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Cell volume regulation following hypotonic stress in the intestine of the eel, *Anguilla anguilla*, is Ca^{2+}-dependent

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Abstract

The involvement of Ca^{2+} in the regulatory volume decrease (RVD) mechanism was studied in both isolated enterocytes and intestine of the eel, *Anguilla anguilla*. Videometric methods and electrophysiological techniques were respectively employed. The isolated enterocytes rapidly swelled following a change from isotonic (315 mOsm/kg) to hypotonic (180 mOsm/kg) saline solutions. Afterwards, they tended to recover their original size. This homeostatic response was inhibited both in the absence of extracellular Ca^{2+} and in the presence of TMB8, an inhibitor of Ca^{2+} release from intracellular stores. It is likely that Ca^{2+} entry through verapamil-sensitive Ca^{2+} channels is responsible for RVD since the blocker impaired the ability of the cell to recover its volume after the hypotonic shock. The observation that a 10-fold increase of K^+ concentration as well as the presence of quinine in the hypotonic solution completely abolished RVD indicated the involvement of K^+ in this response. Experiments performed with the isolated intestine suggested that the opening of basolateral K^+ channels facilitates K^+ loss (and hence water efflux) from the cell during RVD and that this opening is probably due to Ca^{2+} entry into the cell through both the mucosal and the serosal membranes.

Keywords: Fish intestine; Regulatory volume decrease; Ca^{2+}; K^+ channels

1. Introduction

Most animal cells, when exposed to hypotonic media, rapidly swell but thereafter tend to return towards their original volume by eliminating cellular osmolytes and hence water. This regulatory mechanism is termed regulatory volume decrease (RVD). The osmolytes responsible for RVD are inorganic solutes as K^+ and Cl^-, specific amino-acids and organic bases (see Hoffmann and Mills, 1999). Usually, Ca^{2+} plays an important role in RVD. In many cell types the removal of extracellular Ca^{2+} (Ca_e^{2+}) compromises RVD, in other cells insensitive to Ca_e^{2+} changes of intracellular Ca^{2+} (Ca_i^{2+}) concentrations are able to affect the homeostatic response (see Hoffmann and Mills, 1999); in rabbit TALH cells, calcium release from intracellular stores induced by extracellular Ca^{2+} participates in cell volume regulation (Tinel et al., 2002). On the other hand, there are cells in which neither Ca_e^{2+} nor Ca_i^{2+} appear to be important for RVD (Grinstein and Smith, 1990; Harbak and Simonsen, 1995; Kanli and Norderhus, 1998).

It is known that for aquatic animals volume perturbations can be elicited not only by changes in extracellular and intracellular solute concentrations as in terrestrial species, but also by variations of external medium. In fish, gills and intestine are the main interfaces with the environment and, together with kidney, participate in the maintenance of the constancy of the composition of the "internal milieu". Consequently, they are frequently exposed to osmotic stress. Fish intestine represents a good model for studying the mechanisms underlying cell volume regulation. Nevertheless, there are only few studies on this topic (Lionetto et al., 2001, 2002; Trischitta et al., 2004). Our previous studies performed with *Gobius niger* intestine (Trischitta et al., 2004) showed that the homeostatic mechanisms responsible

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for volume recovery after hypotonic stress can be studied in isolated cells, by analysing volume changes using video imaging. The present work was undertaken to study RVD and its Ca^{2+} dependence in the intestine of the eel, *Anguilla anguilla*, adapted to seawater. For this purpose, we exposed the isolated enterocytes to a large step change of osmolarity in order to unmask volume regulatory mechanisms. This experimental manoeuvre has been utilized in many studies on both fish (Terreros et al., 1990; Joyner and Kirk, 1994; Bursell and Kirk, 1996; Kültz, 1996; Kanli and Norderhus, 1998; Lionetto et al., 2002) and mammals (Welling et al., 1985; Haddad and Graf, 1989; Park et al., 1994; Best et al., 1996; Bond et al., 1998).

With the aim to support the conclusions suggested by the experiments with isolated enterocytes, we performed some experiments in which the hypotonic stress was applied to native tissues mounted in an Ussing chamber; electrophysiological techniques were applied.

2. Materials and methods

All the experiments were carried out on eels, *A. anguilla* (120–150 g). Prior to the experiments they were kept in large tanks containing running natural seawater for at least 3 weeks at room temperature (16–20 °C) for acclimation.

2.1. Cell experiments

Intestinal cells were isolated by the method described by Mürer et al. (1974) and by Vilella et al. (1996). Briefly, the intestine was isolated and stripped off the longitudinal and circular muscle layers by dissection with two pairs of fine forceps. Then it was cut longitudinally and washed four times in ice-cold NaCl solution (1.1%) to remove mucus and food particles. Pieces of intestine were placed in citrate buffer and gently stirred by a glass rod for 15 min to facilitate cell detachment. The suspension produced by the mechanical agitation was filtered through 225, 75 and 50 μm nylon meshes and then centrifuged at 1700 rpm for 10 min. The supernatant was discarded and the cell pellet was suspended in isotonic control solution (Table 1, solution 1) and centrifuged again. This procedure was repeated twice. The resulting enterocytes were used for the morphometric analysis. The viability of the cells was evaluated by the Trypan blue exclusion method.

The isolated cells were visualised and measured by a method described by La Spada et al. (1999). One drop of cell suspension was placed on a glass slide pre-treated with polylysine to facilitate cell adhesion. Two thin strips of double-sided adhesive were placed on the upper and lower edges of the glass slide to support the cover slip and to create an interspace in which the experimental solutions were added. They were placed at one side of the cover slip with a pipette and were absorbed at the opposite side with strips of filter paper. This allowed a rapid change (a few seconds) of the solution in the interspace. Cells were observed with a light microscope (Leitz Diaplan). Cell images were digitized using a color video camera (Sony) which was connected to an Apple Macintosh computer. Individual cells were selected and images taken at various time intervals. The profile of the cells was drawn with the aid of 3.1 Aldus Free Hand. The area of the profile was subsequently measured with the aid of 1.56 NIH image software. The areas of the cells in every experimental condition (A_{exp}) were compared to the areas measured in isotonic solution (A_i) at the beginning of the experiment. Consequently, the data are reported as relative area A_{exp}/A_i.

2.2. Tissue experiments

The intestine was removed and stripped and mounted vertically in a modified Ussing chamber (membrane area, 0.3 cm^2). The tissues were continuously perfused on both sides by a gravity flow of electrolyte solutions from reservoirs kept at 18 °C by water jackets. The luminal and

Table 1
Ionic composition of the solutions (concentration in mM)

	Isotonic, control (1)[a]	Isotonic, 50% NaCl (2)[a]	Hypotonic, 50% NaCl (3)[b]	Isotonic, Ca^{2+}-free (4)[a, c]	Hypotonic, Ca^{2+}-free (5)[b, c]	Hypotonic, 40 mM K^+ (6)[b]
NaCl	133	67	67	133	67	31
KCl	3.2	3.2	3.2	3.2	3.2	39.2
$MgCl_2$	1.4	1.4	1.4	1.4	1.4	1.4
$CaCl_2$	2.5	2.5	2.5	–	–	2.5
$NaHCO_3$	20	20	20	20	20	20
KH_2PO_4	0.8	0.8	0.8	0.8	0.8	0.8
Glucose	20	20	20	20	20	20
HEPES	–	–	–	–	–	–
Na^+ gluconate	–	–	–	–	–	–
Mannitol	–	132	–	–	–	–

All solutions were bubbled with a mixture of 1% CO_2 and 99% O_2 to yield a pH of 8.0±0.1.

[a] π=315±3 mOsm/kg.

[b] π=190±3 mOsm/kg.

[c] +EGTA 10^{-4} M.