Short communication

Volume Sensitive ^{36}Cl Fluxes in L6 Myoblasts

V. ŠAJTER[1], J. ZACHAR[2] and O. HURŇÁK[2]

1 *Postgraduate Medical School, Limbová 12, 83303 Bratislava, Slovakia*
2 *Institute of Molecular Physiology and Genetics, Slovak Academy of Sciences, Vlárska 5, 833 34 Bratislava, Slovakia*

Abstract. Efflux of ^{36}Cl ions was followed in undifferentiated L6 myoblasts at rest and after exposure to hypotonic saline in order to test participation of the chloride exit in regulatory volume decrease mechanism. The efflux curve can be fitted at least with two time constants. After the hypotonic stress the slow rate constant increases almost threefold, from 0.021 ± 0.019 min^{-1} normal saline to 0.060 ± 0.019 min^{-1} in hypotonic solution.

Key words: Isotopic fluxes — Chloride efflux — Muscle cell line — L6 myoblasts — Osmotic cell swelling — Hyposmotic stress — Regulatory volume decrease — Cell volume regulation

We have demonstrated recently (Zachar and Hurňák 1995) the existence of regulatory volume decrease in L6 myoblasts, first discovered in blood cells (Kregenow 1981) and since then observed in a number of different cells (for a recent review see e.g. Hoffmann and Simonsen 1989; Hoffmann 1992; Lang et al. 1993). Most probably the volume recovery is due to the exit of K^+ and Cl^- ions by concomitant loss of water, as follows from the effect of substitution of K and Cl ions on the volume recovery in these cells. The mechanism of exit of K^+ and Cl^- remains to be elucidated. There are in general at least three possibilities how this may occur: 1) via separate K^+ and Cl^- channels, 2) by means of neutral K-Cl cotransport, and 3) by parallel operation of cation and anion exchange mechanisms (Hoffmann and Simonsen 1989).

We are reporting here on experiments with labeled ^{36}Cl fluxes which were aimed to test the participation of chloride exit in regulatory volume decrease mechanism in undifferentiated L6 myoblasts. We have demonstrated that the ^{36}Cl efflux curve can be fitted at least with two rate constants. After the hypotonic stress the slow rate constant increases almost threefold.

The L6 cells were obtained from American Type Culture Collection (A.T.C.C.;

Rockville, MD, USA). Cells for experiments were subcultured at regular time intervals to prevent the cultures to reach confluence. The details were published elsewhere (Hurňák and Zachar 1992, 1994). Cells were seeded in plastic or glass dishes on 6 cover slips (10 × 10 mm) at a maximum average density of about 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. Before the experiments, the culture medium was exchanged for saline of the following composition (in mmol/l): 135 NaCl; 5 KCl; 1 $CaCl_2$; 2 $MgCl_2$; 10 HEPES; 5 glucose; pH 7.4 at 25 °C. The calculation of cells in each of the six compartments containing the cover slips was performed from video frames grabbed and analyzed by the image analysis software (JAVA Jandel, USA), as described in detail elsewhere (Zachar and Hurňák 1995). The number of cells on cover slips varied a great deal (Fig. 1) in spite of the fact that the cells on all six coverslips were cultivated in the same Petri dish.

The cover slips were placed in a dish with separate six compartments (25 × 25 mm each) and fixed gently by a perspex pin to the bottom of every compartment. Such an arrangement enabled quick and safe exchange of solutions when measuring the efflux. The uptake of $^{36}Cl^-$ was initiated by adding $^{36}Cl^-$ containing saline at room temperature to the cells for a period of 10–90 min. $Na^{36}Cl$ was obtained from Amersham (UK). The uptake process was efficiently stopped by aspirating the radioactive medium and washing the monolayers twice with a tracer-free saline; this washing period lasted 1 minute. Afterwards, 1 ml of normal (non-radioactive) saline at 25 °C was added to the monolayers in each of the six compartments and replaced at 1 min 4 times and then every 2 min over a 15 min period. At the end of the efflux, the monolayers were solubilized in 0.2 N NaOH or trypsin. The radioactivity present in each of the effluent samples and the remaining radioactivity in the cells were measured in a liquid scintillation counter. The time course of the tracer wash-out was calculated by summing in retrograde order the amount of tracer remaining in the cells at the end of the efflux and the amounts of tracer collected during the successive time intervals. This time course became monoexponential after about 3 min, and the amplitude of this slowly exchanging fraction was used to estimate the cellular $^{36}Cl^-$ content.

Fig. 2 shows an average efflux of $^{36}Cl^-$ (from six compartments in the same trial) from normal L6 myoblasts exposed for 90 min to a radioactive saline. Circles represent the data points and the solide lines represent the best fit lines computed on the assumption of a two compartment model (Lin and Gruenstein 1988; Mastrocola et al. 1991):

$$y = a \exp(-f \cdot t) + b \exp(-s \cdot t),$$

where y is the amount of Cl^- remaining in cells at time t; a and b are amounts of

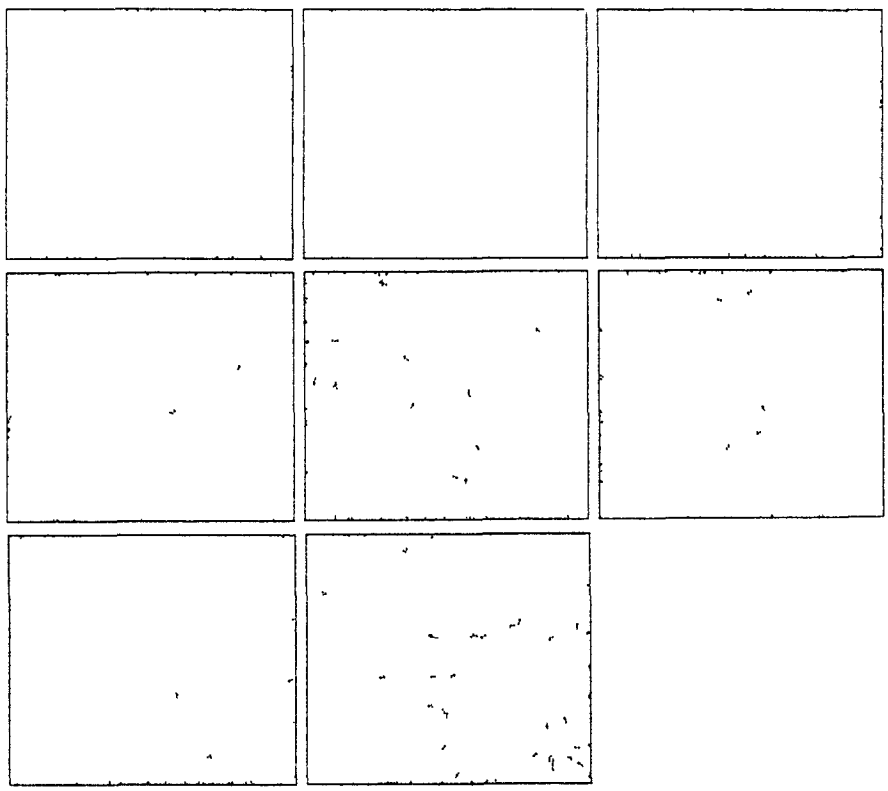

Figure 1. Number of cells in different dishes from the same passage of L6 myoblasts cultured exposed to radioactive saline Counting was performed automatically (see Methods) after trypsinization of the cells at the end of experiment Following numbers of cells were obtained (from left to right) *upper row* 942 343 962 *middle row* 733 1039 713 981 *lower row* 981 1029 Mean 843 The number of cells/mm^2 in each dish was estimated as the average from several fields (5 6) such as those shown in the Figure The following values were obtained *upper row* 1522 554 1554 *middle row* 1181 1679 1152 *lower row* 1585 1663 Mean 1362 cells/mm^2

radioactivity in the fast and slow compartments, f and s are the rate constants of efflux of the fast and the slow compartments respectively The decay components of the efflux curve (f and s) are shown by dotted lines in Fig 2

The time constant of the fast compartment $\tau_f(= 1/f)$ is 1 2 min i e nearly two orders of magnitude faster in this particular trial, than that of the slow compartment $\tau_s(= 1/s) = 100$ min The nature and/or localization of the *fast* compartment are uncertain (as discussed later on) In agreement with other similar studies (Lin and Gruenstein 1988 Mastrocola et al 1991 Rugolo et al 1992) we

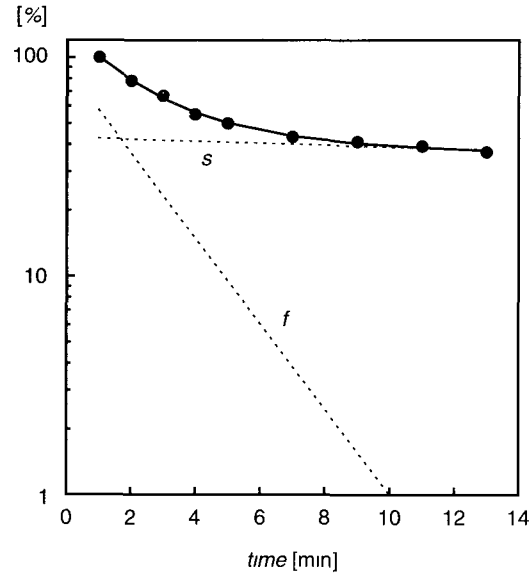

Figure 2. Efflux of $^{36}Cl^-$ from normal L6 myoblasts exposed for 90 min to radioactive saline at 25 °C; semilog plot. Circles represent the data points (averages from six compartments in the same trial). The interrupted lines are best fit lines computed on the assumption of a two compartment model (see equation in text). *f* and *s* denote the fast and the slow components of the efflux, respectively.

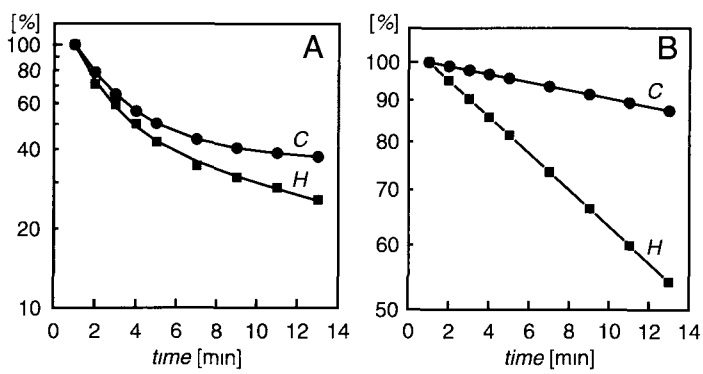

Figure 3. The effect of hyposmotic stress (2/3 of control osmolality) on efflux of $^{36}Cl^-$ from L6 myoblasts. *A*: The time course of efflux in control (*C*) and hyposmotic (*H*) solutions. *B*: The slow components of efflux in control and hyposmotic saline computed by a double fit procedure from the curves in *A*.

consider the *slow* phase as corresponding to the efflux via the cell membrane. It was the particular phase which did show changes during the experimental procedures.

Fig. 3 shows the changes in $^{36}Cl^-$ efflux due to application of hypotonic solution (2/3 of control osmolality). Fig. 3*A* shows the time course of $^{36}Cl^-$ efflux in control (*C*) and hypotonic solution (*H*). The solid curves through the experimental points represent the best fit curves computed on the assumption of a two time-constant model of decay in radioactivity in L6 cells after exposure to hypotonic saline. Fig. 3*B* shows the corresponding slow rate constant lines in control and hypotonic saline. Fig. 4 shows average values obtained in hypotonic solutions (0.0604 ± 0.0191) as against control values in isotonic conditions (0.0214 ± 0.0191); $n = 12$. The control values are comparable to the efflux constants in human fibroblasts, ascribed by Lin and Gruenstein (1988) to cation cotransport (0.020 min^{-1}) or to electrically conductive pathway (0.016 min^{-1}); in their observations the efflux constant of the anion exchange pathway had an approximately twice as high value (0.040 min^{-1}).

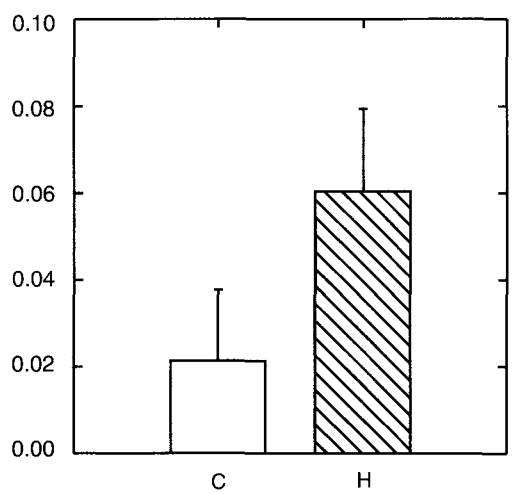

Figure 4. Average values (± SD) of slow rate constants (min^{-1}) in control (*C*) and hyposmotic (*H*) solutions.

There are several interpretations of the rapid phase of the $^{36}Cl^-$ efflux in different cultures. In human fibroblasts the rapid phase is interpreted as being due to the presence of transient P_{CO_2} gradient (Lin and Gruenstein 1988). Another likely explanation is that the fast phase represents the efflux from the extracellular space beneath and between cells; Mastrocola et al. (1991) ruled out, however, this possibility in keratinocytes by measuring the extracellular space. It is also possible that some loosely attached cells are removed with the first efflux buffers. This mechanism could also explain isolated sudden increases in fractional effluxes, which were sometimes observed during the first few minutes of collection in L6 myoblasts.

There is a general agreement that the second slow phase of the efflux curve represents the actual transmembrane chloride efflux from the cell. The total rate constant of Cl^- efflux may, however, represent a sum of several separate transport pathways:

$$r_{Cl} = r_e + r_c + r_a,$$

where r_{Cl} is the observed Cl^- efflux rate constant, and r_e, r_c, r_a are the rate constants of the assumed components of the electrically conductive transport (r_e), chloride-cation cotransport (r_c) and chloride-anion exchange (r_a) respectively. In human fibroblasts these components make up $\approx 95\%$ of the total Cl^- efflux (m); with $m_e : m_c : m_a = 20\% : 25\% : 50\%$ (Lin and Gruenstein 1988). The proportion of these components in L6 myoblasts has not been determined so far, nor was even addressed the question of their presence in these cells.

The existence of individual chloride efflux components is usually proved by means of transport inhibitors. As a rule, anion exchange component is assessed by means of stilbene derivatives DIDS or SITS. Bumetanide is a potent and quite specific inhibitor of the Na^+-K^+-Cl^- co-transport system (Lauf et al. 1984). It should be, however, remembered that the all commonly used transport inhibitors have side inhibitory effects, so that the exclusion of a transport system based on their effects must be checked by other means. From this point of view are interesting the effects of variation of $[K^+]$ and $[Cl^-]$ in the external environment of L6 myoblasts. The abolition of RVD in high KCl and the increase of RVD in $NaNO_3$ saline show that the Cl^- ions are involved in the mechanism of volume recovery. From the results (Zachar and Hurňák 1995) with the effects of transport inhibitors in isotonic conditions it would follow that all these systems are present in L6 myoblasts as well. The data are not sufficient, however, for a quantitative estimation of their contribution in the chloride movements.

It is to be noted that DIDS also blocks the high-conductance chloride channels in L6 myoblasts (Zachar and Hurňák 1995), so that they cannot be used to prove the presence of either anion exchange or a maxi-Cl channel chloride efflux component.

Although the channel mosaics in L6 undifferentiated myoblasts is fairly scant, as can be judged from the patch-clamp evidence, the possibility still remains that the changes in osmolality influence some small conductance chloride channels and/or activate some quiescent transport system. The small conductance channels with less than 10 pS channel conductance might remain undetected and their activation ascribed to the early stages in build-up of maxi-Cl channels. Also we have no evidence whether L6 myoblasts possess few-picosiemens and/or sub-picosiemens channels, which cannot be detected by the single channel patch-clamp technique (as single channel open-close events) and require other methods for detection (Marty et al. 1984; Matthews et al. 1989; Doroshenko et al. 1991; Sakai et al. 1992). To check their presence in L6 myoblasts becomes important in view

of the fact that they were shown recently as volume-sensitive in bovine chromaffin cells (Doroshenko and Neher 1992), in T lymphocytes (Lewis et al. 1993) and human endothelial cells (Nilius et al. 1994). Also, the relation of the volume sensitive ^{36}Cl fluxes to volume-sensitive intermediate-conductance Cl^- channel (Kubo and Okada 1992) remains to be checked, especially if the blocking effect of arachidonic acid on Cl^- channels in L6 myoblasts is taken into account (Zachar and Hurňák 1994).

Acknowledgements. This work was performed using the equipment and radiochemicals of the former Centre of Physiological Sciences (Slovak Academy of Sciences). The authors wish to thank Mr. A. Melota for his skilled technical assistance, and Mrs. G. Gajdošíková for careful cell culture maintenance.

References

Doroshenko P., Neher E. (1992): Voltage-sensitive chloride conductance in bovine chromaffine cell membrane. J. Physiol. (London) **449,** 197—218

Doroshenko P., Penner R., Neher E. (1991): Novel chloride conductance in the membrane of bovine chromaffin cells activated by intracellular GTPγS. J. Physiol. (London) **436,** 711—724

Hoffmann E. K. (1992): Cell swelling and volume regulation. Can. J. Physiol. Pharmacol. **70,** S310—S313

Hoffmann E. K., Simonsen L. O. (1989): Membrane mechanisms in volume and pH regulation in vertebrate cells. Physiol. Rev. **69,** 315—382

Hurňák O., Zachar J. (1992): Maxi-chloride channels in L6 myoblasts. Gen. Physiol. Biophys. **11,** 389—400

Hurňák O., Zachar J. (1994): Conductance-voltage relations in large - conductance chloride channels in proliferating L6 myoblasts. Gen. Physiol. Biophys. **13,** 171—192

Kregenow F. M. (1981): Osmoregulatory salt transporting mechanisms: control of cell volume in anisotonic media. Annu. Rev. Physiol. **43,** 493—505

Kubo M., Okada Y. (1992): Volume-regulatory Cl^- channel currents in cultured human epithelial cells. J. Physiol. (London) **456,** 351—371

Lang F., Ritter M., Volkl H., Haussinger D. (1993): The biological significance of cell volume. Renal Physiol. Biochem. **16,** 48—65

Lauf P. K., Adragna N. C., Garay R. P. (1984): Activation by N-ethylmaleimide of a latent K^+- Cl^- flux in human red blood cells. Amer. J. Physiol. **246,** C385—C390

Lewis R. S., Ross P. E., Cahalan M. D. (1993): Chloride channels activated by osmotic stress in T lymphocytes. J. Gen. Physiol. **101,** 801—826

Lin P., Gruenstein E. (1988): Pathways of Cl^- transport in human fibroblasts. Amer. J. Physiol. **255,** (Cell Physiol. 24), C112—C122

Marty A., Tan Y. P., Trautmann A. (1984): Three types of calcium dependent channel in rat lacrimal glands. J. Physiol. (London) **357,** 293—325

Mastrocola T., Deluca M., Rugolo M. (1991): Characterization of volume transport in cultured human keratinocytes. Biochim. Biophys. Acta **1097,** 275—282

Matthews G., Neher E., Penner R. (1989): Chloride conductance activated by external agonists and internal messengers in rat peritoneal mast cells. J. Physiol. (London) **418,** 131—144

Nilius B., Oike M., Zahradník I., Droogmans G. (1994): Activation of a Cl^-current by hypotonic volume increase in human endothelial cells. J. Gen. Physiol. **103,** 787—805

Rugolo M., Mastrocola T., Deluca M., Romeo G., Galietta L. J. V. (1992): A volume-sensitive chloride conductance revealed in cultured human keratinocytes by Cl^-36-efflux and whole-cell patch clamp recording. Biochim. Biophys. Acta **1112,** 39—44

Sakai H., Okada Y., Morii M., Takeuchi N. (1992): Arachidonic acid and prostaglandin E2 activate small-conductance Cl^- channels in the basolateral membrane of rabbit parietal cells. J. Physiol. (London) **448,** 293—306

Zachar J., Hurňák O. (1994): Arachidonic acid blocks large-conductance chloride channels in L6 myoblasts. Gen. Physiol. Biophys. **13,** 193—213

Zachar J., Hurňák O. (1995): Regulatory volume decrease in cultured myoblasts L6. Gen. Physiol. Biophys. **14,** 179—190

Final version accepted October 2, 1995