XVII International Congress of Eye Research (ICER). The International Society for Eye Research, ISER, Buenos Aires, 2006.

REGULATION OF EXTRACELLULAR ATP IN THE VERTEBRATE RETINA: ECTONUCLEOTIDASES LOCALIZATION AND ACTIVITY.

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INTRODUCTION

ATP is released by neurons and glia acting as a neurotransmitter and neuromodulator via purinergic receptors (P_2). Extracellular ATP concentrations are precisely regulated by membrane glycoproteins called ecto-nucleoside triphosphate diphosphohydrolases (ENTPDases). These enzymes hydrolyze extracellular nucleoside tri- and/or diphosphates whereas nucleoside monophosphates are not substrates. Resulting AMP is further metabolized to adenosine by an ecto-5´-nucleotidase. Adenosine, in turn, has been described as a potent inhibitory neuromodulator through type P_1 receptors. Activity of ENTPDases can be defined by their dependence on Ca^{2+} or Mg^{2+} and insensitivity to inhibitors of P-type, F-type, V-type ATPases, and phosphatases. ENTPDases may serve two major roles: 1) modulation of P2-receptor-mediated signaling by removal of extracellular ATP and ADP and 2) to generate extracellular adenosine and P1-receptor-mediated signaling. Several subtypes of ENTPDases have been described based on cloning. ENTPDases 1 and 2 can also be distinguished because ENTPDase 1 produces AMP in much higher rate than the subtype 2.

OBJECTIVE

To describe the presence of ENTPDas-like activity and characterize the expression pattern of vertebrate ENTPDase subtypes throughout retinal layers.

EXPERIMENTAL PROCEDURE

Zebrafish (*Danio rerio,* **ZF**) and mouse (*Mus musculus*) were used as indicated in figure legends.

Western blot: ENTPDases protein expression was detected with specific primary[1] and secondary antibodies (Ab.) coupled to peroxidase and a chemiluminescent substrate (Perkin Elmer).

Immunohistochemistry: Paraformaldehyde fixed cryostat sections (10 µm) were incubated with primary antibodies[1] directed against ENTPDases or retinal cells markers. Secondary flourophore conjugated Ab. (Jackson) were used to detect primary antibody binding.

Activity in retinal membrane homogenates: ATPase activity was started by adding 2 mM ATP and [γ^{32}P]-ATP. Reaction was stopped at fixed times by transferring aliquots to a stop solution. Released phosphate forms a complex that is extracted with isobutanol.

RESULTS

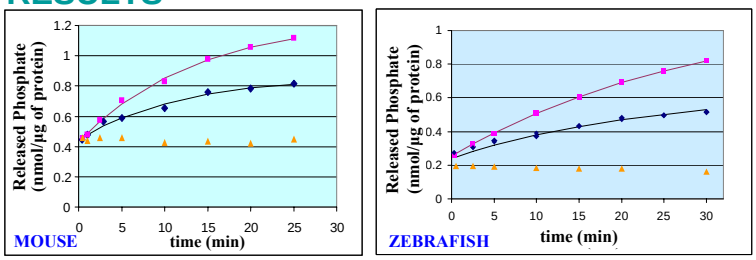

Figure 1. ENTPDase-like activity in membrane homogenates of vertebrate retinas. Squares show total ATPase and phosphatase activity, which was reduced to 59 % (ZF) and 55 % (Mouse) in presence of inhibitors (1 mM): Vanadate (P type -ATPases), N-ethylmaleimide (V type -ATPases), levamisol (Phosphatases) and 5 µg/ml oligomycin (F Type -ATPases) (diamonds). Lines depict fitting of experimental data by least squares analysis. Activity in the presence of inhibitors was completely abolished by a divalent cations chelator (EDTA, triangles). Vi: initial rate of enzymatic activity.

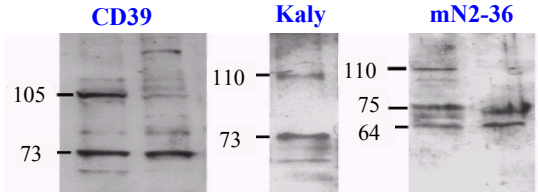

Figure 2. ENTPDase protein expression is detected by Western blot in the adult retina of zebrafish and mouse by using specific antibodies against mammalian ENTPDases 1 and 2. Antibodies Kaly and CD39 recognize ENTPDase subtype 1. mN2-36 Ab. recognizes ENTPDase 2. Bands of 73-75 kDa match previously described apparent molecular weights for mammalian ENTPDases in several tissues. ZF: zebrafish, ME: mouse epididyme as a positive control.

CONCLUSIONS

1. Vertebrate retina exhibits immunoreactivity for ENTPDase subtypes 1 and 2.
2. ENTPDases 1 and 2 present a different distribution among vertebrate species, as well as throughout retinal layers of the same animal.
3. Vertebrate retina demonstrates ENTPDase-like activity which was resistant to inhibitors of all known enzymes with ATPase or phosphatase activity. Such activity can be abolished by chelating divalent cations.
4. A regionalized localization of ENTPDase subtypes may suggest a differential regulation for ATP and ADP extracellular signaling as well as adenosine production in synaptic spaces.

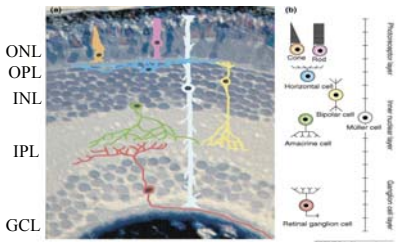

Figure 3. Photomicrograph of a retinal section from a 5 d-old ZF, with cell types scheme overlaid. ONL: outer nuclear layer (cone and rods nuclei); OPL: outer plexiform layer (synapses); INL: inner nuclear layer (interneurons nuclei); IPL: inner plexiform layer (synapis); GCL: ganglion cell layer.
Cayouette et al., Lineage in the vertebrate retina. Trends Neurosci. 2006 Oct; 29(10):563-70. Epub 2006 Aug 21.

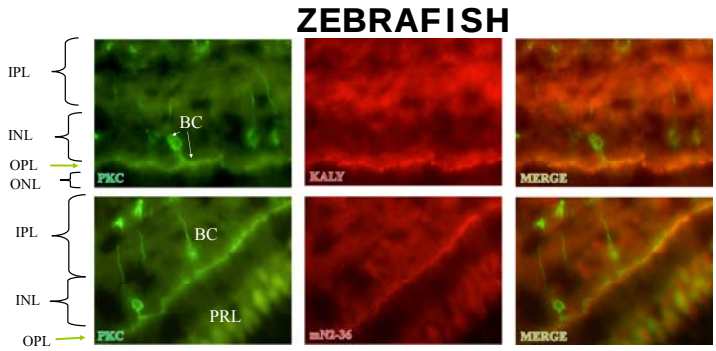

Figure 4. ENTPDases 1 and 2 detected by immunohistochemistry in ZF retina. Polyclonal antibody Kaly specifically detected ENTPDase 1, likely in photoreceptors feet. Polyclonal antibody mN2-36 specifically detects ENTPDase 2, also showed labeling in the OPL (with different distribution). Colabeling with PKC alpha (ON-bipolar cells, BP) showed no colocalization either with Kaly or mN2-36. Controls without either primary or secondary antibody, or with preimmune serum were systematically done (not shown). Magnification in all images 1000x, NA:1.3 . PRL: Photoreceptor layer.

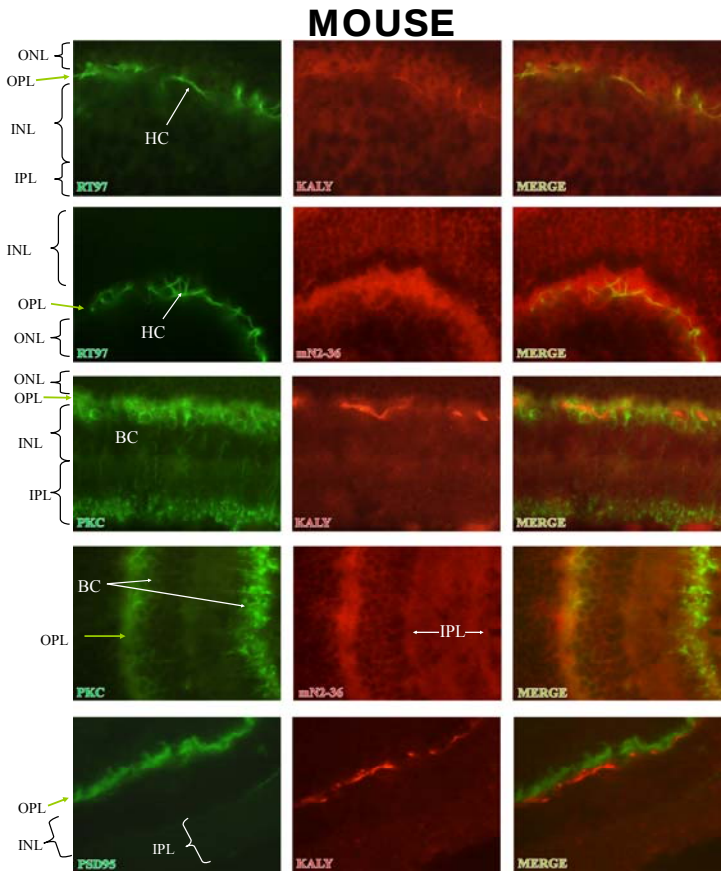

Figure 5. ENTPDases 1 and 2 detected by immunohistochemistry in mouse retina. Labeling of ENTPDases 1 (Ab. Kaly) and 2 (Ab. mN2-36). Colabeling with a Horizontal cell marker (RT-97) showed that ENTPDase 1 is expressed by Horizontal cells (HC). mN2-36 immunoreactivity showed a more diffuse distribution in the OPL and weaker labeling in the IPL. Colabeling with PSD95 (photoreceptor terminals) did not colocalize with ENTPDase 1. ENTPdase 2 expression may partially co-localize with PKCalpha (ON- bipolar cells). Controls without either primary or secondary antibody, or with preimmune serum were systematically done (not shown). Magnification in all images 1000x, NA:1.3.

Acknowledgments:

1. Antibodies Kaly and mN2-36 were kindly donated by Dr. Jean Sevigny (Université Laval, Québec, Canada). This work was supported by grants from Fundación Antorchas and CONICET. RT-97 (was obtained from Develop. Studies Hybridoma Bank, University of IOWA). CD-39, PSD-95, PKCalpha were purchased from different companies.