

Short communication

A Note on the Cellular Effects of Nystatin in Single Myoballs

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Abstract. Volume changes in single L6 myoblasts (myoballs) exposed to nystatin solutions were followed on single cell level by means of quantitative video image analysis. The myoblasts swelled in nystatin solutions. The volume change was dependent on the nystatin concentration, the threshold concentration being 12.5 $\mu\text{mol/l}$ of nystatin freshly dissolved in Krebs solution. The threshold effect was triphasic: a slight initial volume decrease (shrinkage) for about 2 min followed by a volume increase and, after about 10 min by a significant volume decrease. At twice as high nystatin concentration (25 $\mu\text{mol/l}$) the final shrinkage phase was lacking. At 50 $\mu\text{mol/l}$ concentration the volume increased continually after a delay of about 1–2 min. and reached a plateau of about 350% of the original volume. At 100 $\mu\text{mol/l}$ concentration of nystatin the myoblasts increased their volume in about five min to more than 500% of the original value. The effects of nystatin diminished upon prolonged storage of nystatin Krebs solution. Nystatin solutions (50 $\mu\text{mol/l}$) prepared 3 hours before use were still active to about 80%. Volume changes in 100 $\mu\text{mol/l}$ nystatin solutions were, however, substantially diminished (to about 20%) 5 hours after the preparation of the nystatin solution. By replacing external Na^+ by TEA^+ in the presence of external Cl^- a regulatory volume decrease was observed to subnormal values; the myoblast volume shrank to about half of the control value. The volume changes were reversible after reintroduction of Krebs solution. The regulatory volume decrease to subnormal values was also observed after replacing external Cl^- by glutamate anion in the presence of external Na. The volume changes were, however, not reversible after reintroduction of Krebs solution. The swelling of myoblasts in 50 $\mu\text{mol/l}$ nystatin Krebs solution continued after a definite enlargement of the whole myoblast was reached with the formation of several blebs, which eventually coalesced to form a continuous layer around the myoballs. The enlarged vesicles in nystatin solutions were able to start and fulfill

the mitotic cycle. Cell volume measurements represent a handy means for checking the activity of nystatin solutions for the perforated patch experiments.

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

The polyene antibiotic nystatin is widely used to permeabilize the cell membrane for the recording of whole-cell membrane currents without mechanical rupturing the cell membrane, in the so called perforated patch recording (Horn and Marty 1988). The low-resistance pathway is formed in this technique by incorporating voltage-independent pores (~ 0.8 nm) into the membrane patch that are permeable for small monovalent cations (Na^+ , K^+) and moderately permeable for small anions (Cl^-), but impermeable to divalent cations and most intracellular substances and metabolites. The nystatin method of the whole-cell patch clamp recording can thus be used for the inquiry of mechanisms important to intracellular cell signalling and channel regulation as the main components of these processes are not dialyzed from the cytoplasm (for recent reviews of the method and criteria see e.g. Rae 1991; Korn et al. 1991; Rae and Fernandez 1991; Grantyn et al. 1992; Chung and Schlichter 1993; Oleson et al. 1993).

Nystatin seems to interfere with the seal formation. It is therefore applied into the pipette by backfilling after the tip of the pipette was filled with the antibiotic-free solution. The length of the antibiotic-free solution in the tip is critical. The tip should be filled to a length, which allows the antibiotic to diffuse to the tip in few seconds. The optimal length (under about $500\text{ }\mu\text{m}$) should be determined by each investigator. It follows that the permeabilization of the patch membrane by nystatin may be considerably (more than hour) delayed. Another drawback is the inactivation of the nystatin solution about 3 hours after the stock solution was prepared. It would be therefore of interest to have a method for checking the perforating activity of the nystatin solution. For this purpose, changes in cell volume were used, which are expected to occur in physiological saline containing nystatin, as follows from the Donnan equilibrium (Boyle and Conway 1941).

Fig. 1 shows the time course of volume changes in single L6 myoblasts (myoballs) exposed to different concentrations of nystatin dissolved in the standard saline. The standard saline had the following composition (in mmol/l): NaCl 135; KCl 5.0; CaCl_2 1.0; glucose 5; HEPES 10.0; pH 7.4. (referred to as isotonic 135 mol/l NaCl saline). Nystatin solution was prepared as recommended by Horn and Marty (1988) and reviewed by Korn et al. (1991). Nystatin (Sigma Chemical Co., St. Louis, MO) was dissolved by means of vortex and sonication in dimethylsulfoxide (DMSO) to make a stock solution (50 mg/ml); it was kept for 3 days

in a freezer. Immediately before the experiment the stock solution was thawed and diluted to the required concentration of nystatin, which was either used for perforated patch-clamp recording (published elsewhere) or applied to the bath of proliferating myoblasts (myoballs) in the experiments reported in this paper.

The L6 cells were obtained from American Type Culture Collection (A.T.C.C.; Rockville, MD; U.S.A.). Their maintenance and preparation of myoballs followed the same procedure as described elsewhere (Hurňák and Zachar 1994; Zachar and Hurňák 1995). The cell volume of rounded myoblasts (myoballs) was measured as described previously by means of a video image analysis system set-up for continuous observation, recording and measurement of volume changes in single cells (Zachar and Hurňák 1995). Cells were viewed at $\times 320$ 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 in a video recorder. Later on, the records were played back and images were grabbed with a PCVISION Plus frame grabber, resolution 512×480 pixels, at minute intervals, and stored on a hard disk of an IBM PC.

As follows from Fig. 1 the myoblasts swelled in nystatin solutions. The volume change was dependent on the nystatin concentration. The threshold concentration of freshly prepared nystatin solution was about $12.5 \mu\text{mol/l}$ (Fig. 1A, *filled circles*).

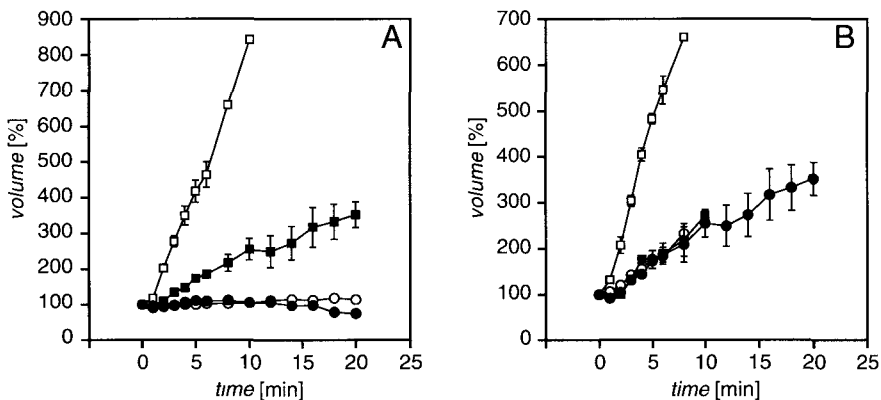


Figure 1. Volume changes in single L6 myoblasts (myoballs) exposed to different concentrations of nystatin dissolved in isotonic standard saline. A : $12.5 \mu\text{mol/l}$ (*filled circles*), $25 \mu\text{mol/l}$ (*empty circles*), $50 \mu\text{mol/l}$ (*filled squares*), and $100 \mu\text{mol/l}$ (*empty squares*). Changes in the effectiveness of nystatin solutions with time are shown in B. *Empty symbols*: freshly prepared nystatin solutions; *filled symbols*: nystatin solutions prepared 3 (*filled circles*) or 5 (*filled squares*) hours before their application into the bath. Volume changes were recorded continuously before and during application of isotonic nystatin solutions. No volume recovery was ever observed after reintroduction of control saline. *Ordinate*: cell volume in relative units. *Abscissa*: time in min after the start of application of the nystatin challenge.

The threshold effect was triphasic: a slight initial volume decrease (shrinkage) for about 2 min followed by a volume increase and, after about 10 min, by a significant volume decrease. At twice as high nystatin concentration (25 $\mu\text{mol/l}$) the final shrinkage phase was lacking (Fig. 1A, *empty circles*). At 50 $\mu\text{mol/l}$ concentration (Fig. 1A; *filled squares*) the volume increased continuously after a delay of about 1–2 min. and reached a plateau of about 350% of the original volume. At 100 $\mu\text{mol/l}$ concentration of nystatin (Fig. 1A, *empty squares*) the myoblasts increase their volume in about five min to more than 500% of the original value. No volume recovery was ever observed after reintroduction of control saline.

The effects of nystatin diminished upon prolonged storage of nystatin solution. Nystatin solutions (50 $\mu\text{mol/l}$) prepared 3 hours before use were still active to about 80% (Fig. 1B, *filled circles*). Volume changes in 100 $\mu\text{mol/l}$ nystatin solutions were, however, substantially diminished (to about 20%) 5 hours after the preparation of the nystatin solution (Fig. 1B, *filled squares*) in contrast with freshly prepared nystatin solution (*empty squares*).

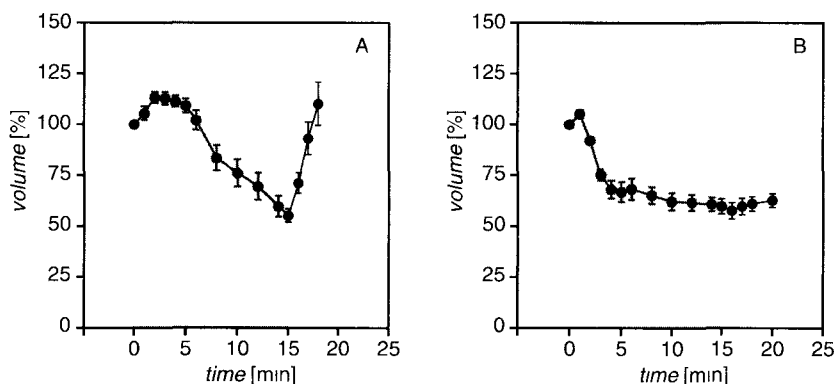


Figure 2. The time course of volume changes in 50 $\mu\text{mol/l}$ nystatin solutions after replacing Na^+ ions with TEA^+ ions (A) or Cl^- ions with glutamate anions (B). Note the regulatory volume decrease with undershoot (A) in nystatin TEA-Cl saline as well as the recovery after the reintroduction of the standard isotonic NaCl solution. In Na-glutamate solutions (B) the recovery is virtually lacking. Volume changes were recorded continuously before, during and after application of isotonic nystatin solutions. *Ordinate*: cell volume in relative units. *Abscissa*: time in min after the application of the nystatin solutions.

If Na^+ ions were replaced with TEA^+ in the presence of external Cl^- a regulatory volume decrease was observed to subnormal values. The time course of the volume change in these conditions is shown in Fig. 2A. The myoblast volume shrank to about half of the control value. The volume changes were reversible after

reintroduction of Krebs solution. A fast regulatory volume decrease to subnormal values was also observed after replacing external Cl^- by glutamate anion, which is impermeant through nystatin channels. The transient initial volume increase was, however, very small if any, and the final shrunken volume was not reversible after introduction of standard saline without nystatin. The dependence of the volume changes on the presence of various small cations and anions in the external (bath) saline can be explained if the Donnan, osmotic and electric equilibria (Boyle and Conway 1941; Adrian 1956, 1960; Hodgkin and Horowicz 1959) are taken into account (Eqs. 1–3; for a review see Zachar 1971).

$$\frac{[\text{K}]_o + b[\text{Na}]_o}{[\text{K}]_i + b[\text{Na}]_i} + \frac{[\text{Cl}]_i}{[\text{Cl}]_o} \quad (1)$$

$$[\text{K}]_i + [\text{Na}]_i + [\text{Cl}]_i + \frac{A}{v} = C \quad (2)$$

$$[\text{K}]_i + [\text{Na}]_i + [\text{Cl}]_i + \frac{zA}{v} = 0 \quad (3)$$

where $[\text{K}]$, $[\text{Na}]$, $[\text{Cl}]$ are internal (*i*) or external (*o*) concentrations of the respective ions; $b = P_{\text{Na}}/P_{\text{K}}$ is the ratio of the permeability for Na^+ and K^+ ions; A is the number of non-diffusible particles; v is the cellular volume and C is the total concentration of all particles in the bath solution; z is the mean valence of non-diffusible particles. The equilibrium potential for Cl ions may be considered to be equal to the resting potential (V), since Cl ions are distributed passively across the membrane, so that:

$$V = \frac{RT}{F} \ln \frac{[\text{Cl}]_i}{[\text{Cl}]_o} \quad (4)$$

where R , T and F have their usual meaning in the Nernst Eq. (4).

The outcome of the volume change is dependent on the actual values of the equation parameters which are mostly unknown in the present case (L6 myoblasts). The differences in these parameters may explain the fact that nystatin applied into the bath did not lead to swelling in pituitary cells (Horn and Marty 1988) in contrast to L6 myoblasts reported here. The cell interior of L6 myoblasts behaved as a hypertonic solution to standard saline, but as a hypotonic solution to the bath saline either in the absence of Na^+ or Cl^- ions.

The volume changes in myoblasts monitored by the video analysis system are illustrated in Fig. 3. The individual pictures represent frames from a continuous record of a group of cells from two different dishes (*A – C* and *D – F* respectively) subjected to $50 \mu\text{mol/l}$ – nystatin solution.

Formation of blebs was noticed in a great proportion of myoballs, especially when higher concentration of nystatin were used. The individual blebs protruding from the surface membrane eventually began to confue and at the end formed a

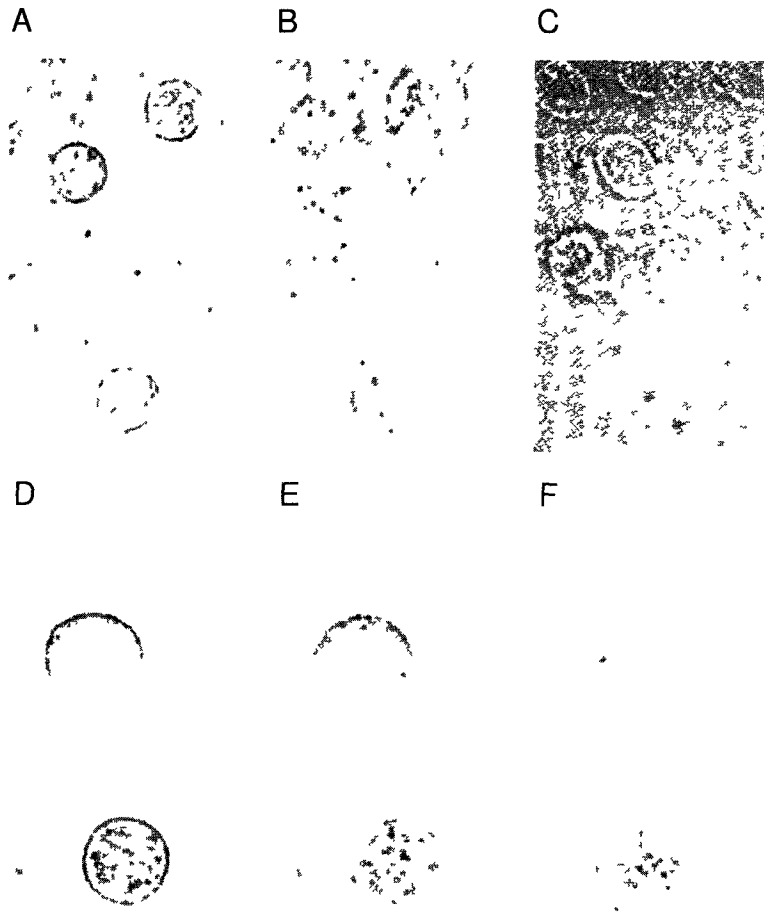


Figure 3. Myoballs as seen with the light microscope on the monitor of the video analysis system. The pictures represent frames from a continuous record of a group of cells from two different dishes (A–C and D–F) subjected to 50 $\mu\text{mol/l}$ nystatin isotonic saline. A: three myoballs in control saline. B: the same cells 5 min after application of isotonic 50 $\mu\text{mol/l}$ nystatin saline. C: another field in the same dish 5 min later. D: blebs formation in the enlarged myoballs subjected to nystatin saline for 6 min. E, F: enduring confluence of blebs to form a continuous layer around the myoballs (as seen in C). Note the spindle shaped myoblasts attached to the bottom of the chamber in A. Calibration (as represented by the width of the frame in A): 65 μm .

continuous layer around the myoballs (as seen in Fig. 3C). Note the spindle shaped myoblasts attached to the bottom of the chamber in A.

The cells were functional in nystatin solutions as also evidenced by the fact that they were able to divide and it even seemed that the nystatin procedure facilitated

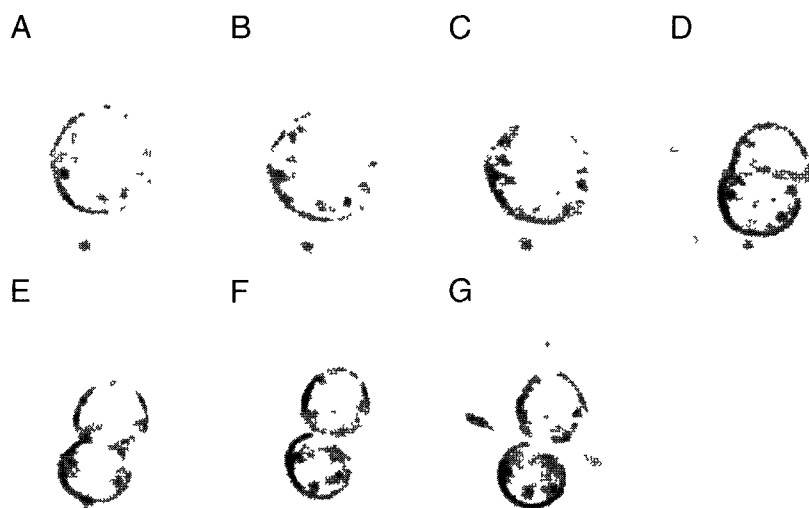


Figure 4. Mitosis in nystatin solution. Frames from a continuous record of a dividing myoblast in a $25 \mu\text{mol/l}$ nystatin isotonic NaCl saline. *A* 5 min *B* 8 min *C* 12 min *D* 18 min *E* 21 min *F* 25 min *G* 30 min after the introduction of nystatin saline.

cell division (Fig. 4). Frames are from a continuous record of a dividing myoblast in a $25 \mu\text{mol/l}$ nystatin isotonic NaCl saline. The shown cell division occurred in 30 min.

It follows from the experiments that the activity of nystatin solutions can conveniently be checked by measuring the cell volume at least in L6 myoblasts. The method can also be used to find out such a combination of ions in the external saline which would keep the cell volume in required limits. As an interesting by-product of the observations reported is the ability of the myoblasts to divide in the presence of the antibiotic nystatin which contrasts with the effects of this antibiotic on unicellular microorganisms.

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