Regulatory Volume Decrease in Cultured Myoblasts L6

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Abstract. A method is described for measuring volume changes in single L6 myoblasts at a stage of proliferating "myoballs", which allows to follow volume changes on single cell level by means of quantitative video image analysis. Myoblasts exposed to hypoosmotic or hyperosmotic challenges for up to 3–5 min behave as osmometers. The relative cell volume is a linear function of the reciprocal of the relative osmolality in the range 0.5–2T. Cells exposed to hypotonic Krebs solution with Na^+ and Cl^- ions as the main ions exhibit volume readjustment towards the original level. The regulatory volume decrease (RVD) was complete after about 15 min in hypotonic solution with C'_{max} (maximum RVD) increasing with the decrease in osmolality in the test solution. By replacing external Na^+ by K^+ in the presence of external Cl^- regulatory volume decrease was reversed; myoblast volume continued to increase. RVD was present after replacing Cl^- with NO_3. Quinine (0.5 mmol/l) partially blocked RVD. It is suggested that RVD in L6 myoblasts is mediated mainly by separate K^+ and Cl^- channels.

Key words: Muscle cell line — L6 myoblasts — Cell volume regulation — Regulatory volume decrease — Volume measurement — Video image analysis — Myoballs

Introduction

Maintenance of a constant cell volume under resting conditions belongs to basic vital functions of animal cells. When exposed to a hypotonic challenge cells are able to regulate the swollen volume toward original values. This phenomenon, first investigated in red cells, was termed regulatory volume decrease, RVD (Kregenow 1981). After a hypotonic challenge the volume of exposed cells increases; the cells initially behave nearly as ideal osmometers. After a definite period, however, they down regulate their cell volume by a net loss of KCl and a concomitant loss of cell water. The mechanism of activation of RVD remains unknown (Hoffmann 1992). As far as the mechanism of KCl loss is concerned, three principal types of transport systems are considered as possible pathways for ion fluxes through

the cell membrane. 1) Volume activated ionic channels, realising conductive K^+ flux functionally coupled to conductive Cl^- flux. 2) Volume-sensitive electroneutral K^+-Cl^- cotransport system. 3) Parallel operation of electroneutral cation (K^+-H^+) exchange and anion (Cl^--HCO_3^-) exchange. For a review of these and other possible pathways see Lang et al. (1993).

The phenomenon of RVD was very soon found to exist in a number of different cells (for recent reviews see e.g. Hoffmann and Simonsen 1989; Hoffmann 1992; McCarty and O'Neil 1992; Lang et al. 1993) with different participation of the above membrane transport systems in the mechanism of RVD. Basic data on RVD were collected on blood cells, mainly erythrocytes, due to the relative ease of the cell volume measurement. Several methods are now available for measuring the cell volume in different cells (see review by Kimelberg et al. 1992). All these methods mostly inform on average changes of the cell volume in vast sets of cells assuming homogeneity.

The purpose of the experiments reported shortly in this paper was to find out whether RVD exists in myoblasts, in particular at the very early stages of development, when the channel and the transport system mosaics in the membrane are relatively scarce (Hurňák and Zachar 1992). Volume changes in cultured myoblasts (L6 line) were followed in single proliferating "myoballs" by means of microscopical video recording and subsequent image analysis of selected frames.

The present study brings evidence for the existence of RVD in single L6 myoblasts and suggests that the mechanism of RVD might be due to volume activated ionic channels, realising conductive K^+ flux functionally coupled to conductive Cl^- flux.

Materials and Methods

Cell culture

The L6 cells were obtained from American Type Culture Collection (A.T.C.C.: Rockville, MD; U.S.A.). Cells for experiments were subcultured at regular time intervals to prevent the cultures to reach confluence. Cells were seeded in plastic or glass dishes on cover slips (10×10 mm) at a maximum density of 1500 cells/mm^2 in Dulbecco's modified Eagle's medium containing 20% fetal bovine serum and antibiotics: streptomycin, kanamycin (100 mg/ml each) and penicillin (100 units), and were kept in a humidified atmosphere under 5% CO_2/95% O_2 at 37 °C. After reaching proliferation stage with a high density of "myoballs" (Fig. 2) the culture medium was exchanged for Krebs solution. The experiment was started after equilibration of the culture dish in this solution for at least 30 min.

Solutions

The Krebs solution had the following composition (in mmol/l): 135 NaCl; 5 KCl; 1 CaCl$_2$; 1 MgCl$_2$; 20 HEPES; 5 glucose; pH 7.4 at 23 °C. In experiments with anisotonic solutions the concentration of NaCl in Krebs solution varied from 30 to 300. The osmolality of the

solutions was measured using a Knauer semi-micro-osmometer (Oberursel, Germany) In some experiments the Cl^- content was substituted equimolarly with NO_3^-, and Na^+ with K^+ Transport inhibitors (0 01 DIDS and 0 05 bumetanide) were added to hypotonic perfusion media (for further details, see legends to Figures) Quinine was used in concentrations of 0 5 and 1 mmol/l
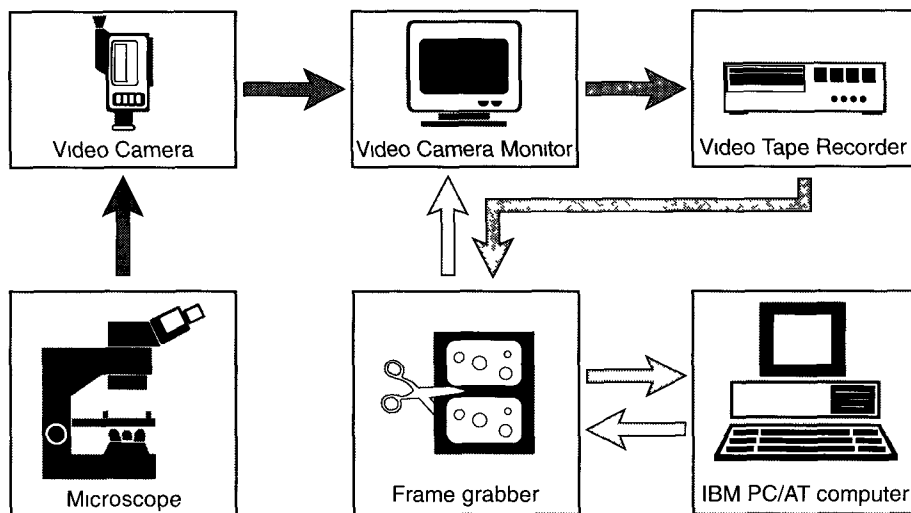
Figure 1. Block diagram of the video image analysis system set up The system includes a light source, an inverted microscope a low-light-level video camera, a video camera monitor a video tape recorder, an image processor (frame grabber) and a (computer) For details, see text

Measurement of cell volume

The block diagram of the video image analysis system set-up assembled for continuous observation, recording and measurement of volume changes in single cells is shown in Fig 1 Cells were viewed at ×320 in bright field illumination of Leitz Fluovert inverted microscope The image was recorded with a Panasonic camera (WV-CD110E) The image was played back onto a Panasonic monitor (WV-CM110G) and stored on a Sony Video 8 video recorder The records were later played back and images were grabbed with a PCVISION Plus frame grabber, resolution 512×480 pixels, every minute and stored on a hard disk of an IBM PC 486 JAVA Jandel Video analysis software was used to determine cross-sectional area Highly contrasted images permitted clear discrimination between the cell border and the background JAVA automatically traces the edge and places the digitized values in the data worksheet The area of a closed figure was calculated from the data in the worksheet by a software Assuming spherical shape of the cells, cell volume ($V = 4/3\pi\ r^3$) can be related to cross-sectional area (A) by the following equation

$$V = \frac{4}{3}\pi^{(\ 1/2)}\ A^{(3/2)}$$

Figure 2. Rounded proliferating myoblasts ("myoballs") as seen under the light micro-scope on the monitor of the video analysis system. The pictures represent frames from a continuous record of a group of cells with one (A) or three (B) rounded myoblasts ("myoballs") respectively which were later subjected to hypoosmotic challenge. The my-oballs were loosely attached to the bottom and did not change their position during the superfusion of the dish. Note the spindle shaped myoblasts attached to the bottom of the chamber in A. Both the myoball and the attached myoblasts are intentionally a little out of focus in order to bring them into view. White bars represent 10 μm.

In protocols the calculated volumes were taken as percentages of initial volume (at time $t = 0$). The radius (r) of the myoballs was $9.4 \pm 1.3 \ \mu m$ (mean $\pm$ SD).

Materials

DIDS (4,4'-diisothiocyanatostilbene-2,2'-disulphonic acid) and other compounds were either from SIGMA or Aldrich.

Results

Cell cultures of L6 myoblasts were exposed to solutions with reduced osmolarity in proliferative phase with a number of newly formed (dividing cells) which are rounded, but attached to the bottom of the culture dish (usually a coverslip) or to quiescent myoblasts. Several developmental stages of these myoballs including mitotic division of the cells can be observed in the light and especially in the scanning electron microscopic field. As communicated in a separate paper (Hurnak and Zachar 1994) these cells are fully functional at least as far as the existence of the resting potential and the large conductance chloride channels which are typical of this stage of myoblasts development (Hurnak and Zachar 1992) is concerned. They were therefore chosen for measurements of volume changes after an osmotic challenge by means of video image analysis (see Materials and Methods). For this purpose a light microscopic field with a number of such myoballs was chosen as shown in Fig. 2. The cells were continuously followed by means of a video camera and a video monitor where they were simultaneously projected. A number of individual frames was then chosen from the video records for image analysis of the cells at various intervals before and after the introduction of the test solution and after the reintroduction of control saline.

Volume recovery in hypotonic solutions

Figure 3 (filled circles) shows the average time course of volume changes in seven myoblasts exposed to hypoosmotic Krebs solution (130 mOsm/l ≈ 0.5 of normal osmolality). After exposure to hypotonic saline the volume increased to about 150% in 3–4 min. After reaching a maximum the size of the myoball started decreasing showing all signs of the regulatory volume decrease. The return to control values was complete in about 15 min and was followed at this osmolality by a slight undershoot of the myoball volume. After switching back to control Krebs solution the fibre volume decreased followed by a very prolonged return if any to control values.

The extent of the regulatory volume decrease depends on the osmolality of the hypotonic test solution. The half time of decay of RVD, $t_{0.5}$, was 7 ± 0.5 min in 0.5 T hypotonic saline and increased with the decreasing relative tonicity (T). The value of $t_{0.5}$ is intermediate between that found for erythrocytes (hours) and that observed for thymocytes (s) (Arrazola et al. 1993).
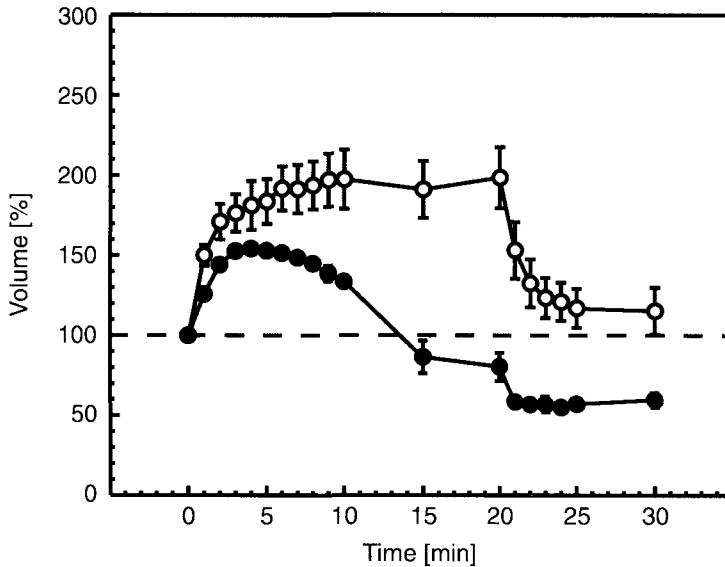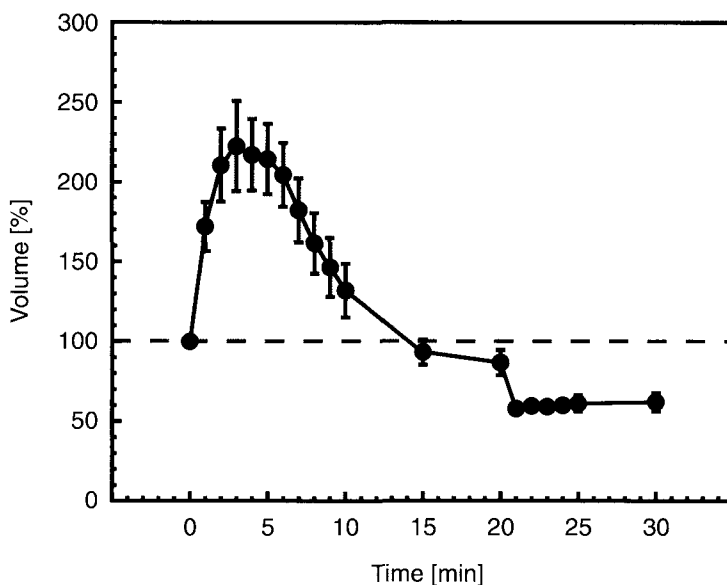
Figure 3. Regulatory volume decrease in L6 myoblasts in response to a maintained decrease of osmolarity by 1/2 from the control (270 mOsm/l). Filled circles: in NaCl containing hypotonic Krebs solution. Note the spontaneous decrease of cell volume in the hypoosmotic solution, the undershoot and the volume increase after switching back to normal saline. Volume changes were recorded continuously before and during the pulse of decreased osmolarity, which lasted for 20 min as indicated. Empty circles: volume behavior of myoblasts exposed to hypoosmotic Krebs solution (189 mOsm/l) after complete replacement of Na^+ with K^+ (65 mmol/l). The increase in cell volume continued throughout the exposure period. After the reintroduction of the normal Krebs saline the cell volume returned within 3 min to original values. *Ordinate*: cell volume in relative units. *Abscissa*: time in min after the start of application of hypoosmotic challenge.

Initial volume changes, before the onset of volume recovery, obey the Boyle-van't Hoff linear relationship of 1/p vs. volume. Extrapolation of y intercept yields a nonsolvent volume of $\approx 65\%$, which is rather high.

Changes of RVD by ion substitutions

As shown in Fig. 3 (open symbols), RVD can be reversed by replacing Na^+ with K^+ in hypotonic solutions. The value attained in the 3 min after the introduction of the test solution is slightly higher than the maximum value in Na^+ – containing hypotonic Krebs solution (filled circles). The volume of the myoball, however, continues to increase instead of decreasing as it happens in Na^+ – containing saline. The rise in volume continued during the whole period of exposure to KCl Krebs solution. After the reintroduction of normal (Na^+ – containing) Krebs solution the

volume of the cells quickly attained the original values with no RVI (regulatory volume increase). The rise of volume is remarkable, and may attain values twice the initial "osmotic" changes in volume. The reversal of RVD by replacing external Na^+ by K^+ is considered as evidence for the operation of K^+-Cl^- co-transporter (Arrazola et al. 1993).

Figure 4. Regulatory volume decrease in L6 myoblasts exposed to hypotonic Krebs solution (130 mOsm/l) after complete replacement of Cl^- with NO_3^-. *Ordinate*: cell volume in relative units. *Abscissa*: time in min after the start of application of hypoosmotic challenge.

The K^+-Cl^- co-transporter is incapable of functioning after the replacement of Cl^- with NO_3^- (Lauf 1985; Eveloff and Warnock 1987; Garcia-Romeu et al. 1991; Schield et al. 1991). It was therefore interesting to see the osmotic behavior of L6 myoblasts after replacing Cl^- with NO_3^- in Krebs solution. Fig. 4 shows the existence of the regulatory volume recovery also in Krebs solution after replacement of Cl^- with NO_3^-, contrary to the above assumption of RVD being due to the operation of the K^+-Cl^- co-transporter. The volume increase in hypotonic $NaNO_3$ solutions is substantially higher ($220 \pm 30\%$) in comparison with NaCl solutions of the same osmolality ($150 \pm 8\%$). These anion substitution experiments suggest that the permeability to anions is an essential factor in the magnitude of the cell swelling in hypotonic Na solutions. The possibility that RVD might be due to the exit of K and Cl ions via separate pathways, i.e. through a kind of potassium or
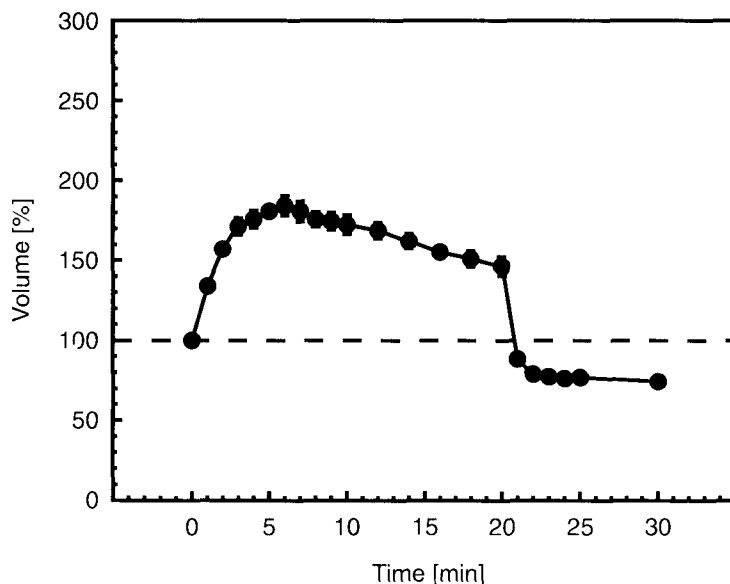
Figure 5. Regulatory volume decrease in L6 myoblasts exposed to hypotonic Krebs solution (130 mOsm/l) containing quinine (0.5 mmol/l), which blocked RVD to a great extent as compared with control time course

chloride channels, was strengthened by the effect of quinine, a potassium channel blocker. As shown in Fig. 5 quinine (0.5 mmol/l) blocked to a great extent RVD initiated by 130 mOsm/l hypotonic solution, a fact which may be taken as evidence for the participation of separate potassium channel in RVD.

The effect of transport inhibitors

Figure 6 shows the effect of DIDS (100 μmol/l) and bumetanide (0.5 mmol/l) on RVD. DIDS (hollow circles), an inhibitor of $Cl^--HCO_3^-$ was virtually without any effect on the osmotic behavior of L6 myoblasts. The same concerns the effect of bumetanide (filled circles), a potent and quite specific inhibitor of the $Na^+-K^+-Cl^-$ cotransporter system (Lauf et al. 1984).

Discussion

The aim of the experiments reported in this paper was twofold: first to describe the feasibility of the method which was developed for observing volume changes in single cells, and second to find out, whether myoblasts possess the mechanism of volume regulation after exposure to hypotonic media.
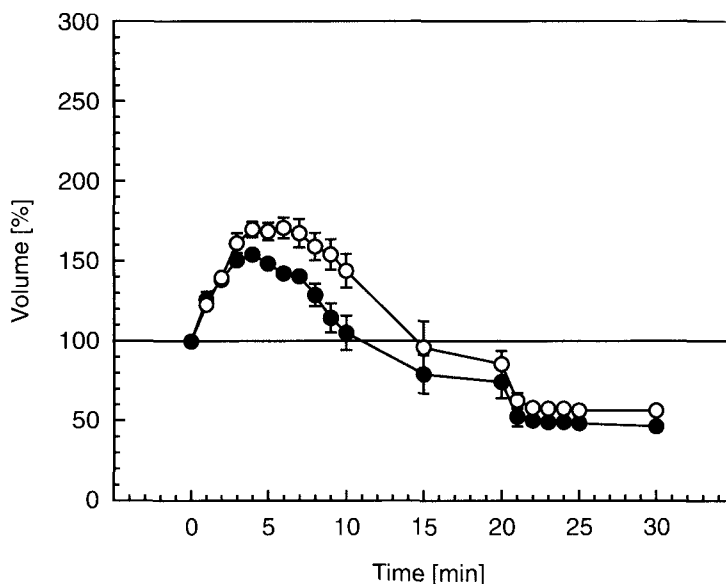
Figure 6. The effect of DIDS (100 μmol/l) (empty circles) and 0.5 mmol/l bumetanide (filled circles) on RVD initiated by 130 mOsm/l hypotonic solution in L6 myoblasts. RVD was little changed in comparison with the hypotonic challenge in the absence of transport inhibitors. *Ordinate*: cell volume in relative units. *Abscissa*: time in min after the start of application of hypoosmotic challenge.

Measurement of volume changes in single myoblasts

There are several methods available, which enable to measure the volume of cells in culture (for a review see Kimelberg et al. 1992). All these methods record, however, average volume changes of a large population of cells, which might be relatively uniform, as in the case of blood cells, or less homogeneous as in the case of cell cultures. There were attempts to estimate and/or record volume changes in single cells, which are subject to several uncertainties, when the cells are attached to the observation dish, or have other than spherical shape, e.g. in isolated myocytes (Drewnowska and Baumgarten 1991). To observe volume changes in myoballs, when the cell is detached from the bottom of the dish can only be made by fixing the cell in the view by means of fixing devices, mostly micropipettes (e.g. Haddad et al. 1991, Kubo and Okada 1992, Farrugia and Rae 1993). After several unsuccessful efforts to get reproducible results with attached cells, we made use of the natural process of spherically shaping cells during the proliferation phase. As we report elsewhere (Hurňák and Zachar 1994) the myoballs of the myoblasts behave, at least as far as their membrane potential and channel activity is concerned, the same way as myoblasts attached to the bottom of the observation chamber do. We preferred

this natural process of rounding the cell to trypsinization and similar procedures which might also change other surface properties of the cell. Although cells in culture appear homogeneous, neighboring cells may differ quantitatively from each other in their response to the same experimental procedure. We therefore made records from at least two cells in the visual field, and made calculations from at least three different dishes. Any dish was only used for one experimental test, because of the long lasting recovery from the hypotonic challenge.

Regulatory volume decrease in L6 myoblasts

The results show that L6 myoblasts exhibit regulatory volume decrease (RVD) in hypotonic media at a very early stage of development. From the three mechanisms considered in the Introduction the most probable candidate for the mechanism of volume recovery seems (by exclusion) volume activation of ionic channels, realising conductive K^+ flux functionally coupled to conductive Cl^- flux.

The fact that RVD was reversed (cell volume increase) by replacing external Na^+ with K^+, might suggest activation of the inward K^+-Cl^- co-transport system similar to that described in other cells (see Hoffmann and Simonsen 1989; Lang et al. 1993). This assumption seems, however, improbable, because of the effect of substitution of Cl^- with NO_3^- in hypotonic solutions. K^+-Cl^- co-transport is namely generally considered (Hoffmann et al. 1986; Garcia-Romeu et al. 1991; Soler et al.1993) unable to carry NO_3^- which, by contrast, is effectively transported by the anion exchanger and the anion channels. Thus, the fact that RVD was preserved in hypotonic $NaNO_3$ solutions supports the assumption on the participation of other pathways for the K and Cl exit during RVD.

Parallel operation of electroneutral cation (K^+-H^+) exchange and anion (Cl^--HCO_3^-) exchange is less probable owing to the effect of DIDS (100 μmol/l), an inhibitor of Cl^--HCO_3^- on RVD, which influenced RVD in L6 myoblasts to a small extent, if at all. On the other hand, the inhibitory effect of quinine on RVD speaks in favor of the assumption of separate K and Cl pathways (Grinstein et al. 1982; Hoffmann et al. 1986; Corasanti et al. 1990).

In spite of these facts the elucidation of the RVD mechanism in these cells must await further tests. New support might be expected to come from experiments with labelled ions, and/or electrophysiological evidence.

The decay in volume during spontaneous recovery in hypotonic solutions is very fast and might show a significant undershoot, if the cell is kept in hypotonic solution for a sufficient time (20–30 min). The undershoot is evidently due to loss of osmolytes. In the absence of other evidence, however, the mechanism of volume stabilisation at a subnormal level remains unknown. After returning to isotonic medium, the myoblasts exhibit a post-RVD regulatory volume increase (post-RVD RVI); they shrink below the new level reached at the end of superfusion with hypotonic saline. The originally isotonic medium is now effectively hypertonic

to the cytoplasm due to a loss of cellular osmolytes during RVD. The myoblasts are then capable of regaining osmolytes to allow to swell back up to control value. The drift of the volume was absent (Fig. 3) if external Na^+ was replaced by K^+.

It would be interesting to explore if the transport system responsible for RVD in undifferentiated L6 myoblasts is present at later stages of development, in view of the evidence in other cells that the K^+-Cl^- co-transport system significantly decreases following maturation (Lauf 1988; Arrazola et al. 1993). The time parameters of RVD are faster than e.g. in erythrocytes, but in the order of minutes. In much faster volume readjustment, however, the participation of other transport systems, mainly the maxi-Cl channel (Hurňák and Zachar 1992), found to exist already at this stage of development cannot be excluded.

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