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Fluorescent labelling of Na^+,K^+-ATPase in intact cells by use of a fluorescent derivative of ouabain: Salinity and teleost chloride cells

Stephen D. McCormick

Department of Integrative Biology and Cancer Research Laboratory, University of California, Berkeley, California, USA

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Summary. Anthroylouabain, a fluorescent derivative of ouabain, was used to localize Na^+,K^+-ATPase in transport epithelia of two species of teleosts. Exposure of the opercular membrane of seawater-adapted tilapia (*Oreochromis mossambicus*) and the jaw skin of the long-jawed mudsucker (*Gillichthys mirabilis*) to a 2 μM anthroylouabain solution resulted in the appearance of cells stained bright blue. These were deemed to be chloride cells by their large size, distinct morphology and co-localization of DASPEI fluorescence, a mitochondrial stain. Addition of ouabain (1 mM final concentration) greatly decreased anthroylouabain fluorescent staining of chloride cells of seawater-adapted fish. Exposure of opercular membranes from freshwater tilapia to 2 μM anthroylouabain did not result in significant staining. Anthroylouabain is therefore a useful vital stain for localizing Na^+,K^+-ATPase in chloride cells of seawater-adapted teleosts, and may be useful for fluorescent labelling of ouabain-sensitive Na^+,K^+-ATPase in other tissues and species.

Key words: Na^+,K^+-ATPase – Anthroylouabain – Chloride cells – Ion transport – Ouabain – Fluorescent staining – *Oreochromis mossambicus* (Teleostei) – *Gillichthys mirabilis* (Teleostei)

Na^+,K^+-ATPase, the sodium pump, is ubiquitous in animal cells. This ion-translocating enzyme performs a variety of functions including maintenance of intracellular and extracellular ion homeostasis, acid-base regulation, repolarization of excitable cells, and nutrient transport (Rossier et al. 1989). In transporting epithelia, Na^+,K^+-ATPase is present in large quantities to generate ionic and electrical gradients utilized for net transport of salts and organic compounds. Many transport tissues such as the teleost gill and skin and the vertebrate kidney are heterogeneous in nature, containing many cell types of which those involved in net ion transport may be a minority. Precise localization of these cells, which can be accomplished by labelling Na^+,K^+-ATPase, is important in determining sites of ion transport. Previous methods have relied on autoradiography using radioactively labelled ouabain (Karnaky et al. 1976), cytochemical localization in partially-fixed thin sections (Firth 1987), or immunohistochemistry (Siegel et al. 1984). The use of fluorescence to localize membrane components such as Na^+,K^+-ATPase allows the examination of living tissue directly and instantaneously, which in turn provides flexibility in experimental manipulation of the tissue.

Ouabain (a glycoside also known as g-strophantin) is a potent inhibitor of Na^+,K^+-ATPase, which binds in a highly specific manner to the phosphorylated enzyme; this binding is antagonized by potassium (Yoda and Yoda 1988). Fortes (1977) reported that a fluorescent derivative of ouabain, anthroylouabain, increased in fluorescence upon binding to purified membrane preparations of Na^+,K^+-ATPase. The present study examines anthroylouabain fluorescence in ion transport tissue of two teleost species, where it is shown to bind specifically to Na^+,K^+-ATPase-rich chloride cells. This technique should have applications to other tissues and species where ouabain-sensitive Na^+,K^+-ATPase is present in high concentrations.

Materials and methods

Animals

Tilapia (*Oreochromis mossambicus;* 30–60 g body weight) were maintained in outdoor, freshwater ponds at 27–29° C. Tilapia were gradually adapted to 30–33 parts per thousand (ppt) artificial seawater in 30 gallon tanks with charcoal filtration and aeration at

Send offprint requests to: Stephen D. McCormick, Department of Zoology, University of California, Berkeley, CA 94720, USA

27–29° C in a room with constant light conditions (12L:12D). Long-jawed mudsuckers (*Gillichthys mirabilis;* 8–20 g body weight) were obtained from the wild (San Francisco Bay) and maintained in 5-gallon tanks with charcoal filtration and aeration at 12–14° C and 30–33 ppt in a room with constant light conditions (12L:12D). Fish were adapted to seawater for at least 2 weeks prior to sampling. They were removed from tanks and immediately pithed; opercular membranes of tilapia and jaw skins of the long-jawed mudsucker were dissected as described by Foskett et al. (1981) and Marshall and Nishioka (1980), respectively. Tissues destined for measurement of Na^+,K^+-ATPase activity were placed in ice-cold SEI buffer (0.3 M sucrose, 0.02 M Na_2 ethylene diamine tetraacetic acid, and 0.05 M imidazole, pH 7.3), frozen immediately on dry ice and stored at −80° C.

Solutions

Low-K^+ tilapia Ringer solution (LKTR) was composed of (in mM): 140 NaCl, 15 $NaHCO_3$, 1 $MgSO_4$, 2 $CaCl_2$, 1 NaH_2PO_4, 0.1 KCl and 5.5 glucose adjusted to pH 7.8 with H_2SO_4 following aeration with 100% O_2. A 2 mM anthroylouabain (Sigma) stock solution was prepared by dissolving anthroylouabain in 100% ethanol which can be stored at −20° C for at least 6 months. Just prior to use the stock solution was diluted 1:999 in LKTR to yield a 2 μM anthroylouabain-LKTR solution. Dimethylaminostyrylethyl-pyridiniumiodine (DASPEI, Bereiter-Hahn 1976) was prepared in a stock solution of 8 mM in distilled water and stored in a dark bottle at room temperature. 1 mg/ml phenylenediamine solution was prepared by dissolving 2 mg/ml phenylenediamine in LKTR and diluting 1:1 with glycerol.

Tissue incubation and examination

Following dissection, tissue was rinsed in 4 ml LKTR at room temperature for 1 min, then placed in 4 ml of 2 μM anthroylouabain in LKTR in a Petri dish aerated with 100% O_2 at room temperature for 1 h. The tissue was removed and rinsed twice in 2 ml ice-cold LKTR for 3 min, placed on a glass slide (routinely with apical surface up) and covered with a glass coverslip. Anthroylouabain has an excitation wavelength of 365–368 nm and emission wavelength of 480 nm (Fortes 1977). For routine observation, a Zeiss standard RA microscope modified for epifluorescence with a 100 W mercury illuminator, a 365 nm excitation filter, a 395 chromatic beam splitter and a 420 nm longwave pass filter was used. Pictures were taken on 400 ASA color slide film for 4–20 s or on 3200 ASA black and white print film for 1–4 s, depending on the magnification used. Cell density was measured by counting the number of anthroylouabain-positive cells in at least 10 fields of 0.46 mm^2.

Simultaneous staining with anthroylouabain and the mitochondrial stain DASPEI (Bereiter-Hahn 1976) was achieved by diluting stock solutions to yield a final concentration of 2 μM DASPEI and 2 μM anthroylouabain in LKTR as above. Anthroylouabain was examined as above with the exception that the 420 nm longpass filter was replaced by a 430–490 nm bandpass filter, which excluded DASPEI fluorescence. A 450–490 nm bandpass excitation filter, a 510 nm chromatic beam splitter, and a 520 nm longwave pass filter excluded all anthroylouabain fluorescence and was used to examine DASPEI staining.

Na^+,K^+-ATPase Activity

Five to ten min prior to assay, tissue was rapidly thawed, removed from SEI buffer and homogenized in 85 μl SEI buffer with 0.1% Na deoxycholate. The homogenate was centrifuged at 5000 *g* for 30 s, and the supernatant removed and assayed for Na^+,K^+-ATPase activity and protein content (McCormick and Bern 1989). 20 μl of homogenate were placed in a final volume of 1 ml assay mixture containing 50 mM imidazole, 1 U/ml LDH, 2.5 U/ml PK, 2 mM phosphoenolpyruvate (PEP), 0.05 mM NADH, 0.5 mM ATP, 0.4 mM KCN, 45 mM NaCl, 2.5 mM $MgCl_2$, and 10 mM KCl (pH 7.5). A second cuvette also containing 0.5 mM ouabain was assayed simultaneously. The assay mixture was quickly mixed and the change in absorbance (340 nm) recorded in a Perkin-Elmer recording spectrophotometer maintained at 25° C. The rate of NADH oxidation (equimolar to ATP hydrolysis and ADP production) was calculated from the linear rate between 3 and 9 min after initiation of the reaction. Na^+,K^+-ATPase was calculated as the difference in ouabain-sensitive and -insensitive ATP hydrolysis and expresses as μmoles ADP·mg $protein^{-1}\cdot h^{-1}$.

Results

Exposure of the jaw skin of the seawater-adapted long-jawed mudsucker and the opercular membrane of seawater-adapted tilapia to 2 μM anthroylouabain in low-K^+ Ringer solution results in the appearance of bright blue cells (Figs. 1A, 2B). These are seawater-adapted chloride cells as judged by cell size, morphology and co-localization of mitochondrial staining (Fig. 1B, C). Use of lower concentrations of anthroylouabain or exposure times less than 1 h resulted in decreased staining, which is rapidly lost. No greater contrast is achieved by higher concentrations (up to 50 μM), because of increased intensity of staining of both chloride cells and all other cells in the membrane.

Under low magnification (10× objective) fluorescence lasts for up to 1 h. With a 40× objective, photobleaching of the examined field occurs within 10–30 s. It may be possible to decrease photobleaching by limiting the wavelength of excitation solely to that needed to stimulate anthroylouabain fluorescence. Addition of several drops of 1 mg/ml phenylenediamine solution to the tissue prior to placement of the coverslip did not significantly reduce photobleaching at high magnifications.

Anthroylouabain fluorescence of the chloride cell appeared equally distributed with no obvious areas of high intensity or patchiness (Fig. 1A). No apparent polarity (apical versus basolateral) in the distribution of anthroylouabain fluorescence was observed; the intensity of staining was similar whether examined from the basolateral or apical surface of the membrane, and a nuclear shadow was often visible from either perspective.

Simultaneous exposure of the tissue to 2 μM anthroylouabain and the mitochondrial stain DASPEI results in blue and yellow staining of the same cells when examined through the appropriate light filters (Fig. 1B, C). The use of a 430–490 nm bandpass emission filter to exclude DASPEI staining greatly reduces the signal from anthroylouabain fluorescence (Fig. 1B) and is probably not appropriate for routine staining.

Simultaneous exposure of the tissue to 2 μM anthroylouabain and 1 mM ouabain greatly decreases fluorescence of chloride cells (Fig. 2C), although the back-

Table 1. Na^+,K^+-ATPase activity and chloride (anthroylouabain-positive) cell density of tilapia (*Oreochromis mossambicus*) opercular membrane and long-jawed mudsucker (*Gillichthys mirabilis*) jaw skin. Fish were adapted to either freshwater or seawater for at least 2 weeks prior to sampling. Values are means (*m*), standard errors (*SE*) and sample sizes (*n*)

		Tilapia opercular membrane		Mudsucker jaw skin
		Freshwater	Seawater	Seawater
Na^+,K^+-ATPase activity	m	0.46	1.27*	3.51
(μmoles ADP·mg protein^{-1}·hour^{-1})	SE	0.09	0.18	1.65
	n	6	8	4
Chloride cell density	m	nd	9564	29270
(cells·cm^{-2})	SE		1303	9290
	n	6	6	4

* Significantly different from Na^+,K^+-ATPase activity of opercular membrane of freshwater tilapia ($P<0.01$, Student *t*-test); *nd*, not detectable

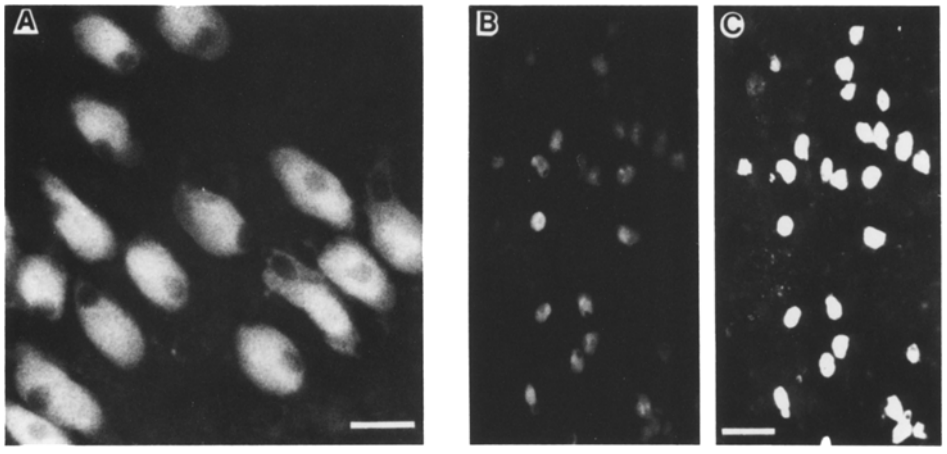

Fig. 1A–C. Fluorescent staining of chloride cells in jaw skin of seawater-adapted long-jawed mudsucker (*Gillichthys mirabilis*). **A** Staining for 1 h with a 2 μM anthroylouabain solution and examined with a 420 nm long-pass filter. *Bar:* 20 μm. **B, C** Simultaneous staining with 2 μM anthroylouabain and 2 μM DASPEI solution; **B** examination with a 430–490 nm barrier filter to exclude DASPEI fluorescence; **C** examination with a 520 nm long pass filter to exclude anthroylouabain fluorescence. *Bar:* 50 μm; same magnification for **B** and **C**

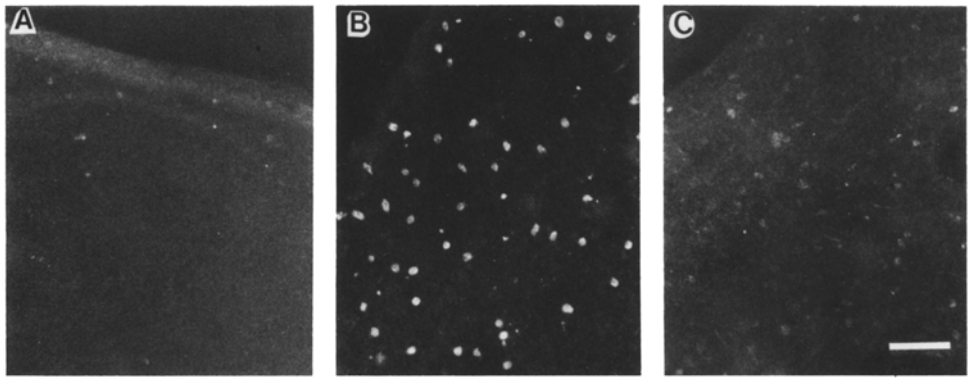

Fig. 2A–C. Anthroylouabain staining of opercular membrane of tilapia (*Oreochromis mossambicus*). **A** Opercular membrane from freshwater tilapia exposed to a 2 μM anthroylouabain solution; **B** opercular membrane from seawater-adapted tilapia exposed to a 2 μM anthroylouabain solution; **C** opercular membrane from seawater-adapted tilapia exposed to a 2 μM anthroylouabain, 1 mM ouabain solution. *Bar:* 100 μm; same magnification for **A, B,** and **C**

ground fluorescence is not affected. A blue background fluorescence, present in all preparations, is due to tissue autofluorescence since it appears in the absence of anthroylouabain.

No staining occurred in the opercular membrane of freshwater tilapia (Fig. 2A). Na^+,K^+-ATPase activity of the opercular membrane of freshwater tilapia is one-third that of seawater-adapted tilapia (Table 1). Na^+,K^+-ATPase activity of transport epithelium of long-jawed mudsucker is greater than that of seawater-adapted tilapia, consistent with the greater density of chloride cells in the former (Table 1, Figs. 1A, 2B).

Discussion

The teleost chloride cell is a large, columnar, mitochondria-rich cell with a large apical crypt and an extensive tubular system continuous with the basolateral membrane, which itself has serosal contact (Karnaky 1986; Pisam et al. 1987, 1988). Chloride cells are known to be responsible for net ion excretion by teleosts in seawater (Foskett and Scheffey 1982). DASPEI, a vital mitochondrial stain, has been widely used to identify and enumerate chloride cells (Marshall and Nishioka 1980; Foskett et al. 1981; Karnaky et al. 1984). The co-localization of anthroylouabain and DASPEI fluorescence demonstrates that the anthroylouabain is indeed staining chloride cells. The large size and columnar appearance of anthroylouabain-positive cells are also characteristic of chloride cells (Karnaky 1986; Pisam et al. 1987, 1988).

Previous studies have demonstrated that the high levels of Na^+,K^+-ATPase in the gills of seawater-adapated teleosts can be localized to chloride cells and are intimately involved in net ion secretion (Sargent et al. 1975; Silva et al. 1977; Hootman and Philpott 1979). Karnaky (1986) estimated that the teleost chloride cell has a higher concentration of Na^+,K^+-ATPase relative to other cell types (up to 1.5×10^8 molecules per cell). Using [^{3}H]ouabain autoradiography (Karnaky et al. 1976) and cytochemistry (Hootman and Philpott 1979) Na^+,K^+-ATPase has been localized to the extensive tubular system of the chloride cell, which though continuous with the basolateral membrane, extends throughout the cell even up to the apical membrane. This distribution of Na^+,K^+-ATPase on the tubular system throughout the cell may account for the apparently even distribution of anthroylouabain staining in chloride cells of seawater-adapted fish seen in the present study. The possibility cannot be ruled out, however, that anthroylouabain has been internalized within the cytosol of the chloride cell.

Mitochondria-rich cells have been found in the gills and opercular membrane of freshwater-adapted teleosts (Pisam et al. 1987, 1988), including the tilapia (Foskett et al. 1981). These "freshwater chloride cells" are much smaller than "seawater chloride cells", and their function is unknown. Since gill and opercular membranes have much lower Na^+,K^+-ATPase activity in freshwater- than in seawater-adapted tilapia (Table 1; Young et al. 1988), it can be supposed that "freshwater chloride cells" will have lower Na^+,K^+-ATPase than "seawater chloride cells". The lack of anthroylouabain fluorescence in opercular membranes of freshwater tilapia supports this supposition. It should be noted that since anthroylouabain binds only to the phosphorylated form of Na^+,K^+-ATPase (Moczydlowski and Fortes 1980), only active enzyme molecules will be labelled.

Several lines of evidence indicate that anthroylouabain is binding specifically to Na^+,K^+-ATPase: (1) Previous studies have shown that anthroylouabain and ouabain have the same high specificity of binding to Na^+,K^+-ATPase in isolated membrane preparations of rabbit kidney and eel electroplax (Moczydlowski and Fortes 1980). (2) Anthroylouabain fluorescence appeared in chloride cells of seawater-adapted teleosts, which are known to have high concentrations of Na^+,K^+-ATPase. (3) Anthroylouabain fluorescence did not appear in the opercular membrane of freshwater tilapia which have lower Na^+,K^+-ATPase activity than seawater-adapted tilapia. (4) Addition of 500× (1 mM) cold ouabain drastically decreased anthroylouabain fluorescence of "seawater chloride cells".

The utility of anthroylouabain for localization of Na^+,K^+-ATPase in other tissues will depend on the amount of ouabain-sensitive Na^+,K^+-ATPase and the intensity of blue autofluorescence, i.e., the signal-to-noise ratio. Although the large 'signal' present in teleost chloride cells makes them an ideal system for detecting fluorescently labelled Na^+,K^+-ATPase, anthroylouabain should be useful in identifying Na^+,K^+-ATPase-rich cells in other tissues, particularly ion-transporting and nerve cells. Fluorescent imaging microscopy should permit precise quantification of anthroylouabain fluorescence, and hence direct quantification of Na^+,K^+-ATPase in intact cells.

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