAGE-resolved cell lysates. Phospho-site signals were normalized for total ARPP-HA expression with anti HA antibody.
b. Graph of summary data shows phosphorylation on the different sites expressed in arbitrary densitometric units (a.u.) as mean ± SEM of six independent experiments run in triplicate. *, $p < 0.05$; *** $p < 0.001$, on way ANOVA, multiple comparison test.

Figure 7: cAMP-dependent signaling regulates ARPP-16 phosphorylation via MAST3/PKA in a cell line model



a. The effect of cyclic AMP (cAMP) signaling as a mutual regulator of ARPP16 phosphorylation (S46 and S88) in intact cells was investigated. Hek293T cells were transfected with ARPP16-HA (A16-HA) alone or plus MAST3-HA or MAST3-T389D-HA. After 24 hours cells were treated with 10 µM Forskolin (FSK). Levels of S46 and S88 A16-HA were measured by immunoblotting with phospho-specific antibodies on SDS-PAGE-resolved cell lysates (upper panel). **b.** To address the role of PKA in the regulation of MAST3 activity in living cells A16-HA was co-transfected with wild type MAST3 or MAST3 T389A (unphosphorylatable) in Hek293T cells. Effect of FSK on the transfected cells was measured by immunoblotting with phospho-specific A16 antibodies on cell lysates (upper panel). Phospho-site signals were normalized for total ARPP-HA expression with anti-HA antibody. Graph of summary data (lower panel) shows phosphorylation on the different sites expressed in arbitrary densitometric units (a.u.) as mean ± SEM of four independent experiments run in triplicate. **, $p < 0.01$; *** $p < 0.001$, one way ANOVA, multiple comparison test. ##, $p<0.01$.