Nanokinetics of drug molecule transport into a single cell

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Aims: To analyze drug transport at a single cell level, a mast cell line, RBL-2H3, was treated with cell-permeable fluorescent compounds, such as quinacrine, and was monitored by a fluorescence video microscope. **Methods:** Small areas in the video that corresponded to granules and part of the cytosol in a cell were chosen and the signal intensity in these areas was monitored sequentially. **Results:** The initial rate of quinacrine uptake through the cell membrane calculated from the fluorescent signal was correlated with quinacrine concentration, and it decreased at a lower temperature, showing that the transport was an energy-requiring process, such as active transport. The kinetics of the transport through the microgranular membrane did not depend on the temperature but the pH in the cytosol, therefore this process should be passive transport by pH gradient. **Conclusion:** These data indicate that the observation of video microscope-mediated drug transport using fluorescent dye is useful in kinetic analysis at the nanometer scale.

The molecular dynamism in cells or tissues is one of the essential bases for nanomedicine; therefore, analytical methods should be developed to study it. The video microscopic method is useful not only for visualizing the cell morphologically [1–5], but also for analyzing molecular dynamism, such as molecular movement and content variations, using fluorescent probes or other probing techniques [6–9].

We have studied video microscopic imaging analysis, which provides morphological observations of object-oriented cellular responses, including molecular mechanisms [5,10,11]. This indicated that the signal intensity of a certain area in a video image could be used for quantitative analysis at a high magnification and even a single molecule can be seen as a faint dot [12,13].

The rat basophilic leukemia cell line, RBL-2H3, is used frequently as a model of mucosal mast cells [14,15] and has been studied widely to analyze the mechanism of the exocytotic process [16]. The cells have many acidic microgranules, which stably contain basic neurotransmitters, such as histamine and serotonin. We found that these granules stained easily with a cell-permeable fluorescent compound, quinacrine (QC), which is a weakly basic molecule. As acidic granules can be stained with QC, this compound was used as an indicator for cellular pH [17]. In addition, a QC derivative was used formerly as an

antimalarial drug and is now used as an inhibitor of phospholipase A₂ in cell biology experiments [18].

In this report, we applied the video microscopic method to analyze the dynamics of drug transport into a single cell and further to sub-micrometer size (0.2–0.8 µm diameter) acidic granules in which drug molecules are specifically concentrated. This kinetic analysis at the nanometer scale is called 'nanokinetics' [19] and we think that an understanding of nanokinetics is important for nanomedicine.

Materials & methods

Cell culture

The rat basophilic leukemia cell line, RBL-2H3, was cultured in minimal essential medium (Invitrogen, Carlsbad, CA, USA) supplemented with 15% fetal bovine serum (Irvine Scientific, Santa Ana, CA, USA), penicillin G (100 U/ml; Invitrogen) and streptomycin (100 µg/ml; Invitrogen) in a humidified incubator with 5% CO₂ and 95% air at 37°C. The day before the experiment, cells were inoculated onto glass cover slips at 5 × 10⁴ cells/cm², as described previously [10].

Fluorescent microscopic observation

An inverse-type incident-light fluorescence microscope (Axiovert 135-TV; Carl Zeiss Oberkochen, Germany) with a differential interference contrast (DIC) illumination system

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Figure 1. Scheme of quinacrine uptake analysis.

and silicon-intensified target (SIT) video camera (C-2741; Hamamatsu Photonics, Hamamatsu, Japan) was used for fluorescence video imaging. The whole microscopic system was placed in a temperature-controlled room. The image was recorded by a video tape recorder and analyzed by graphic analysis system (GBP-1, Sharp Semiconductor, Tokyo, Japan) installed on a personal computer. This system allows a sequential analysis of signal intensity in a certain area of a video picture, with optical resolution in the X-Y plane at 200 nm and focus depth (Z-axis) of 400 nm, by using an objective lens for 100-times magnification. Using image processing methods, such as the video-enhancement contrast method, the image can be modified to provide a more resolved image, however, we cannot overcome the theoretical optical wavelength limitation in the resolution. To obtain reproducible data without compensation, we always used the same system conditions, such as experimental temperature, SIT camera control, including gain and amplification value, and neutral density filters. After obtaining the video image, small areas (9 × 9 pixels square each) corresponding to microgranules or part of the cytosol were

chosen and the value of the fluorescent intensity was obtained sequentially and used for further analysis.

Quantification of QC

In order to analyze the QC concentration in the cells, a standard curve of QC concentration and fluorescence intensity was constructed by measuring the fluorescence of QC solutions (0.001, 0.01, 0.1, 1 and 10 μmol/l) in culture dishes using the video microscope, as described above. Since the fluorescence intensity of a QC solution changes according to the pH, we analyzed the relevance of the fluorescence of QC to pH. The fluorescence of the QC solution adjusted to various pH by Britton-Robinson wide-range buffer was measured by a fluorescence spectrometer and the correlation coefficient was calculated using a pH–fluorescence correlation curve.

Compartmental analysis

Two-compartmental analysis was used for this QC transport system as shown in Figure 1. Equation 1 represents the transport across the cell membrane between extracellular medium and cytoplasm, where C0, C1 and C2 are the concentration of QC in medium, cytoplasm and a granule, respectively, and k01, k10, k12 and k21 are the transport kinetic constants of uptake from the medium to the cytosol, feedback from the cytosol to medium, uptake from the cytosol to a granule and feedback from a granule to the cytosol, respectively.

Equation 1:

$$dC1/dt = k01C0 - k10C1 - k12C1 + k21C2$$

Equation 2 represents the transport between cytoplasm and granules across the granular membrane:

$$dC2/dt = k12C1 - k21C2$$

The concentration of QC was measured by the fluorescence intensity in the video image. We measured the starting increasing rate, so the inverse reaction can be neglected. Therefore, these equations are changed to:

Equation 3:

$$dC1/dt = k01C0 - k12C1$$

Equation 4:

$$dC2/dt = k12C1$$

Since dC1/dt, dC2/dt and C1 are measured by video signal intensity analysis and C0 is known, k01 and k12 can be estimated.

Figure 2. 0.1 and 1 μ M quinacrine uptake into RBL-2H3 cells.

The upper pictures show DIC images of analyzed RBL-2H3 cells before QC addition and time-lapse fluorescent images after QC addition. Cytosolic and granular fluorescence increased gradually after QC addition. In the lower graphs, fluorescence intensity of selected areas in the cytosol and microgranules (three areas each) after QC addition was plotted. The arrows show the time of QC addition. Data are representative of three independent experiments. DIC: Differential interference contrast; QC: Quinacrine.

Results & discussion

Figures 2 & 3 (upper columns) show QC transport into RBL-2H3 cells in a DIC image and time-lapse fluorescence images. The fluorescence intensity was estimated by the video image intensity in each area (in the cytosol and granules), as described above. After adding QC to the culture buffer, the fluorescence intensities were monitored according to time. At lower concentration, QC transported into the cells rather slowly through the cell membrane; however, above 1.0 μ M of QC, the rate of transport had almost started to maximize and the intensity of fluorescence of QC became saturated within 1 min (Figure 2 and data not shown). The estimated uptake rates of QC by fluorescence intensity at the early step (0–15 s; $n = 3$) are summarized in Table 1. The rate of increasing fluorescence was concentration dependent both in the cytosol and in granules. With these rates, and Equations 3 & 4, k_{01} and k_{12} were evaluated as in Table 1. The value of k_{01} decreased according to the increase in the outside concentration of QC. The transportation was not speedy enough even when the medium concentration was high. By contrast, the inner transport from

cytosol to microgranules was almost constant and not dependent on the concentration of medium QC.

In order to investigate the requirement of energy for its transport, the same analysis was performed at two different temperatures (24 and 14°C), as shown in Figure 3. The initial rates of QC transport measured by the fluorescence at two different temperatures were decreased drastically to 1/20 and 1/10 for cytosol and granules by the decrease in temperature of 10°C (Table 2). However, only the rate constant of the first transport step (k_{01} : from medium to cytosol) decreased ten-times, with k_{12} remaining unchanged. This showed that transport through the cell membrane was an energy-requiring process, such as active transport, but the second step was not energy dependent and is an autodiffusion process.

Since the interiors of the microgranules of this mast cell type are known to be acidic [20], the weakly basic molecule, QC [21], appeared to be transported by the pH gradient between the cytosol and granules. In order to reduce this pH

Table 1. The kinetics of quinacrine uptake at various quinacrine concentrations.

<i>The initial rate of QC uptake</i>		
Quinacrine (μ M)	Cytoplasm (SD)	Granules (SD)
0.001	0.057 (0.006)	0.13 (0.01)
0.01	0.16 (0.02)	0.28 (0.03)
0.1	0.85 (0.08)	1.48 (0.13)
1	2.92 (0.08)	6.0 (0.8)
10	9.02 (0.09)	19.4 (0.2)
<i>(Fluorescence intensity/sec)</i>		
<i>The constant of QC uptake</i>		
Quinacrine (μ M)	K_{01}	K_{12}
0.001	0.183	0.31
0.01	4.4×10^{-2}	0.44
0.1	2.3×10^{-2}	0.21
1	8.9×10^{-3}	0.25
10	2.8×10^{-3}	0.19
<i>(1/sec)</i>		

The rate of QC uptake was calculated from the initial slope (within 60 s). The data shown are the average of three independent experiments. The constants were calculated using the formula from Figure 1. SD: Standard deviation of the values for initial rates of QC uptake.

gradient, ammonium chloride was introduced to the medium to neutralize the acidic environment of the microgranule, as seen in Figure 4 (upper panel). The transport into the cytosol was stimulated, judging from the starting rate, as shown in Figure 4 (lower right); however, k01 showed a small increase and k12, transport through the microgranule membrane, decreased by half. This shows that the transport into the microgranule seems to be pH dependent.

In this analysis, we have not measured the intracellular pH exactly, but have used the reported pH value of the cytosol and microgranules [20]. In addition, we compensated the fluorescence value simply by pH, but it is possible that another factor, such as oxidation or degradation of QC, might affect the fluorescence. The further analysis of QC molecules using mass spectrometry might provide the information on QC status inside the cytosol and microgranules.

Conclusions

The dynamic aspect of drug transport in the cytosol and microgranules in a single cell was analyzed to quantitate its ‘nanokinetics’ by

using a 2D video detector. It was found that the quinacrine uptake through this RBL-2H3 cell membrane is an energy-requiring process, such as active transport. However, the transport through the microgranular membrane depended on the pH in the cytosol, as opposed to the temperature, as with passive transport by a pH gradient. The video microscopic method of visualizing drug transport in a cell is useful in kinetic analysis at the nanoscale.

Future perspective

When observing the behavior of nanoparticles in solutions at the submicron scale, movements of the nanoparticles are very dynamic. Nanoparticles move rapidly and are randomly driven by Brownian motion. Such a dynamic image of molecular movement could be produced in any nanoscale biosystem and showed an approach towards the quantitative estimation of the nanodynamics of molecules in a single cell. The nanokinetic understanding of medicinal molecules inside cells or in tissues, even in microorgans, will help the development of future nanomedicine applications, especially for more dynamic applications.

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Table 2. The kinetics of quinacrine uptake at various temperatures.		
The initial rate of QC uptake		
Temperature (°C)	Cytoplasm (SD)	Granules (SD)
24	0.85 (0.12)	1.5 (0.1)
14	0.043 (0.002)	0.14 (0.02)
(Fluorescence intensity/sec)		
The constant of QC uptake		
Temperature (°C)	K01	K12
24	2.2×10^{-2}	0.21
14	1.8×10^{-3}	0.16
(1/sec)		

The rate of QC uptake was calculated from the initial slope (within 60 s). The data shown are the average of three independent experiments. The constants were calculated using the formula from Figure 1. SD: Standard deviation of the values for initial rates of QC uptake.

Figure 4. 1 μ M quinacrine uptake into RBL-2H3 cells with or without 10 mM ammonium chloride. **Executive summary****Nanokinetics: subcellular kinetics in a single cell**

- Subcellular molecular dynamism, such as drug transport through a cell membrane and micro-organ membrane in a single cell should be clarified for future nanomedicine design.

Video microscope as a tool for nanokinetic analysis

- Video microscopic imaging of a fluorescent drug molecule, quinacrine, in a single cell of mast cell model, RBL-2H3, was performed.
- 2D light-intensity data of video images were useful for nanokinetic analysis.

Results of nanokinetic analysis

- Quinacrine uptake through the RBL-2H3 cell membrane is an energy-requiring process, such as active transport.
- Transport through the microgranular membrane did not depend on the temperature but on the pH in the cytosol, such as passive transport by pH gradient.

Future perspective

- Nanokinetic understanding of molecular transport will be the basis for future designing in nanomedicine, such as micro-shot drug delivery and micro-organ-targeted treatment.

Bibliography

- Martin PM, Magdelenat HP, Benyahia B, Rigaud, O, Katzenellenbogen JA: New approach for visualizing estrogen receptors in target cells using inherently fluorescent ligands and image intensification. *Cancer Res.* 43(10), 4956–4965 (1983).
- Wadsworth P, Sloboda RD: Microinjection of fluorescent tubulin into dividing sea urchin cells. *J. Cell Biol.* 97(4), 1249–1254 (1983).
- Weiss D, Galfé G: Video-microscopic techniques to study the living cytoplasm. In: *Image Analysis in Biology*. Hader DP (Ed.). CRC Press, Boca Raton, USA, 135–158 (1992).
- Terakawa S, Suzuki Y: Exocytosis in colonic goblet cells visualized by video-enhanced light microscopy. *Biochem. Biophys. Res. Commun.* 176(1), 466–472 (1991).
- Suzaki E, Kobayashi H, Kodama Y, Masujima T, Terakawa S: Video-ratedynamics of exocytotic events

- associated with phagocytosis in neutrophils. *Cell Motil. Cytoskeleton* 38(3), 215–228 (1997).
6. Saengkhae C, Loetchutinat C, Garnier-Suillerot A: Kinetic analysis of fluorescein and dihydrofluorescein effluxes in tumour cells expressing the multidrug resistance protein, MRP1. *Biochem. Pharmacol.* 65(6), 969–977 (2003).
7. Csala M, Staines AG, Banhegyi G *et al.*: Evidence for multiple glucuronide transporters in rat liver microsomes. *Biochem. Pharmacol.* 68(7), 1353–1362 (2004).
8. Schwartz JW, Novarino G, Piston DW, DeFelice LJ: Substrate binding stoichiometry and kinetics of the norepinephrine transporter. *J. Biol. Chem.* 280(19), 19177–19184 (2005).
9. Petrasek J, Mravec J, Bouchard R *et al.*: PIN proteins perform a rate-limiting function in cellular auxin efflux. *Science* 312, 914–918 (2006).
10. Ozawa K, Kobayashi H, Kawai E, Suzuki E, Nonomura Y, Masujima T: Quantitative analysis of exocytosis visualized by a video-enhanced light/fluorescence microscope reveals two distinct components of exocytosis in RBL-2H3 cells. *FEBS Lett.* 398(1), 67–73 (1996).
11. Ozawa K, Masujima T, Ikeda K, Kodama Y, Nonomura Y: Different pathways of inhibitory effects of wortmannin on exocytosis are revealed by video-enhanced light microscope. *Biochem. Biophys. Res. Commun.* 222(2), 243–248 (1996).
12. Funatsu T, Harada Y, Tokunaga M, Saito K, Yanagida T: Imaging of single fluorescent molecules and individual ATP turnovers by single myosin molecules in aqueous solution. *Nature* 374(6522), 555–559 (1995).
13. Harada Y, Funatsu T, Murakami K, Nonoyama Y, Ishihama A, Yanagida T: Single-molecule imaging of RNA polymerase-DNA interactions in real time. *Biophys. J.* 76(2), 709–715 (1999).
14. Beaven MA, Maeyama K, Wolde-Mussie E, Lo TN, Ali H, Cunha-Melo JR: Mechanism of signal transduction in mast cells and basophils: studies with RBL-2H3 cells. *Agents Actions* 20(3–4), 137–145 (1987).
15. Seldin DC, Adelman S, Austen KF *et al.*: Homology of the rat basophilic leukemia cell and the rat mucosal mast cell. *Proc. Natl Acad. Sci. USA* 82(11), 3871–3875 (1985).
16. Kawasaki Y, Saitoh T, Okabe T, Kumakura K, Ohara-Imaizumi M: Visualization of exocytotic secretory processes of mast cells by fluorescence techniques. *Biochim. Biophys. Acta* 1067(1), 71–80 (1991).
17. Korf BR, Schuh BE, Salwen MJ: Optimum pH for nuclear sex identification using quinacrine. *Clin. Genet.* 8(2), 145–148 (1975).
18. Nakadate T, Yamamoto S, Ishii M, Kato R: Inhibition of 12-*O*-tetradecanoylphorbol-13-acetate-induced epidermal ornithine decarboxylase activity by lipoxygenase inhibitors: possible role of product(s) of lipoxygenase pathway. *Carcinogenesis* 3(12), 1411–1414 (1982).
19. Suzuto M, Hirakawa Y, Ohnishi H *et al.*: Nano-kinetics of probe-particles in solution visualized by a pin-fiber video scope. *Anal. Sci.* 19(1), 43–47 (2003).
20. Sussman Y, Reck B, Pecht I: Mutual relationship among cytosolic pH, Na⁺ and Ca²⁺ ions in the degranulation of rat leukemic basophils. *Immunol. Lett.* 13(4), 215–219 (1986).
21. Liao X, Wiedmann TS: Solubilization of cationic drugs in lung surfactant. *Pharm. Res.* 20(11), 1858–1863 (2003).