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

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INTRODUCTION

ATP is released by neurons and acts as a neurotransmitter and neuromodulator via purinergic receptors (type P_2 receptors). Extracellular ATP concentrations are precisely regulated by membrane glycoproteins, with extracellular catalytic domains, 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 membrane receptors. Activity of ENTPDases can be defined by their: 1) dependence on Ca^{2+} or Mg^{2+}; 2) insensitivity to inhibitors of P-type, F-type, V-type ATPases, and phosphatases. ENTPDases may serve at least two major roles: 1) terminate ATP/ADP-induced extracellular signal transduction; 2) regulate extracellular adenosine production and hence signaling. Several subtypes of ENTPDases have been characterized in mammals based on cloning. Two major subtypes: ENTPDases 1 and 2 can also be distinguished because ENTPDase 1 produces AMP in much higher rate than the subtype 2. Therefore, heterogeneous distribution of different ENTPDases among retinal layers suggests a localized regulation of extracellular adenosine levels.

OBJECTIVE

To characterize the expression pattern of vertebrate ENTPDase subtypes throughout retinal layers and to correlate this pattern to the localized enzyme activity.

EXPERIMENTAL PROCEDURE

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

Western blot: Total proteins from neural retinas (pigment epithelium free) were separated by SDS-PAGE and blotted. ENTPDases expression was detected by using specific primary antibodies[1] and a secondary antibody coupled to peroxidase and a chemiluminescent substrate (Perkin Elmer).

Immunohistochemistry: 10 µm-thick cryostat sections were obtained from paraphormaldehyde fixed eyecups. After blocking, retinal sections were incubated with several specific primary antibodies[1] directed against ENTPDases. A biotynilated antibody, avidin-biotin-peroxidase complex and DAB (Vector) were used to detect primary antibody binding.

Activity: 1. Autoradiography: Total ATPase activity was assayed on 10 µm-thick cryostat retinal sections in a buffer containing $CeCl_3$, ATP and [γ^{32}P]-ATP. Released phosphate forms an insoluble precipitate with cerium. After washing, dried slides were dipped in autoradiographic emulsion (Amersham) and exposed for a week. Sections were developed and inspected under the light microscope.

2. In retinal membrane homogenates: Total or Ecto-ATPase activities were measured in the buffer (omitting $CeCl_3$) used for autoradiography. Activity was started by adding cold and radioactively labelled ATP. Reaction was stopped at fixed times by transferring aliquots to a molibdate percloric acid solution. Released phosphate forms a phosphomolibdic complex that is extracted with isobutanol. Isobutanolic phase was separated and counted for radioactivity.

RESULTS

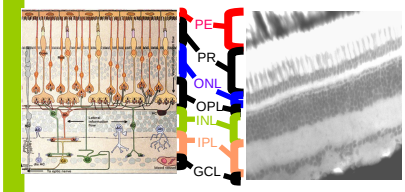

Figure 1. Schematic representation and photomicrograph of a retinal section of zebrafish. PE: pigment epithelium; PR: photoreceptor layer (cone and rods); ONL: outer nuclear layer (cone and rods nuclei); OPL: outer plexiform layer; INL: inner nuclear layer (interneurons nuclei); IPL: inner plexiform layer; GCL: ganglion cell layer; BC: bipolar cells; AC: amacrine cells; HC:horizontal cells; MC: Müller cells; GC: ganglion cells

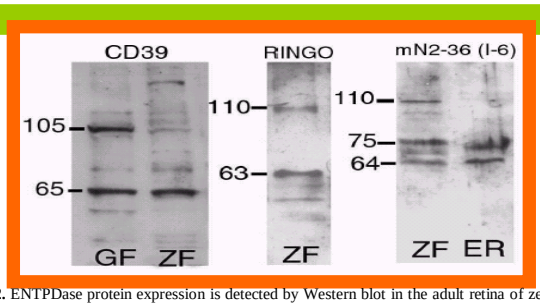

Figure 2. ENTPDase protein expression is detected by Western blot in the adult retina of zebra- and goldfish by using specific antibodies against mammalian ENTPDase 1 and 2. Antibodies CD39 and Ringo recognize ENTPDase subtype 1. I_6 antibody recognizes ENTPDase subtype 2. Bands of 63-65 kDa match previously described molecular weights for mammalian ENTPDases in several tissues. Higher molecular weight bands likely represent glycosylation states. GF: goldfish, ZF: zebrafish, ER: rat epididyme as a positive control.

CONCLUSIONS

- Vertebrate retina exhibits immunoreactivity for two major subtypes of ENTPDases.
- ENTPDases 1and 2 present a different distribution pattern among retinal layers. Subtype 2 is more prominently expressed in both synaptic layers in the outer and inner retina. Subtype 1 is mainly present in the inner half of the retina.
- Fish retina demonstrates ENTPDase activity which was resistant to inhibitors of all known enzymes with ATPase or phosphatase activity. Such activity can be abolished by chelating divalent cations necessary for ENTPDase activity.
- Total ATPase activity can be detected *in situ* on retinal sections.
- An heterogeneous ENTPDase distribution pattern may suggest a differential ATP/ADP extracellular signaling regulation as well as adenosine production throughout retinal layers.

ZEBRAFIS H

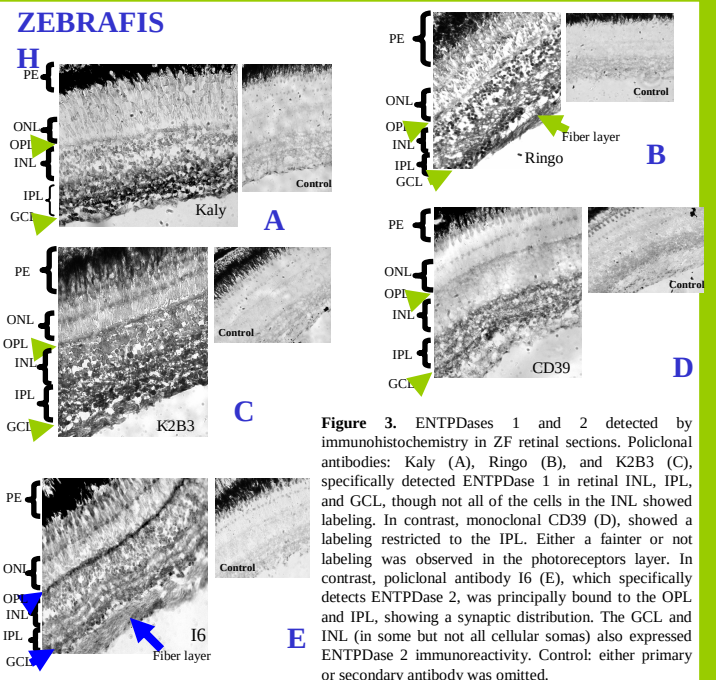

Figure 3. ENTPDases 1 and 2 detected by immunohistochemistry in ZF retinal sections. Policlonal antibodies: Kaly (A), Ringo (B), and K2B3 (C), specifically detected ENTPDase 1 in retinal INL, IPL, and GCL, though not all of the cells in the INL showed labeling. In contrast, monoclonal CD39 (D), showed a labeling restricted to the IPL. Either a fainter or not labeling was observed in the photoreceptors layer. In contrast, policlonal antibody I6 (E), which specifically detects ENTPDase 2, was principally bound to the OPL and IPL, showing a synaptic distribution. The GCL and INL (in some but not all cellular somas) also expressed ENTPDase 2 immunoreactivity. Control: either primary or secondary antibody was omitted.

MOUSE

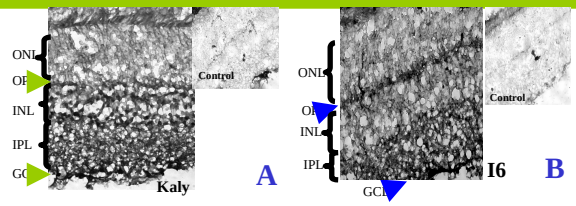

Figure 4. ENTPDases 1 and 2 detected by immunohistochemistry in mouse retinal sections. Labeling of ENTPDases 1 (Kaly, A) and 2 (I6, B) showed similar patterns as the ones observed for zebrafish retinas. ENTPDase 2 showed stronger immunoreactivity at the synaptic layers level (OPL and IPL) whereas ENTPDase 1 showed a darker labeling at the inner retina including IPL and retinal somas (INL and GCL). Again, as in fish, the immunoreactivity pattern showed a heterogeneous distribution, generally some cells were deeply labeled while other ones were not labeled at all.

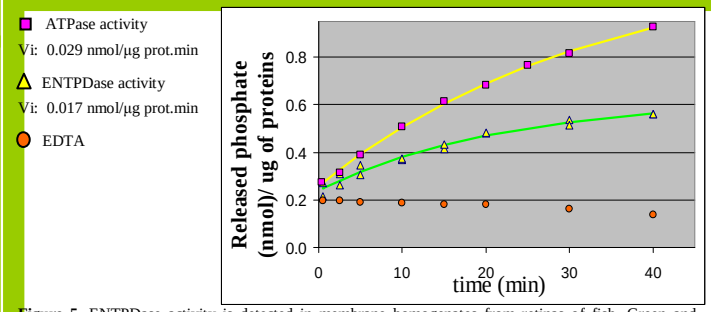

Figure 5. ENTPDase activity is detected in membrane homogenates from retinas of fish. Green and yellow lines depict fitting of experimental data by least squares analysis. Pink squares show total ATPase and phosphatase activity, which was reduced to 41 % in the presence of inhibitors: 1 mM Vanadate (P type -ATPases), 1 mM N-ethylmaleimide (V type -ATPases), 1mM levamisol (Phosphatases), and 5 µg/ml oligomycin (F Type -ATPases) (yellow triangles). Remaining activity is probably due to ENTPDases. This is further supported because activity in the presence of inhibitors was completely abolished by a divalent cations chelator (EDTA, orange circles). Vi: initial velocity (enzyme activity).

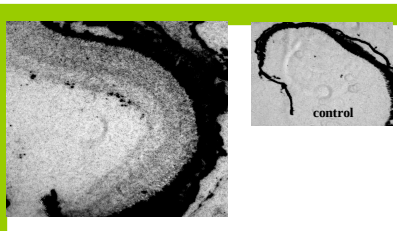

Figure 6. Autoradiograms showing total ATPase activity on retinal sections of zebrafish. Preliminary experiments showed ATPase activity detected *in situ* by autoradiography. The identity of enzymes responsible for this activity is currently under study. Sections previously treated with percloric acid served as controls.

Acknowledgments:

1. Antibodies (excepted CD39, purchased) were kindly donated by Dr. Jean Sevigny (Université Laval, Québec, Canada).

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