Video-microscopy for analysis of molecular dynamics in cells[1]

Koichiro Ozawa, Atsushi Tamura, Kayo Ikeda, Eriko Kawai, Tadashi Kondo, Yasufumi Fukano, Seiji Nomura, Yuko Ishihara, Tsutomu Masujima *

Analytical Chemistry Laboratory, Institute of Pharmaceutical Sciences, Hiroshima University School of Medicine, 1-2-3 Kasumi, Minami-ku, Hiroshima 734, Japan

Abstract

Real-time analysis of molecular dynamics in living cells was studied by developed video-microscopes. Two new detective methods were reported, one is for analysis of ciliary movement and the other is the quantitative analysis of exocytosis of insulin-containing granules with a video-enhanced light/fluorescent microscope. For analysis of ciliary movement, glass beads were migrated in the flow. The migration speed parallel to the flow produced by ciliary beating was used as an index of the beating activity. When tracheal epithelium isolated from mouse was incubated with ambroxol, an expectorant known to activate ciliary beat frequency, the floating speeds of glass beads were changed with 1 min of incubation. The results suggest that the present method is useful not only for screening of expectorants but also for the study of molecular mechanisms underlying ciliary beat of tracheal epithelium. Visualization of the moment of the release of contents from insulin-containing granules was achieved using video-enhanced fluorescent microscopy in MIN6 cells of mouse insulinoma cell line. A fluorescent amino acridine dye, quinacrine, was found to be incorporated into low-pH secretory granules, including insulin, in the cells. The granules which incorporated quinacrine emitted a slightly blue-green fluorescence. Upon stimulation with glucose, release of the quinacrine fluorescence from granules were observed. The present method would be useful for quantitative analysis of secretion of insulin from MIN6 cells as well as pancreatic β-cells. © 1997 Elsevier Science B.V.

Keywords: Video-microscope; Cell dynamics; Ciliary movement; Expectorant; Insulin secretion; MIN6 cells; Pancreatic β-cells

1. Introduction

Chromato-videoscopy has been developed for the analysis of separating processes in a column and many fundamental aspects have been found in high-performance liquid chromatography [1,2] and capillary electrophoresis [3]. The dynamic aspects and mechanisms of superoxide anion generation have also been reported in neutrophils [4]

and of exocytosis in rat basophilic leukemia (RBL-2H3) cells, a tumor analog of rat mucosal mast cells [5] using video-microscopes.

Physiological and pharmacological properties of expectoration responses are complex and are modified by many factors, including endogenous factors and expectorants [6–8]. Ciliary movement of tracheal epithelium affects the expectorate responses which are influenced mainly be ciliary beat frequency and mucous secretion from epithelium. In order to study the ciliary movement of tracheal epithelium, a photo-electric registration device [9] and transmission electron microscopy [10] have been used. Few analytical methods, however, have been reported for studying ciliary movement in vitro.

Functional studies of pancreatic β-cells have been performed in vitro using short-term islet cell cultures [11,12] or cell lines [13,14]. Although short-term islet cultures have been used for the study of molecular mechanisms for glucose-induced secretion of insulin, the use of this culture has been limited by the disadvantage that it is a mixture of several cell types. Cell lines have been widely used for studies on the regulation of insulin secretion, but these cells do not appear to show altered response to glucose stimulation, including glucose-induced secretion of insulin [15–17]. An insulinoma cell line derived from a transgenic mouse, mouse insulinoma cell line (MIN6) cells [18], retains glucose-stimulated insulin secretion as well as normal islets. The MIN6 cell line, therefore, has been used especially to analyze the molecular mechanisms by which pancreatic β-cells regulate insulin secretion, in response to extracellular mechanisms by which pancreatic β-cells or MIN6 cells, radioimmunoassay has been mainly used to assay [12–21]. Although studies with radioimmunoassay allow the properties and mechanisms underlying secretion of insulin from these cells to be studied visualization of exocytotic events of insulin has not been reported.

The authors have therefore tried to develop a new detection method for ciliary beat frequency by using a video-enhanced microscope. The method has been examined as to whether it would be a useful technique for screening for expecto-

rants. Furthermore, a novel analyzing system for exocytosis of insulin-containing granules has been developed and it is suggested that the visualization of secretory processes by the video-enhanced fluorescence microscope should be quite useful for the study of molecular mechanisms underlying exocytosis in β-cells and MIN6 cells.

2. Materials and methods

2.1. Materials

Stock solutions of ambroxol HCl, bromhexine HCl, S-carboxymethylcysteine were prepared in dimethyl sulfoxide and diluted to give $< 0.1\%$ v/v dimethyl sulfoxide with Krebs-Ringer buffer (120 mM NaCl, 4.7 mM KCl, 1.2 mM $MgCl_2$, 25.0 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 2.5 m $CaCl_2$, 14.0 mM glucose; pH 7.2) saturated with 95% O_2 and 5% CO_2. All other reagents were dissolved directly in Krebs-Ringer buffer. MIN6 cells, a glucose-sensitive insulin-secreting cell line, were generously supplied by Dr Jun-ichi Miyazaki, Molecular Embryology, Institute of Development, Aging and Cancer, Tohoku University Sendai 980-77, Japan.

2.2. Cell culture

MIN6 cells were cultured in Dulbecco's modified Eagle's medium containing 25 mM glucose and 10% FCS under a humidified condition of 95% air and 5% CO_2 [18].

2.3. Observation of isolated tracheal epithelium with video-enhanced light microscope

Tracheal epithelium isolated from mouse was mounted in a perfusion chamber and continuously perfused with Krebs-Ringer buffer (pH 7.2), including glass beads (0.44 μm in diameter), against the direction of the ciliary movement. Addition of expectorants was performed by perfusion. Differential interference contrast (DIC) images were observed with a Zeiss axiovert 135TV microscope (Carl Zeiss, Oberkochen, Germany) equipped with a CCD camera (CS8330; Tokyo

Electronic Industry, Tokyo, Japan), an image processor (PIP-4000; A.D.S., Tokyo, Japan), and a video tape recorder (AG-7355; Panasonic, Osaka, Japan). The video signals of the camera or the recorded data were contrast-enhanced with the image processor. All measurements were performed at room temperature (25–26°C).

2.4. Observation of exocytosis of insulin from MIN6 cells with video-enhanced fluorescence microscope

MIN6 cells on a glass cover-slip were washed twice with HEPES-balanced Krebs-Ringer bicarbonate buffer (119 mM NaCl, 4.74 mM KCl, 2.54 mM $CaCl_2$, 1.19 mM $MgCl_2$, 1.19 mM KH_2PO_4, 25 mM $NaHCO_3$, 10 mM HEPES, pH 7.4) containing 0.5% bovine serum albumin (BSA) without glucose three times. Then the cells were incubated with quinacrine to stain low-pH granules, which include insulin in MIN6 cells. Quinacrine itself, even at 5 μM did not affect secretion of insulin from MIN6 cells measured by either radioimmunoassay kits (Phadesefinsulin; Pharmacia) or the authors' video-microscope. After washing with HEPES-balanced Krebs-Ringer bicarbonate buffer containing 0.5% BSA and 5 mM glucose, the cover-slip was mounted in a perfusion chamber and continuously perfused with the same buffer. Fluorescence images of quinacrine were observed with an Olympus IX70 microscope (Olympus, Tokyo, Japan) equipped with a silicon intensified target (SIT) camera (C2741; Hamamatsu Photonics, Hamamatsu, Japan), an image processor (PIP-4000; A.D.S., Tokyo, Japan), and a BETACAM-video tape recorder (UVW-1400; Sony, Tokyo, Japan). The video signals of the camera or the recorded data were contrast-enhanced with the image processor. All measurements were performed at room temperature (25–26°C).

2.5. Statistical analysis

Statistical significance was determined using the Student's t-test or one-way ANOVA followed by the Post-hoc Dunnet's procedure.

3. Results

3.1. Observation of ciliary movement in tracheal epithelium isolated from mouse

Ciliary movement of tracheal epithelium isolated from mouse was not clearly observed by video-enhanced light microscopy (Fig. 1A), because the beating frequency was faster than the video rate (1/30 s). The authors therefore tried to develop a different detection method for ciliary movement by using a video-enhanced microscope and found that, when glass beads (0.44 μm in diameter) were caused to migrate against the cil-
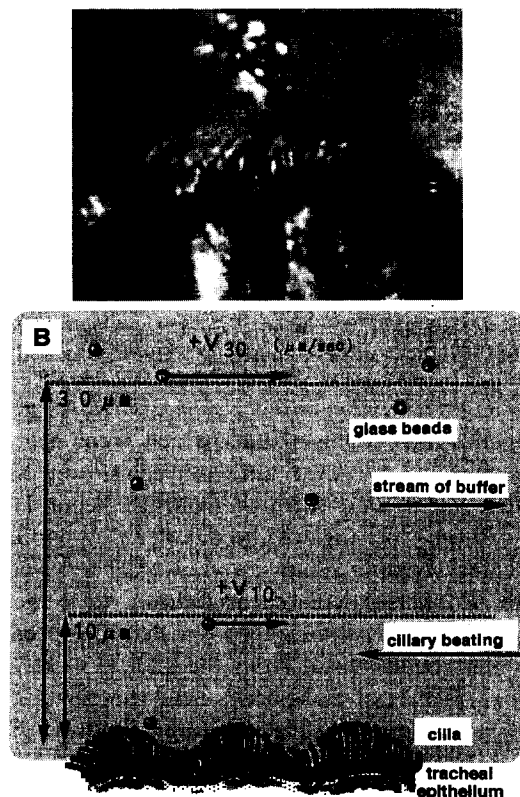

Fig. 1. (A) Nomarski image of mouse isolated tracheal epithelium; and (B) diagram of ciliary beating of the epithelium and stream of buffer including glass beads, in a perfusion chamber. Tracheal epithelium isolated from mouse was mounted in a perfusion chamber and continuously perfused with Krebs-Ringer buffer, including glass beads (0.44 μm in diameter), in a direction against the ciliary movement. Values of R_v were calculated according to a formula described in the text.
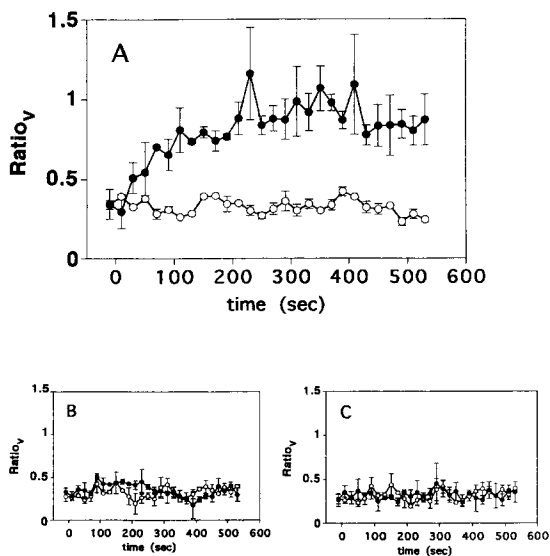
Fig. 2. Effects of expectorants on ciliary movement of tracheal epithelium. Tracheal epithelium isolated from mouse was mounted in a perfusion chamber and continuously perfused with Krebs-Ringer buffer, including glass beads (0.44 μm in diameter), in a direction against the ciliary movement. Addition of ambroxol (A), bromhexine (B), and S-carboxymethylcysteine (C) was performed by perfusion at the time 0 s. Tracheal epithelium was perfused without (○) or with 1 μM of each expectorant (●). Values of R_V were calculated according to the formula described in the text. Values shown are the mean ± S.E. (error bars) of three separate experiments.

iary movement, glass beads which were near to the cilia layer (< 10 μm) moved slower than beads which were further from the cilia layer (25–30 μm), because of the counter flow induced by ciliary beating (data not shown). The velocity ratio, R_V, was calculated as follows: $R_V = (v_{30} - v_{10})/v_{30}$, where v_{30} (μm s^{-1}) is the floating speed of the glass beads distal from the cilia (25–30 μm) and v_{10} is that of glass beads near the cilia (< 10 μm) (see Fig. 1B). The floating speed of glass beads and calculated R_V were measured (see Fig. 1B) and the values used as an index of ciliary movement. As shown in Fig. 2A, values of R_V were around 0.4 during control experiments, then the values were quickly increased by the addition of 1 μM ambroxol, an expectorant which is known to activate tracheal ciliary movement, suggesting that the frequency of ciliary beating was increased. However, bromhexine and S-car-

boxymethylcysteine, which are expectorants known not to affect the tracheal ciliary beat frequency but to increase mucous secretion, did not change the R_V values (Fig. 2B and C). Furthermore, the concentration-response curve of ambroxol showed a bell-shape (Fig. 3), but bromhexine and S-carboxymethylcysteine did not show a change in the R_V values through the concentration range examined (Fig. 3).

3.2. Visualization of exocytotic process in a single MIN6 cell

When MIN6 cells were observed under a video-enhanced fluorescent microscope through a SIT camera, an image processor, and a BETACAM-video tape recorder, the release and disappearance of quinacrine fluorescence from low-pH granules, which contain insulin in MIN6 cells, were visualized in individual cells stimulated with increase of extracellular concentration of glucose, from 5 mM
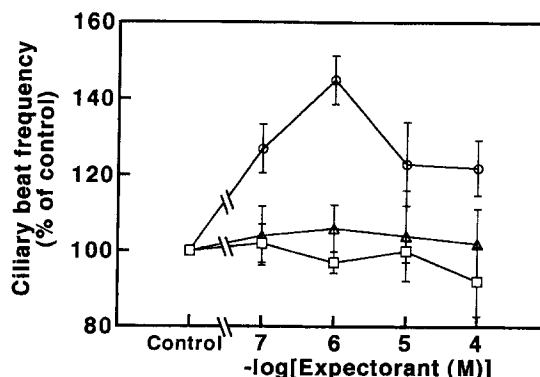

Fig. 3. Effects of different concentrations of expectorants on ciliary movement of tracheal epithelium. Isolated mouse tracheal epithelium was mounted in a perfusion chamber and continuously perfused with Krebs-Ringer buffer, including glass beads (0.44 μm in diameter), in a direction against the ciliary movement. Addition of ambroxol (○), bromhexine (△), and S-carboxymethylcysteine (□) was performed by perfusion at the time 0 s. Values of R_V were calculated at 10 min after addition of the expectorants. The data are shown as % of control values, i.e., R_V values just before the addition of expectorants. Mean values of control R_V were 0.41 ± 0.018 (ambroxol), 0.49 ± 0.036 (bromhexine), and 0.41 ± 0.024 (S-carboxymethylcysteine). Values represent the mean ± S.E. (error bars) of four separate experiments. Significance levels: *$P < 0.05$ or **$P < 0.02$ vs. control (expectorant free).
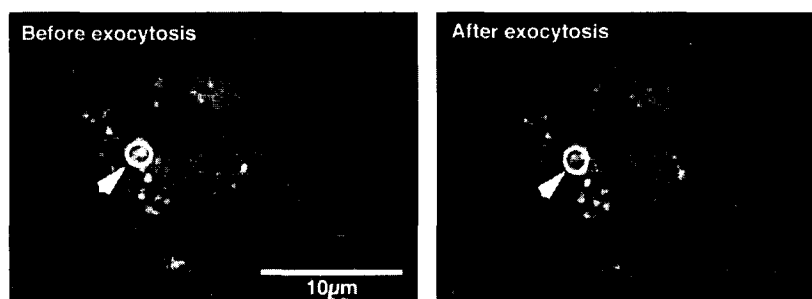
Fig. 4. Release of quinacrine from low-pH granules including insulin in MIN6 cells. MIN6 cells were incubated with quinacrine to stain low-pH granules, which include insulin in MIN6 cells. Fluorescence images of quinacrine were observed with a Olympus IX70 in a cell reproduced from video frames at 33 ms intervals. A granule (arrow) shows release of quinacrine fluorescence from a granule (for details see text).

to 25 mM (Fig. 4). The initial steps of the exocytotic process were quicker than 33 ms (Fig. 4). The beginning of exocytosis was mostly observed at about 40 s after stimulation with glucose (data not shown). When the extracellular concentrations of glucose were increased step by step, 10, 15, 20, and 25 mM, the number of cells showing release and disappearance of quinacrine fluorescence from the granules increased (data not shown), indicating that the MIN6 cells showed the so-called 'all or nothing' type of response.

4. Discussion

In the present study, a novel system is reported for the analysis of tracheal ciliary movement using a video-enhanced microscope. The following observations were found: (1) the values of R_V increased quickly after the addition of ambroxol [22], an expectorant which is known to activate tracheal ciliary beat frequency; (2) the effect of ambroxol on R_V showed concentration-dependent relationship; (3) bromhexine [23] and S-carboxymethylcysteine [24], which are expectorants known not to affect the tracheal ciliary movement but to increase mucous secretion, did not change the R_V values.

It is worth noting that, as shown in Fig. 2A, the effect of ambroxol on ciliary movement was observed 20 s after the addition. However, it has been reported that the effects observed were more

marked after 60 min incubation with ambroxol [22]. The difference between these two results may be based on the possibility that the present method could be more sensitive than the previous methods. Although the present method could not be shown to be useful for screening, of expectorants, such as bromhexine and S-carboxymethylcysteine, which are known not to affect the ciliary movement but to increase mucous secretion in tracheal epithelium, the present results suggest that the proposed method would be a useful technique not only for the screening of expectorants known to activate ciliary beat frequency, but also for the study of the molecular mechanisms underlying ciliary beating of tracheal epithelium.

The present study also successfully demonstrated the visualization of the secretory processes in a pancreatic β-cell line, MIN6 cells, which were pre-stained with quinacrine and observed by video-enhanced fluorescence microscopy. With this system, it was also found that the exocytosis of MIN6 cells showed the so-called 'all or nothing' type of response. For a quantitative analysis of this process, the number of granules which showed the release of quinacrine fluorescence were counted. Early results show a good correlation between results of the rate of exocytosis calculated by this method and results of insulin secretion measured by radioimmunoassay (data not shown, but similar results were reported in Refs. [18–21]), suggesting that the proposed

method is useful for the quantitative analysis of secretion of insulin from MIN6 cells and also for the study of the dynamic molecular mechanisms underlying exocytosis in MIN6 cells, as well as in pancreatic β-cells.

Acknowledgements

References

[1] A. Tamura, K. Tamura, K. Wada, T. Masujima, Chem. Pharm. Bull. 42 (1994) 704–706.

[2] A. Tamura, K. Tamura, S. Razee, T. Masujima, Anal. Chem., 68 (11) (1996) 4000–4005.

[3] S. Razee, A. Tamura, M. Khademizadeh, T. Masujima, Chem. Lett. 1996 (1996) 93–94.

[4] E. Suzaki, E. Kawai, Y. Kodama, T. Suzaki, T. Masujima, Biochem. Biophys. Acta 1201 (1994) 328–332.

[5] K. Ozawa, T. Masujima, K. Ikeda, Y. Kodama, Y. Nonomura, Biochem. Biophys. Res. Commun. 222 (1996) 243–248.

[6] J. Sade, A. Silberberg, A.C. Nevo, Am. Rev. Respir. Dis. 102 (1970) 48–52.

[7] R.A. Gelman, F.A. Meyer, Am. Rev. Respir. Dis. 120 (1979) 553–557.

[8] K. Takeuchi, Y. Majima, K. Hirata, A. Morishita, M. Hattori, Y. Sakakura, Ann. Otol. Rhinol. Laryngol. 98 (1989) 443–446.

[9] H.J.M. Donk, J. Zuidema, F.W.H.M. Merkus, Rhinology 18 (1980) 93–104.

[10] M. Inagaki, Y. Sakakura, T. Shimizu, Y. Majima, K. Ukai, Ann. Otol. Rhinol. Laryngol. 97 (1988) 313–317.

[11] G.M. Grodsky, R.E. Fanska, Methods Enzymol. 39 (1975) 364–372.

[12] Y. Okamoto, H. Ishida, T. Taminato, K. Tsuji, T. Kurose, Y. Tsuura, S. Kato, H. Imu, Y. Seino, Diabetes 41 (1992) 1555–1561.

[13] A.F. Gazdar, W.L. Chick, H.K. Oie, H.L. Sims, D.L. King, G.C. Weir, V. Lauris, Pro. Natl. Acad. Sci. USA 77 (1980) 3519–3523.

[14] R.F. Santerre, R.A. Cook, R.M.D. Crisel, J.D. Sharp, R.J. Schmidt, D.C. Williams, C.P. Wilson, Proc. Natl. Acad. Sci. USA 78 (1981) 4339–4343.

[15] G.A. Praz, P.A. Halban, C.B. Wollheim, B. Blondel, A.J. Strauss, A.E. Renold, Biochem. J. 210 (1983) 345–352.

[16] H. Ohlsson, T. Edlund, Cell 45 (1986) 35–44.

[17] O. Karlsson, T. Edlund, J.B. Moss, W.J. Rutter, M.D. Walker, Proc. Natl. Acad. Sci. USA 84 (1987) 8819–8823.

[18] J. Miyazaki, K. Araki, E. Yamamoto, H. Ikegami, T. Asano, Y. Shibasaki, Y. Oka, K. Yamamura, Endocrinology 127 (1990) 126–132.

[19] H. Ishihara, T. Asano, K. Tsukuda, H. Katagiri, M. Anai, M. Kikuchi, Y. Yazaki, J. Miyazaki, Y. Oka, Diabetologia 36 (1993) 1139–1145.

[20] N. Inagaki, H. Yoshida, M. Mizuta, N. Mizuno, Y. Fujii, T. Gonoi, J. Miyazaki, S. Seino, Proc. Natl. Acad. Sci. USA 91 (1994) 2679–2683.

[21] S. Hagiwara, T. Sakurai, F. Tashiro, Y. Hashimoto, Y. Matsuda, Y. Nonomura, J. Miyazaki, Biochem. Biophys. Res. Commun. 214 (1995) 51–59.

[22] B.G. Disse, Eur. J. Respir. Dis. 153 (1987) 255–262.

[23] D.T. Brown, W.P. Potsic, R.R. Marsh, M. Litt, Ann. Otol. Rhinol. Laryngol. Suppl. 117 (1985) 3–15.

[24] C. Marriott, Symp. Soc. Exp. Biol. 43 (1989) 163–177.