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Journal of Chromatography B, 689 (1997) 321–325

JOURNAL OF
CHROMATOGRAPHY B

Monitoring single-cell pharmacokinetics by capillary electrophoresis and laser-induced native fluorescence

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Received 18 April 1996; accepted 9 July 1996

Abstract

The quantification of insulin released from single cells of the insulin-secreting cell line βTC3 permeabilized by digitonin is demonstrated. A simple method for monitoring the on-column release process by using capillary electrophoresis and laser-induced native fluorescence detection is described. Quantitative measurements of both the amount of insulin released and the amount remaining in the cell can be achieved simultaneously. This protocol provides an alternative approach to the study of cell secretion in the fields of neuroscience and endocrinology.

Keywords: Insulin; Digitonin

1. Introduction

Capillary electrophoresis (CE), with its compatibility with extremely small sample volumes, high separation efficiency and multiple analyte determination, has become a powerful tool for single-cell analysis [1–3]. Various chemical species, from inorganic ions to neurotransmitters to proteins, have been analyzed at the level of a single mammalian cell [4–21]. Electrochemical [4,7–10] and laser-induced native fluorescence (LINF) detection schemes [13,20] have proven to be the most direct techniques for single-cell analysis with CE, since they measure the native properties of the analytes. This avoids problems with incomplete reaction or slow kinetics.

While the quantitation of the total amount of intracellular components is very important, in some cases, however, monitoring the dynamic chemical changes of a single cell (e.g., exocytosis, endocytosis, metabolism, and ion regulation) is more relevant to the understanding of the interaction of the cell with its environment. In many living organisms, the cellular environment is highly heterogeneous. The cells one is interested in usually coexist with other cells and therefore are exposed to a mixture of hormones and neurotransmitters released from neighboring cells. Dynamic monitoring of single cells has the inherent advantage that it provides a controlled environment and precludes the influences of other cells. This is very important in the understanding of cell biology and the pathogenesis of certain kinds of diseases. Intracellular fluorescent probes have been developed to measure the level of calcium and other ions inside single cells [22–24]. Microelectrodes are also used to measure the release of oxidizable compounds [25–27,27–29]. Recently, neurotransmitter uptake has been imaged in single living astrocytes

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PII S0378-4347(96)00317-9

using a UV laser-based optical microscope and CCD detection system [30]

In most of the above methods, it is difficult to perform quantitative measurement of the absolute amount of species secreted or uptaken. It is also difficult to simultaneously measure the amount of analytes secreted and the amount remaining in the cell after release, which are pertinent information for pharmacokinetic studies. The quantitative nature of CE makes it an interesting alternative approach to the study of single cell secretion.

Kristensen et al. [16] used CE with amperometric detection to directly identify and measure the neurotransmitter, dopamine, in two vesicular compartments in a single nerve cell of *Planorbis corneus*. Insulin is traditionally determined by radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) [31,32]. CE-based immunoassay have also been used for insulin analysis [33,34]. However, the CE–LINF method we recently developed for the analysis of insulin in single pancreatic cells [35] is more suitable for studying release because it is a direct method that does not involve derivatization. In the present work, we have developed an on-column protocol to simultaneously monitor the release of insulin from a single βTC3 cell and the residual amount of insulin in the same cell. Insulin is selected here because type II diabetes is characterized by improper insulin release from the pancreatic β-cells. The underlying causes of this improper release are still not clear. Digitonin is used in the present work to cause insulin release. Digitonin reacts with cholesterol in the cell membrane to permeate the cell by producing pores on the membrane. Digitonin has been used to cause the release of catecholamines from adrenal medullary cells [29].

2. Experimental

2.1. CE apparatus

The CE setup was laboratory-made and similar to that described previously [12]. Briefly, a high-voltage power supply (Series MJ, 0–30 kV, Glassman High Voltage, Whitehouse Station, NJ, USA) was used to drive the electrophoresis. A 22 μm I.D., 360 μm O.D. fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) was used for separation. The total length was 63 cm and the detection window was 46 cm from the injection end. For single-cell measurements, a bare capillary was used after rinsing with 0.1 *M* NaOH for 5 min and equilibrating with running buffer for 10 min. For lysed-cell measurements, a non-bonded poly-(ethylene oxide) (PEO) coated capillary was used. The capillary was treated with 0.1 *M* HCl, 0.2% PEO (in 0.1 *M* HCl) for 5 min, respectively, before finally being flushed with the running buffer. The entire electrophoresis and detection system was enclosed in a sheet-metal box with HV interlocks. The buffer reservoir at the high-voltage end was enclosed in a plexiglass box.

The 275-nm line from an Ar ion laser (Model 2045, Spectra Physics, Mountain View, CA, USA) was isolated from other lines with an external prism and focused with a 1-cm focal length quartz lens onto the detection window of the capillary. One WG-305 (Melles Griot, Irvine, CA, USA) and one UG-1 filter (ESCO, Oak Ridge, NJ, USA) were used to block the scattered light and room light from reaching the photomultiplier tube. A low-pass (1 Hz) filter was employed to limit the output signal bandwidth. Data were collected at 5 Hz by a 24-bit A/D conversion interface (ChromPerfect, Justice Innovation, Palo Alto, CA, USA). The data was stored in an IBM/PC-compatible computer.

2.2. Reagents

A balanced salt solution (PBS) was composed of 136 m*M* NaCl, 5.4 m*M* KCl, 0.5 m*M* NaH_2PO_4, 0.34 m*M* Na_2HPO_4, 0.8 m*M* $MgSO_4$, 1.3 m*M* $CaCl_2$ and 10 m*M* HEPES and adjusted to pH 7.4 with NaOH. Bovine insulin and tricine were purchased from Sigma (St. Louis, MO, USA). Digitonin was obtained from Fluka (Ronkonkoma, NY, USA) and poly(ethylene oxide) of M_r 8 000 000 was from Aldrich (Milwaukee, WI, USA). All other chemicals were purchased from Fisher (Fair Lawn, NJ, USA).

2.3. Methods

Because of the difficulties in isolating large numbers of highly purified pancreatic β-cells, insulin-producing β-cell lines are usually used as a model

for wild type pancreatic β-cells [36]. βTC3 cells [37] were generous gifts from Dr.W. Hsu, College of Veterinary Medicine, Iowa State University. Before analysis, the cells were washed five times with 5-ml portions of balanced salt solution. Hydrodynamic injection similar to that described in [12] was used to inject a single cell into the end of the capillary. Once the cell adheres to the capillary wall (Fig. 1A), 20 μM digitonin (dissolved in the balanced salt solution) is hydrodynamically injected by lifting the injection end (digitonin vial) 20 cm above the outlet buffer reservoir for 1 min. This corresponds to a 3.2-mm plug of digitonin, which is enough to cover the whole cell. The injection end of the capillary is then put back into the running buffer reservoir and the cell is incubated for 15 min (Fig. 1B). During this time, digitonin will dissolve the cholesterol in the cell membrane and permeable pores are produced. The insulin-containing granules permeate through the pores and give off their insulin. The release process is not linear with respect to time. A 15-min time period was chosen to produce sufficient material for quantitation yet allow experiments to proceed at a convenient pace. Then, CE is run at 3 kV for 5 min to separate the released insulin zone from the cell (Fig. 1C). Alternatively, buffer solution (20 mM tricine, pH 8.5) is introduced into the capillary hydrodynamically to achieve the same separation. Finally, the running voltage was increased to 30 kV. The cell will lyse and give off the rest of the insulin. Two separated insulin zones will then migrate towards the detection window with the aid of electroosmotic flow (Fig. 1D). Quantitation is achieved by measuring the peak areas and comparing them with those obtained for calibration runs with standard solutions of insulin between cell injections. Fig. 2A shows that the LOD is 100 amol of insulin.

In the off-column experiment, two equal portions of cell suspensions are used. One portion of cells is incubated with digitonin for 15 min and the other portion is just incubated with the balanced salt solution. The cells are then spun down and lysed ultrasonically in H_2O. The two lysates, which correspond to the average amounts of insulin left in the cells and total amount of insulin, are analyzed with CE–LINF.

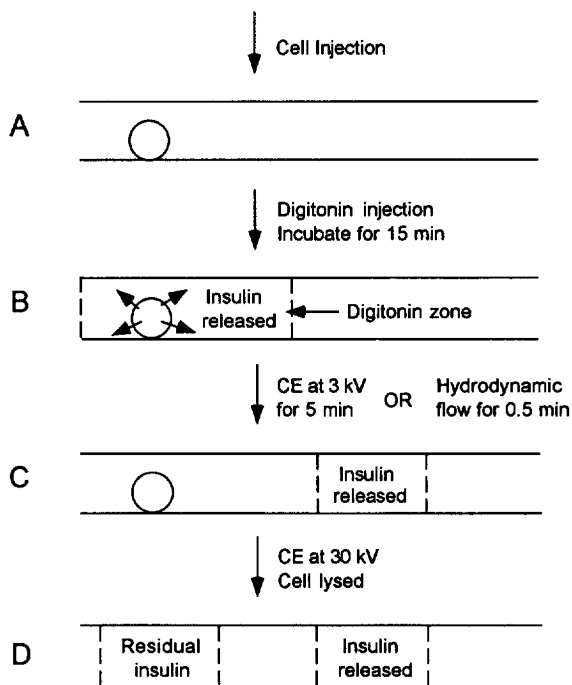

Fig. 1. Schematic diagram of on-column monitoring of insulin release from a single cell.

3. Results and discussion

The key aspects of this work are the processes of injection, on-column release, and on-column lysis of single cells. The adhesion of the cell to the capillary wall soon after injection helps to prevent the cell from further migration down the capillary, so that later on the injected releasing reagent can pass and cover the whole cell. Adhesion does not affect quantitation of the released material, since the contact area is negligibly small. The cell is rather rugged and remains unlysed in 20 μM digitonin for at least 30 min. This is probably because the cell membrane is relatively poor in cholesterol [38].

Fig. 2B is a typical electropherogram obtained with the present protocol for a single βTC3 cell. Since we run CE at 3 kV for 5 min to separate the released insulin zone from the intact cell, the released insulin peak (peak 1) should separate from the residual insulin (peak 2) by 0.5 min (electromigration at 30 kV). However, the peak separation here is only about 0.2 min. The time is also not reproduc-

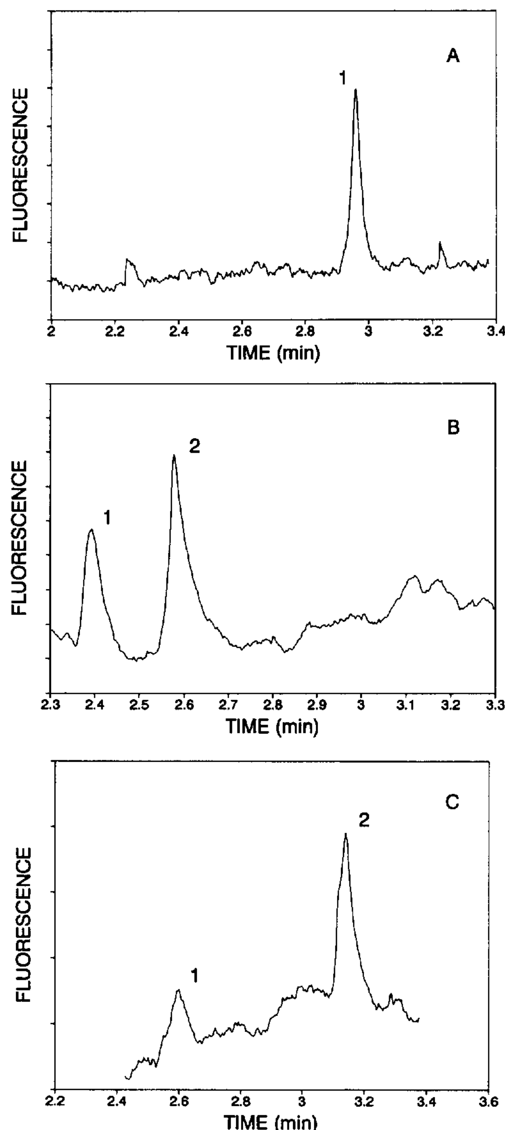

Fig. 2. Electropherograms of insulin standard (A) and insulin from a single βTC3 cell (B and C). (A) Normal CE–LINF, 30 kV, 500 amol insulin injected. (B) On-column release CE–LINF; peak 1: insulin released from the cell due to digitonin; peak 2: residual insulin, which is released due to lysis. The two peaks are separated electrophoretically after release. (C) Same as (B), except that the two peaks are separated hydrodynamically after release. Running buffer: 20 m*M* tricine (pH 8.5).

ible from run to run. This is because the cell sometimes moves or even lyses during the 3 kV CE step, as confirmed by monitoring under a microscope. As a result, the peak for the residual insulin actually reaches the detection window earlier than expected.

Hydrodynamic flow experiments can be used to confirm that peaks 1 and 2 correspond to the released insulin and residual insulin (Fig. 2C). In this experiment, after incubating the cell with digitonin, the injection end of the capillary is lifted by 30 cm for 15 min. The cell will not lyse or move during this period. The calculated peak separation for peaks 1 and 2 is about 0.5 min, which is very close to the experimental values. For a set of five experiments, the temporal separation is 0.57±0.04 min. In addition, if the cell is not incubated with digitonin and CE is simply run at 30 kV after cell injection or if hydrodynamic flow is first applied for the same amount of time and at the same height, we only observe peak 2 (at about 3 min with a larger peak area). The migration times for single-cell analysis are not highly reproducible. This is because cellular species may adsorb on the capillary wall and gradually change the rate of the electroosmotic flow [13].

Table 1 summarizes the fraction of insulin released by digitonin for several batches of cells from different culture dishes, as determined by CE–LINF. On the average, 41% and 21% of insulin are released for batches 1 and 2, respectively. These are smaller percentages compared to the 59% and 47% release obtained by off-column measurements. Apparently, the spinning process caused some additional intracellular insulin to leak out to distort the measured release percentages. This highlights the importance of studying release on column and on a single-cell basis. In general, Table 1 shows that the inter-batch variations are larger than the intra-batch variations. This is expected from cultured cells.

Table 1
On-column insulin release determined by CE-LINF

Cell No.	Peak 1 (area)	Peak 2 (area)	Insulin released (%)
Batch 1			
1	2027	2821	41.8
2	2087	3210	40.2
Batch 2			
3	1712	6376	21.2
Batch 3			
4	1336	787	62.9
Batch 4			
5	1971	3660	35.0
6	3076	3642	45.8
7	2524	4843	34.3

Other chemicals (e.g., glucose, KCl and carbachol) which stimulate the physiological release of insulin are also tested. Off-column experiments showed insulin release of less than 15% [39]. We have not been able to reliably quantify such small amounts of release by the present on-column protocol due to the marginal signal-to-noise ratio. Further improvements in instrumentation will be needed for such studies.

4. Conclusion

A simple method for monitoring the release of insulin from single cells by using capillary electrophoresis and laser-induced native fluorescence is demonstrated. Quantitative measurements of the amount of species released and the residual amount in the cell can be performed simultaneously. This will provide a useful alternative approach to the quantitative study of cell secretion in the field of neuroscience, pharmacokinetics and endocrinology.

Acknowledgments

The authors thank Dr.W.H. Hsu for the generous gift of βTC3 cells. We also thank Dr.T.-H. Chen for help in preparing the cells. The Ames Laboratory is operated for the U.S. Department of Energy by Iowa State University under contract No. W-7405-Eng-82. This work was supported by the Director of Energy Research, Office of Basic Energy Sciences, Division of Chemical Sciences.

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