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REGULATION OF EXTRACELLULAR ATP IN THE ZEBRAFISH RETINA: ECTONUCLEOTIDASES LOCALIZATION AND ACTIVITY
Lionel D. Alfie, Pablo J. Schwarzbaum and Maria Paulia Faillace

INTRODUCTION
ATP is released by neurons and acts as a neurotransmitter and neuromodulator via purinergic receptors (type P receptors). Extracellular ATP concentrations are precisely regulated by membrane glycoproteins, with extracellular catalytic domains, called ecto-nucleoside triphosphate diphosphohydrolases (ENTPDases). These enzymes hydrolyze extracellular nucleotides to nucleosides 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, membrane receptors. Activity of ENTPDases can be defined by: 1) dependence on Ca²⁺ or Mg²⁺; 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-extrasynaptic signaling. Several subtypes of ENTPDases have been characterized in mammals based on cloning. Two main superfamily: 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 (Corvus 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. ENTPDase expression was detected by using specific primary antibodies and a secondary antibody coupled to peroxidase and a chemiluminescent substrate (Perkin Elmer). Immunohistochemistry: 10 µm-thick cryostat sections were obtained from paraffin-embedded fixed eyeups. After blocking, retinal sections were incubated with several specific primary antibodies directed against ENTPDases. A biotinylated antibody, avidin-biotin-peroxidase complex and DAB (Vector) were used to detect primary antibody binding. Activity: 1. Autoradiography: Total ATPase activity assay was carried out on 10 µm-thick cryostat retinal sections in a buffer containing CeCl3, ATP and [32P]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 activity were measured in the buffer (containing CaCl2) used for autoradiography. Activity was started by adding cold and radioactively labeled 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. Isobutanol phase was separated and counted for radioactivity.

RESULTS

CONCLUSIONS
- Vertebrate retina exhibits immunoreactivity for two major subtypes of ENTPDases. ENTPDases 1d and 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.

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