

Evidence for the Involvement of the $\text{Na}^+\text{-K}^+\text{-ATPase}$ in the Mechanism of Axonal Protein and Nucleoside Transport

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[^3H]Proline and [^3H]uridine were injected into both eyes of the goldfish. 1 h before and after this injection 3×10^{-6} M ouabain was administrated unilaterally to the retina. 8 h and 24 h after tracer injection the radioactivity in the retina, optic nerve and tectum was measured. It is suggested that the inhibition of the neuronal $\text{Na}^+\text{-K}^+\text{-ATPase}$ inside the retina is responsible for the reduction of the labelled material transported into the optic nerve.

The mechanism of axonal transport remains unknown. Microtubules are thought to play the most important role in generating the driving force for the rapid movement of substances^{1–3} but recent results indicate a more direct involvement of membranous structures in the transport mechanism^{4, 5}. This may not be a contradiction as would appear, since there is morphological, immunohistochemical and biochemical evidence for many interactions between microtubules and membranes^{6–9}. Moreover, an inhibition of the membrane-bound $\text{Na}^+\text{-K}^+\text{-ATPase}$ by the microtubule inhibitor colchicine was reported by Bos and Emmelot¹⁰.

The present study demonstrates evidence for the inhibition of the axonal transport of proteins and of low-molecular weight [^3H]uridine-compounds in the visual system of the goldfish following intra-ocular application of the specific inhibitor of $\text{Na}^+\text{-K}^+\text{-ATPase}$, ouabain.

Material and Methods

[^3H]proline (10 μCi ; spec. act. 17–22 Ci/mM) and [^3H]uridine (10 μCi ; spec. act. 8 Ci/mM; both tracers from Radiochemical Centre, Amersham, England) were injected into both eyes of 32 goldfishes (*Carassius auratus*). One hour before and one or two hours after this injection ouabain (Serva; 10 μl 10^{-5} M in 0.65% NaCl; final concentration in the eyebulb was approximately 3×10^{-6} M) was injected into the right eye. 10 μl 0.65% NaCl solution were injected into the left eye as control. 8 or 24 h after tracer injection, the animals were decapitated. The retinae were dis-

sected out and the optic tracts were each divided into four segments: the optic nerve (1–3) and the optic tectum (4). After determining the wet weight of the tissue (retina and optic tract), the non-incorporated [^3H]proline-labelled compounds were extracted with 10% Trichloroacetic acid (TCA; 24 h; 2 °C). The specimens were washed in distilled water and solubilized in 0.5 ml Protosol (NEN-Chemicals). After labelling the tissue with [^3H]uridine, it was not treated with TCA overnight because the non-incorporated, soluble compounds transported in the optic system were to be measured (the amount of high-molecular weight [^3H]uridine compounds (RNA and other polynucleotides) following the experimental time used here is only about 5% of the total radioactivity¹¹). For determination of [^3H]uridine radioactivity of the retina some pieces of equal area were delivered from the vitreous body, washed several times in TCA and distilled water (for removal of tracer not taken up, but retaining of tracer not incorporated) and weighed. After solubilization 10 ml Instagel (Packard) were added and the radioactivity was determined in a liquid scintillation counter (Beta-Scint BF 5000). For determination of the blood-borne radioactivity as caused by inevitable damage of blood vessels during the injection, the radioactivity of the cerebellum was also measured. In order to prove the distribution of ouabain in the visual system, 0.1 μCi [^3H]ouabain (spec. act. 9 Ci/mM; Radiochemical Centre Amersham) was injected unilaterally into one eye-bulb of 8 animals. The tracer was transduced from benzene-ethanol (1:9) into water by nitrogen-mediated evaporation. 8 hours and 2 days after injection the animals were sacrificed, the optic tracts segmented and the segments solubilized directly in protosol for determination of radioactivity.

The preparation for electron microscopy was done in the same manner as previously described^{12, 13}.

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Results

Figs 1 a and 1 b show the distributions of TCA-insoluble radioactivity along the optic tract of 7 goldfishes after intraocular injection of [³H]proline

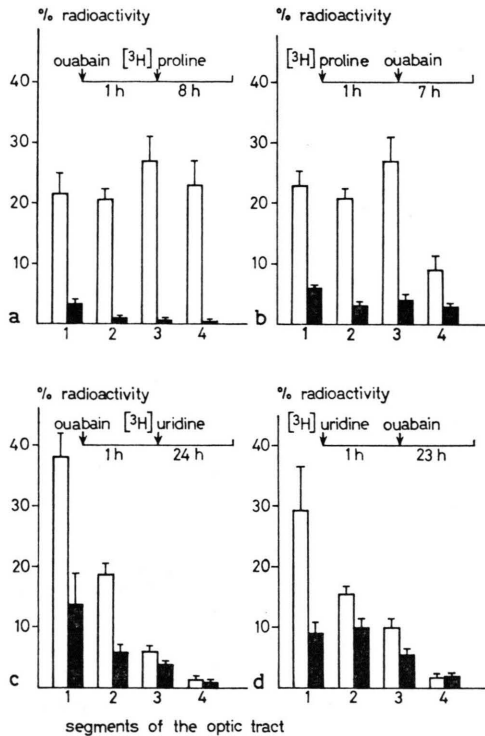


Fig. 1. Effect of 3×10^{-6} M ouabain on the distribution of TCA-insoluble [³H]proline radioactivity (a, b) and of TCA-soluble [³H]uridine radioactivity (c, d) in the visual system of *C. auratus*. The radioactivity of the cerebellum was subtracted from the radioactivity of each segment (cpm/mg fresh weight), which was calculated as percentage of the total radioactivity of the visual system. Experimental arrangement is represented above each graph. White columns: NaCl-injected system, black columns: ouabain-injected system. — In a, c, d 4 animals, in b 3 animals were used.

as well as the effect of ouabain. The fast rate of protein transport is about 100 mm/d¹⁴, so that an experimental time of 8 h after tracer injection is sufficient. By application of ouabain 1 h *before* the tracer the radioactivity in the treated nerve is strongly reduced in comparison with the saline injected control system and not much higher than in the cerebellum (Fig. 1 a), which is labelled only via the blood-stream and serves as a background control. If the drug was injected one h *after* the amino acid (Fig. 1 b), the radioactivity was also reduced, but clearly to a lesser extent than in the experiment shown in Fig. 1 a. The label was distinctly higher than in the cerebellum, indicating a specific accumulation of radioactivity in the nerve following tracer administration to the ganglion cell body. The radioactivity in the retina was only reduced when the inhibitor was applied before the tracer (Table II).

Concerning the soluble, low-molecular-weight, [³H]uridine-compounds, 8 h after [³H]uridine injection only the first nerve segment behind the eyeball is labelled (Table I). For observation of transport to the nerve endings in the optic tectum, a time of at least 24 h is necessary because the rate of nucleoside transport in the axon is only about 30–50 mm/d¹¹. If ouabain is applied 1 h before [³H]uridine the radioactivity in the experimental system is clearly reduced, to about the same extent (Fig. 1 c) as observed when [³H]uridine was injected 1 or 2 h before injection of the drug (Fig. 1 d). The retinal radioactivity shows no difference between pre- and postinjection of ouabain relative to the time of tracer-administration (Table II).

8 hours and 2 days after unilateral intraocular [³H]ouabain-injection no significant radioactivity in the optic tract was found. There was no difference

Table I. Measurements of the distribution of TCA-soluble radioactivity along the optic tract and in the cerebellum of 3 individuals of *C. auratus*. 10 μ l 10^{-6} M ouabain were injected intraocularly 1 h before injection of 10 μ Ci [³H]uridine. Tracer incubation time was 8 h. Only the first segment is clearly labelled, labelling of the first segment of the ouabain treated system is reduced. — c, NaCl-injected system; e, ouabain injected system; numbers in brackets: percentage of radioactivity of the total visual system.

Segments of optic nerve (1–3), optic tectum (4), Cerebellum (C); radioactivity in cpm/mg fresh weight.

	1	2	3	4	C
c	866 (36.5)	169 (7.1)	113 (4.8)	85 (3.6)	73 (3.1)
e	535 (22.6)	253 (10.7)	185 (7.8)	92 (3.9)	
c	740 (36.1)	200 (9.8)	112 (5.5)	76 (3.7)	66 (3.2)
e	540 (26.3)	143 (7.0)	96 (4.7)	77 (3.7)	
c	1012 (39.5)	250 (9.8)	116 (4.5)	113 (4.4)	76 (3.0)
e	505 (19.7)	240 (9.4)	140 (5.5)	113 (4.4)	

2. Injection 1. Injection	Saline	Ouabain	[³ H]uridine	[³ H]proline
saline ^a	—	—	66.2	263.8
ouabain ^a	—	—	57.8	191.0
[³ H]uridine ^b	67.9	68.7	—	—
[³ H]proline ^a	278.5	260.0	—	—

Table II. Measurements of retinal radioactivity (cpm/ μ g tissue), obtained after intraocular injection of ouabain before or after tracer injection. A reduction of radioactivity is only seen, if ouabain was injected before [³H]proline. ^a 1 h before the 2nd injection, ^b 2 h before the 2nd injection, the reduction of radioactivity in the ouabain treated optic tract was nearly the same as shown in Fig. 1 d.

between the radioactivities of the left and right optic nerve and nearly no difference between the visual system and the cerebellum, respectively.

The fine structure of ganglion cell bodies in the retina of *C. auratus* appears normally 8 h after injection of 10 μ l 10⁻⁵ M ouabain. Between 24 and 48 h after injection first alterations are seen in the cytoplasm (myelin bodies, lipid-like bodies, phagolysosomes), whereas the optic nerve remains normal.

Discussion

The results show a clear reduction of both TCA-insoluble [³H]proline labelled proteins and of TCA-soluble [³H]uridine labelled compounds in the goldfish visual system after perikaryal application of ouabain. This reduction might be interpreted in a number of ways: 1. inhibition of tracer uptake mechanism into the retinal ganglion cells, 2. inhibition of perikaryal synthesis, 3. inhibition of the transport mechanism within the intraocular regions of axons. Ouabain at the concentration of 2 $\times$ 10⁻⁶ M is known to inhibit the uptake of different amino acids into mouse brain slices by as much as 31–58%¹⁵. In the study presented here the [³H]-proline radioactivity in the retina was reduced by ouabain-pretreatment, too, but the reduction is not sufficient, to explain the lack of radioactivity in the corresponding visual system of the experiments shown in Fig. 1 a simply by uptake inhibition. In Fig. 1 b an experiment is presented in which the amino acid was allowed to be taken up before application of the drug. The retinal radioactivity was not reduced (Table II), the reduction in radioactivity in the optic tract is less than in Fig. 1 a. It is known from other cells that ouabain can also inhibit protein and nucleic acid synthesis¹⁶. This may explain both the reduced [³H]proline radioactivity

in the retina after pretreatment with ouabain and the reduced amount of the TCA-insoluble labelled compounds in the optic tract, but not the reduction of soluble [³H]uridine radioactivity. These and other nucleosides are transported from the perikaryon directly into the axon^{11, 17–20}. Thus, the effect of ouabain shown in Fig. 1 c–d results neither from uptake inhibition (Table II) nor from an effect on synthetic processes in the retina concerning the nucleotides and the RNA, but is an effect on the transport mechanism within the intraretinal region of the optic neurons.

Anderson and Edström²¹ and Edström²² have reported the inhibition of fast axonal transport by ouabain (10⁻³–10⁻⁴ M) *in vitro* applied directly to the axon. Garcia *et al.*²³ found an inhibition of enzyme accumulation in front of a ligature *in vitro* by 10⁻⁴ and 10⁻⁵ M ouabain treatment. It is known that for many cell systems ouabain can be toxic at low concentrations^{12, 13, 24, 25}. In the experiments presented here, the preservation of the ganglion cells was checked by electron microscopy. From this, it may be assumed that 8 h after tracer injection when the cells are still morphologically intact, no physiological damage should be expected. After this time the radioactivity of the first nerve segment behind the eye-bulb is decreased (Table I). This reduced radioactivity continues to migrate proximodistally. After 24 h, the label has reached both optic tecta. Since ouabain is restricted to the vitreous body and the retina after injection into the eye-bulb, the influence of ouabain on the tracer distribution in the optic nerve is not due to any effects at the axolemma in the optic nerve. The action site to be considered may be the dendritic and/or the perikaryal membrane and the intraretinal parts of optic axons. It is suggested that at each level of the neuron the transport process, at least for nucleosides, perhaps also for faster transported materials

(see Fig. 1 b), is regulated by the Na⁺-K⁺-ATPase. The effects, measured in the nerve, are evoked in the retina, but possibly also could be evoked at all levels of the free axon outside the retina. In order to prove this, non-toxic concentrations of ouabain must be applied locally *in vitro*. Another mode of drug action not yet proved, but not to rule out from these experiments, could be the inhibition of the exportation of transportable material through the gate² of the axon hillock into the axon.

In the axons, in which the nucleoside-transport is inhibited by ouabain, the microtubules remained morphologically intact¹³. In other studies, transport inhibition by colchicine without microtubule dissas-

sembly has been reported²⁶⁻²⁸. Therefore, a direct involvement of microtubules in the transport mechanism may be questioned; microtubular-membranous interactions^{6,7,10}, to disturb probably both by microtubule inhibitors and by inhibitors of membranous Na⁺-K⁺-ATPase, are thought to be of larger importance for the maintenance of axonal transport.

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