



## Transient transfection induces different intracellular calcium signaling in CHO K1 versus HEK 293 cells

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### Abstract

For the controlled production of recombinant proteins in mammalian cells by transient transfection, it may be desirable not only to manipulate, but also to diagnose the expression success early. Here, we applied laser scanning confocal microscopy to monitor transfection induced intracellular  $\text{Ca}^{2+}$  responses. We compared Chinese hamster ovary (CHO K1) versus human embryo kidney (HEK) 293 cell lines, which differ largely in their transfectability. An improved calcium phosphate transfection method was used for its simplicity and its demonstrated upscale potential. Cytosolic  $\text{Ca}^{2+}$  signaling appeared to inversely reflect the cellular transfection fate. Virtually all CHO cells exhibited asynchronous, cytosolic  $\text{Ca}^{2+}$  oscillations, which peaked 4 h after addition of the transfecting solution. Yet, most of the HEK cells displayed a slow and continuous  $\text{Ca}^{2+}$  increase over the time of transfection. CHO cells, when exposed to a transfection-enhancing glycerol shock, strongly downregulated their  $\text{Ca}^{2+}$  response, including its oscillations. When treated with thapsigargin, a  $\text{Ca}^{2+}$  store depleting drug, the number of successfully transfected CHO cells was significantly reduced. Our result points to intracellular store release as a critical component for the transfection fate of CHO cells, and its early detection before product visualization.

### Introduction

Chinese hamster ovary (CHO) cells are the dominant mammalian cell system used for manufacture of biopharmaceuticals (Simonsen and McGrogan, 1994). However, this cell type exhibits a relatively low transfectability. We usually can transfect 90% of HEK cells, versus 40% of CHO cells, when using an improved version of the calcium phosphate transfection method, and GFP as reporter (Preuss et al., 1999).

On the search for possible reasons for this difference, we focused on the intracellular  $\text{Ca}^{2+}$  based second messenger system. We took into account that cytosolic  $\text{Ca}^{2+}$  is crucially involved in processes, which could be well related to the efficiency of transfection. These processes are molecular trafficking

between the extracellular space and the cell interior (Coco et al., 1998), between the nucleus and the cytosol (Greber and Gerace, 1995; Stehno-Bittel et al., 1995; Perez-Terzic et al., 1997; Lee et al., 1998) and the biosynthesis of proteins (Palfrey and Nairn, 1995; Rosen et al., 1995; Bading et al., 1997; Dolmetsch et al., 1998).

Here we report, for the first time, the on-line monitoring of  $\text{Ca}^{2+}$  responses during transfection in CHO versus HEK cells. For this purpose, laser scanning confocal microscopy was used on cells loaded with the  $\text{Ca}^{2+}$  sensitive dye fluo-3. We designed pharmacological experiments to locate the source of transfection-induced  $\text{Ca}^{2+}$  signaling. At first glance, because we use calcium phosphate precipitated DNA for gene transfer into the cell, it may seem that most of

the intracellular  $\text{Ca}^{2+}$  signaling would originate from extra cellular calcium or its phosphate taken up by the cells. Yet,  $\text{Ca}^{2+}$  released from intracellular stores is widely held to be critical for  $\text{Ca}^{2+}$  sensitive regulation of transport and protein synthesis (Greber and Gerace, 1995; Stehno-Bittel et al., 1995, Palfrey and Nairn, 1995; Berridge, 1997). Moreover, we attempted to elucidate the role of this important second messenger for the glycerol shock, which is generally used to enhance the transfectability of mammalian cells (Staedel et al., 1994).

Our work stresses not only the importance of intracellular calcium store release for the transfection fate of CHO cells, but points as well to its early inductance before product visualization. This could open future ways of developing novel diagnostic tools for the product independent and early verification of the transfection success.

## Materials and methods

### *Cell cultures*

Human embryonic kidney (HEK293) and Chinese hamster ovary (CHO K1) cell lines were grown as attached cultures in DMEM/F12 medium (pH 7.3) (Gibco, UK) supplemented with 2.2% fetal calf serum (Sigma, U.S.A.). The cells were kept in an incubator, which had a humidified atmosphere and 5%  $\text{CO}_2$ , at 37 °C. Optimized growing conditions were maintained by splitting the cultures 1:4 to 1:6, 2–3 times per week.

### *Transient transfection*

Exponentially growing cultures were seeded in a density of  $2\text{--}4 \times 10^5$  cells/ml. After 16 to 20 h in the incubator, the cells were transfected using an improved version of the calcium phosphate technique. This approach has been described elsewhere in detail (Jordan et al., 1996). In short, equivolumes of 250 mM  $\text{CaCl}_2$  and 1.5 mM  $\text{Na}_2\text{HPO}_4$  (both pH 7.05) were mixed with 25  $\mu\text{g}$  DNA/ml. Critical for the efficient fate of the forming precipitate was the mixing time of 55–60 s in combination with the following 10-fold dilution in the cell medium. There the precipitate is allowed to continue to grow slowly and to reach the optimum size for its contact with the cells.

For the test of various compounds on the transfection of the cells grown in multi-well plates, we ensured that each sample received the same calcium phosphate DNA co-precipitates, with one identical

mixing time. Half the medium was removed of each well and sampled. Then the chemicals were added to each well, with their concentrations adjusted for the complete medium volume per well. The calcium phosphate DNA mixture (calculated for the complete volume total of all treated wells) was pre-diluted in the sampled medium (half of the total volume of all wells). This transfecting medium was then added to each well, restoring their original medium volumes (and the 1x concentrations of the active chemicals). The transfection efficiency of the precipitate had not been reduced by this two-step dilution procedure. Four hours after transfection onset CHO cells were exposed to a 2.5 min lasting glycerol shock (10% glycerol in growth medium) before medium change.

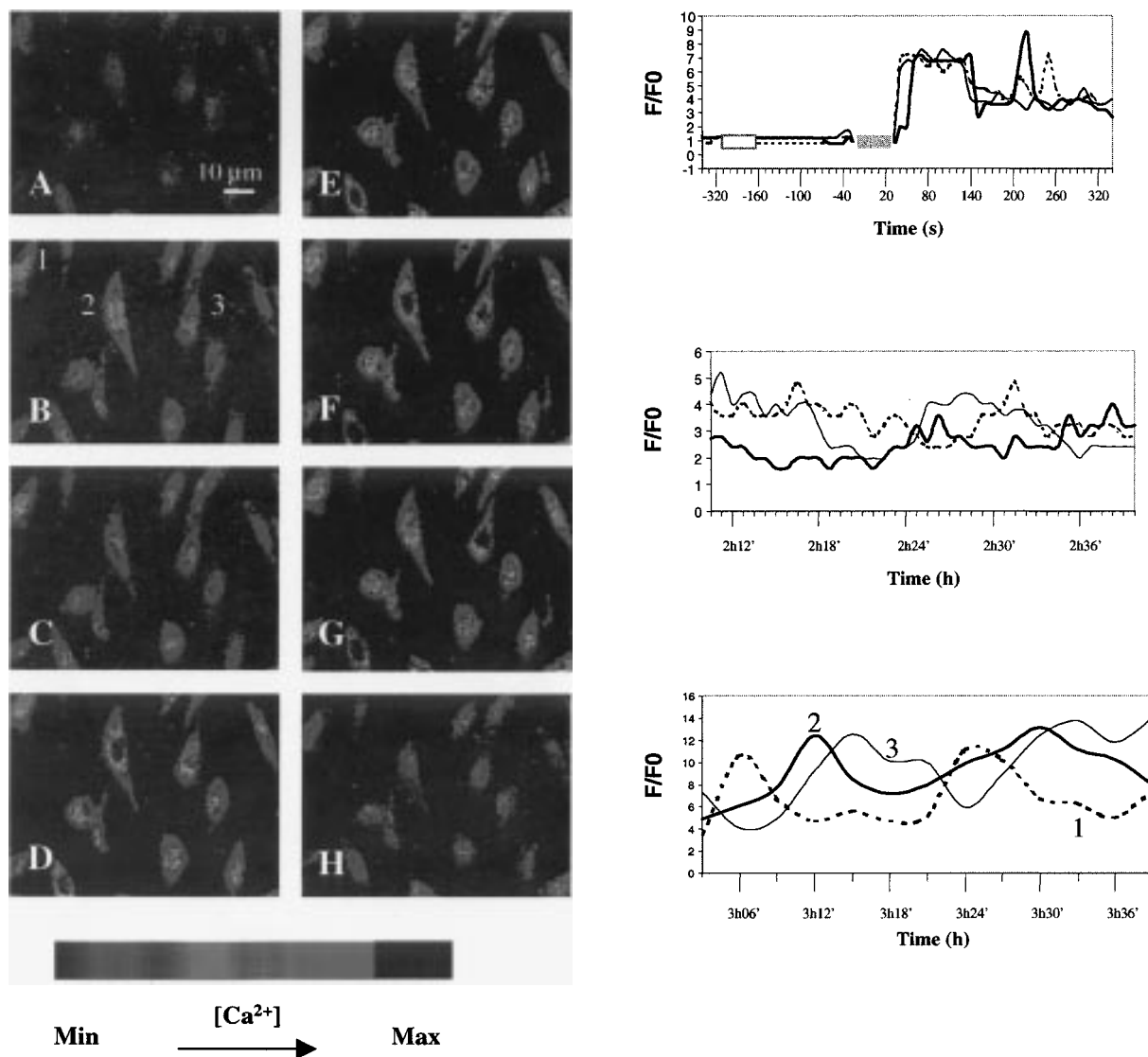
### *Assessment of transfection efficiency and pharmacological treatment*

The transfection efficiency and its dependence on active substances were assessed in cell cultures grown for 16–20 h in the incubator. The active substances were added directly before the transfection with a pEGFP-N1 vector (CLONTECH, U.S.A.). The concentrations of the chemical compounds were: 10  $\mu\text{M}$  nifedipine, 50  $\mu\text{M}$  lasalocid, 5  $\mu\text{M}$  N(G)-nitro-L-arginine methyl ester (L-NAME), 15 nM thapsigargin, 0.5  $\mu\text{M}$  ionomycin, 5  $\mu\text{M}$  dantrolene. At least five wells were measured per compound. Staining with the dead-cell stain TOTO-3 (Molecular Probes, U.S.A.) was used to verify the viability of the cells. Cell viability was not significantly affected by the chemical treatment.

The GFP signal was measured using a fluorescence reader (Cytofluor) with standard fluorescein filter sets. Eight positions were screened per well. Background signals were measured for each well directly after the medium change, 4 h after transfection onset. Wells, which had already GFP expressing cells at that time, were excluded from measurements. After 20 h the plates were measured again. At the end of the experiment cells were trypsinized and counted with a hemacytometer chamber. The transfection efficiency was assessed as the fluorescence intensity minus the background measured before and divided by the number of cells treated with the same compound.

### *Cell loading with $\text{Ca}^{2+}$ indicator*

For confocal microfluorimetric studies, cells were loaded before transfection with the cell-permeant acetoxymethyl ester form of fluo-3 (fluo-3-AM, 10  $\mu\text{M}$ , Molecular Probes, U.S.A.) 70 min in the incubator.



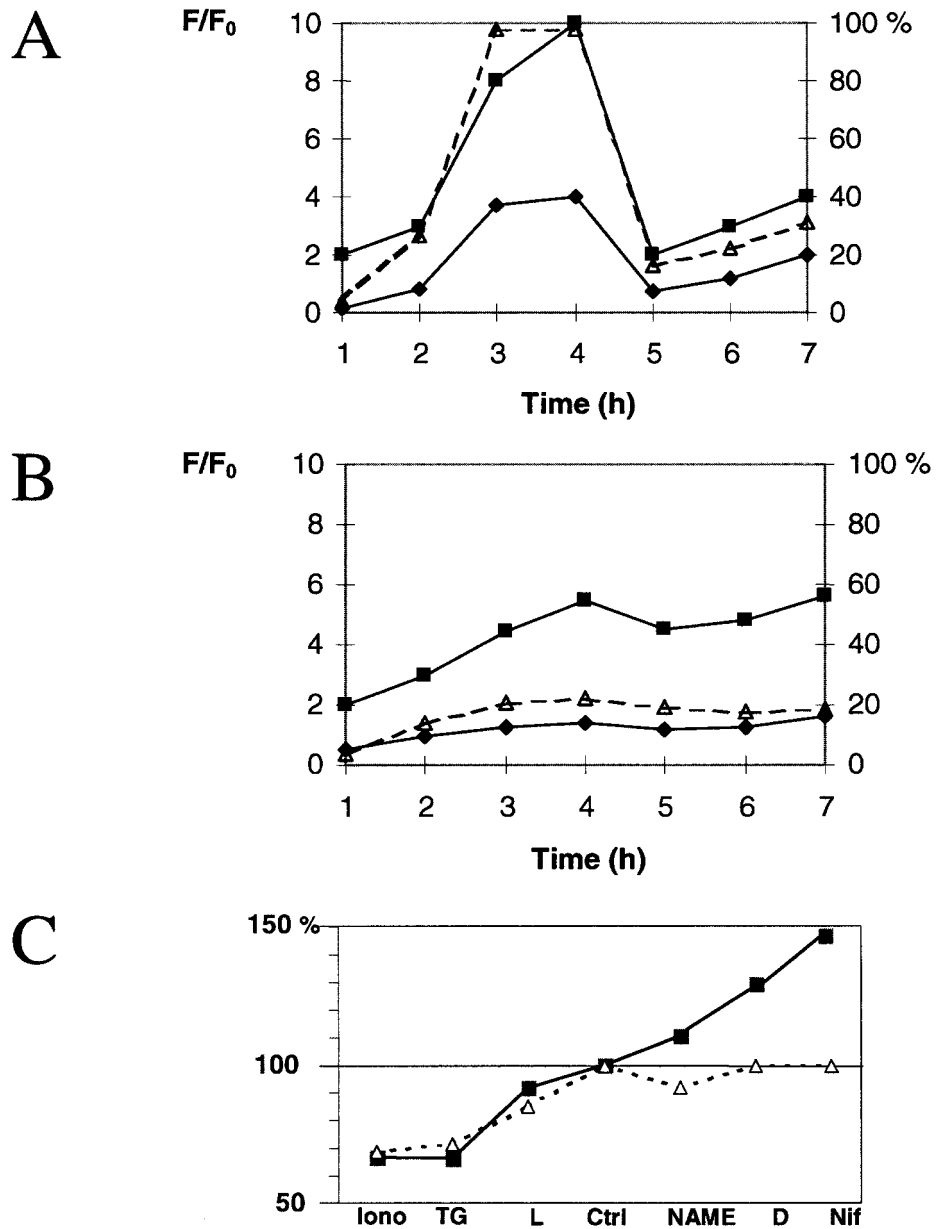
**Figure 1A-H.** False color micrographs indicating the relative cytosolic  $\text{Ca}^{2+}$  concentration changes in 15 CHO K1 cells when subjected to transfection. A: baseline. B: First signal fluctuations exhibiting substantial intercellular differences (240 s after exposure to transfecting particles). C (280 s): After response the intracellular free  $\text{Ca}^{2+}$  concentration fell back to a level 3–4 fold above baseline. D: Strong oscillations, recorded 3 h after transfection onset, and at 9 min intervals thereafter (E–G). H: Five minutes after glycerol shock, which was applied 4 h after transfection onset. Right side: Cytosolic  $\text{Ca}^{2+}$  response curves of three cells (1–3, see B) as measured by the relative fluorescence intensity  $F/F_0$  of the fluo-3 dye. White bar: mock solution. Shaded bar: administration of calcium phosphate/DNA transfection mix.

Then cells were washed twice and placed in new, bicarbonate free medium at room temperature. Under these conditions, no dye compartmentalization as observed.

#### *Laser scanning confocal microscopy*

Cells kept on the confocal stage were transfected in DMEM F12 medium, supplemented with 2.2% fetal

calf serum. The medium lot was free of bicarbonate and supplemented with additional 10 mM HEPES buffer (pH 7.3). Measurements were performed at room temperature, in a humidified chamber, without additional  $\text{CO}_2$ . Calcium signaling in these samples before (10 min – max. 1 h) during, and after transfection was performed using a laser-scanning confocal microscope (Zeiss LSM 510). We used fast scanning rates (pixel time 0.88  $\mu\text{s}$  to 1.75  $\mu\text{s}$ ) at 1 to 3 $\times$  zoom, low



**Figure 2.** Time course of transfection-induced cytosolic  $Ca^{2+}$  signaling as observed by the relative fluorescence intensity  $F/F_0$  of the fluo-3 dye. A: in CHO (n=33) B: in HEK (n=22) cells. ■ represent the average  $[Ca^{2+}]_i$ , ◆ the average amplitude of its oscillations, —△— the percentage of cells which exhibit  $[Ca^{2+}]_i$  oscillations. C: Relative effect of chemicals (x-axis) on transfection efficiency as observed by the sampled GFP fluorescence over 5 wells, each with  $5 \times 10^4$  cells. Description of chemicals see text. Signal of non-treated cells is 100% within each cell group. The transfection rate was measured 20 h after transfection. ■ = CHO cells; △ = HEK cells.

magnification ( $32\times$ ) and low laser power (0.015 mW), to avoid any potentially harmful effects on the cells. Some of the cells were scanned for more than 5 h. Minimum scanning intervals of 40 s were maintained for not longer than 80 min. Staining with the dead-cell stain TOTO-3 at the end of experiments did not reveal

a significant decrease of cell viability ( $<10\%$ ). Almost no cell divisions were observed at room temperature for the time of observation. We used the 488 nm excitation line together with standard fluorescein filter sets. Control experiments on multi-well plates verified that cells transfected under the same temperature

and media conditions as the cells investigated under the microscope are indeed capable of later expressing GFP. They exhibited the same relative sensitivity towards pharmacological treatment as cultures kept in the incubator immediately after transfection. GFP expression did not occur in transfected, 'unloaded' control cells when kept at room temperature for 5 h after transfection.

## Results and discussion

Individual intracellular  $\text{Ca}^{2+}$  responses of CHO cells ( $n=15$ ) were shown in confocal micrographs A-H of Figure 1. Changes of their intracellular  $\text{Ca}^{2+}$  were visualized by false colors, coding the intensity changes of the  $\text{Ca}^{2+}$  dye fluo-3 (see bar below image series). Individual  $\text{Ca}^{2+}$  response curves were calculated from the actual fluorescence intensity ( $F$ ) relative to its baseline signal ( $F_0$ ). Both values have been background subtracted. The response curves of three cells (1–3, Figure 1B) were displayed on the right side of Figure 1. Already 4 min after transfection individual CHO cells began to display their first  $\text{Ca}^{2+}$  fluctuations (Figure 1B and right top curve, shaded bar). These responses appeared to be induced by contact with calcium phosphate-DNA particles. They were macroscopically visible by a slight opacity of the medium. The cells did not respond to a previously applied 'placebo' solution, which had the same volume as the transfection mixture but contained plain cell medium (Figure 1, top curve, empty bar). The individual  $\text{Ca}^{2+}$  fluctuations were preceded by an initial response, which was exhibited by all cells and lasted less than 3 min (Figure 1, top curve, first response after shaded bar). This response appeared to be provoked by the sudden increase of extracellular free  $\text{Ca}^{2+}$  ions stemming from the high  $\text{Ca}^{2+}$  content (250 mM, added in a tenfold dilution step to the medium) of the added calcium phosphate DNA mixture (Figure 1, top, shaded bar). For their uniformity, we refer to this initial response as not related to the transfection process, considering its inability to account for inter-individual differences in the transfection fate of the cells (only 40% of CHO cells transfected successfully). Together with the continuing precipitate formation, which induced a growing opacity of the cell medium, the proportion of oscillating CHO cells increased within the next hours. Figures 2A and B illustrate the time course of the  $\text{Ca}^{2+}$  signaling of these two cell types. It is important to note that HEK cells showed a much

weaker response and less oscillations than CHO cells. They thus appeared to be more uniform in their signaling. Yet, both CHO and HEK cells, regardless of the difference in their  $\text{Ca}^{2+}$  response strength, showed maximum  $\text{Ca}^{2+}$  signal increase 4 h after transfection. At that time, the proportion of oscillating cells within each cell group reached its maximum.  $\text{Ca}^{2+}$  oscillations exhibited by CHO cells had become more sinusoidal (Figure 1, middle and bottom curve). After being exposed to a 2.5 min lasting glycerol shock the calcium response of the CHO cells was dramatically reduced (Figure 2A). It appeared to be 'aligned' to that of HEK cells.

The contrasting signaling behavior between the two cell types seemed to 'inversely reflect' their transfection fate. Twenty hours after administration of the calcium phosphate DNA solution, 90% of HEK cells were visibly transfected, versus only 40% of (glycerol shocked) CHO cells (see also Preuss et al., submitted 1998). Comparison of the overall signal strength of the GFP expression level measured over cell populations plated in wells was approximately 1.5 times higher in HEK cells than in CHO cells.

$\text{Ca}^{2+}$  oscillations are generally related to intracellular store release. Internal  $\text{Ca}^{2+}$  stores play an important role in the regulation of cellular protein synthesis and endo- and exocytotic events (Palfrey and Nairn, 1995; Rosen et al., 1995; Coco et al., 1998). The uptake of calcium phosphate DNA co-precipitate has been found to rely on an active, endocytotic process (Loyter et al., 1982; Orrantia et al., 1990). Both DNA uptake and protein synthesis are central to the function of transfection.

We therefore tested the effect of drugs with regulating effects on the regulation of the store luminal  $\text{Ca}^{2+}$  content. Thapsigargin ('TG'; Figure 2C) is probably the most potent substance for inhibiting the ATP driven  $\text{Ca}^{2+}$  re-uptake pumps, leading to depletion of the affected intracellular stores (Begum et al., 1993; Won et al., 1995). Dantrolene ('D'; Figure 2C), on the other hand, prevents any efflux from  $\text{Ca}^{2+}$  stores (Wei and Perry, 1996).

We applied all  $\text{Ca}^{2+}$  active drugs tested for 4 h from transfection begin until medium change (or shock). Figure 2C shows the effect of various drugs on the GFP expression level within each cell group 20 h after transfection. The fluorescence signals were measured as percentage relative to that of non-treated control cells. We observed that thapsigargin treated CHO cells emitted about half the GFP fluorescence than dantrolene treated cells. HEK cells, on the

other hand, were significantly less susceptible to these drugs.

These results stress the importance of store depletion for the transfection fate of CHO cells. The effects of store depletion have been studied so far only in viral expression systems, or within the scope of regular cellular function (Greber and Gerace, 1995; Greber et al., 1997). These effects range from regulation of protein folding and control of gene expression to the reduction of viral DNA replication in the host cell nucleus to the regulation of molecular trafficking across its pores (Greber and Gerace, 1995; Greber et al., 1997; Stehno-Bittel et al., 1995; Lee et al., 1998).

Ionophores have been shown to prevent viral DNA from entering the nucleus (Greber et al., 1997). This reported finding exemplifies the sensitivity of a multistep process to store depletion, and its possible target sites, endocytosis, membrane penetration and cytoplasmic transport (Greber et al., 1997).

As Figure 2C shows, the  $\text{Ca}^{2+}$  ionophores lasalocid ('L') and ionomycin ('Iono') had varying effects in our cells. Ionomycin strongly reduced the transfection rate in CHO cells. GFP fluorescence was about 35% less than in control CHO cells, and more than 80% less than in dantrolene treated cells.

Dihydropyridine calcium channel antagonist nifedipine ('Nif') is generally used to inhibit the L-type of voltage operated  $\text{Ca}^{2+}$  channels. It also strongly reduced the tendency of CHO cells to transfect, but did not significantly affect HEK cells. Nifedipine has other effects as well, regulating the  $\text{Ca}^{2+}$  release from intracellular stores into the cytosol in polymorphonuclear neutrophils (Rosales and Brown, 1992). Moreover,  $\text{Ca}^{2+}$  influx through nifedipine sensitive channels has been shown to trigger depletion of  $\text{Ca}^{2+}$  influx sensitive  $\text{Ca}^{2+}$  stores (Wang et al., 1998). The very low effect of nitric oxide synthase (NOS) inhibitor L-NAME on the transfection fate of both CHO and HEK shows that transfection induced store depletion in these cells may not trigger (NO mediated) capacitative  $\text{Ca}^{2+}$  entry. According to Parekh and Penner (1997) the dominant  $\text{Ca}^{2+}$  entry pathway in non-excitabile cells is the store-operated one, in which  $\text{Ca}^{2+}$  entry is governed by the  $\text{Ca}^{2+}$  content of the agonist-sensitive intracellular  $\text{Ca}^{2+}$  stores. However, the role and detailed mechanisms of this entry pathway remain obscure.

The strong reduction of  $\text{Ca}^{2+}$  oscillations in CHO cells after shock application appears striking. Still, we cannot rule out that the dye may have been squeezed out of the cells, at least for a short time during the

shock. On the other side, when applying lasalocid or ionomycin after shock treatment, we observed an increase of the fluo-3 signal, which was tenfold as high as the maximal signal before the shock. This circumstance almost rules out substantial dye loss. DNA stain did not enter the cells during shock application, excluding larger membrane permeability changes.

Loyter et al. (1982) have shown that after 4 h after transfection onset most of the DNA is still in the cytosol, but not in the nucleus. Therefore, it could be possible that shocks, when applied 4 h after addition of transfecting DNA solution, are commonly used not to drive the DNA from the outside into the cell, but rather from inside the cytosol into the nucleus. Our observation of a strong reduction of oscillations after the shock, at this moment speculatively, could help in such a process by reducing store release and its sealing of the nuclear membrane. However, we do not know at this moment, whether the  $\text{Ca}^{2+}$  is indeed reverted into the stores by shock treatment, and whether the DNA trapped in the cytosol despite its big amount is still relevant for transfection.

## Conclusion

Our work points to a possible key function of intracellular  $\text{Ca}^{2+}$  store depletion for heterologous, recombinant protein production, complementing its known regulatory role for cellular protein synthesis. The detection of product independent, early signaling may allow a) development of product independent diagnostic tools for the early assessment of the transfection rate in small cell samples b) improving transfection efficiency of calcium phosphate and possibly other transfection methods by manipulating the store luminal  $\text{Ca}^{2+}$  content.

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