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Review

Study of single cells by using capillary electrophoresis and native fluorescence detection

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Abstract

Capillary electrophoresis is known for its compatibility with biological materials and with small samples. It is an ideal tool for the study of single biological cells. Either whole cells or the material secreted from cells can be quantified. By continuously flowing a chemical stimulant over an immobilized cell inside the entrance of the capillary, one can even record the temporal progression of cellular secretion with sub-second resolution. The use of native fluorescence detection in such experiments provides a sensitive, rapid, non-intrusive and quantitative probe of important biomolecules such as catecholamines and proteins.

Keywords: Reviews; Cells; Catecholamines; Serotonin; Proteins; Insulin

Contents

1. Introduction

Capillary electrophoresis (CE) has firmly planted itself as an important separation technique. Through intensive research activity, particularly over the past decade, many of the unique features of CE have been demonstrated. This is not to say that CE will one day surpass liquid chromatography (LC) or gas chromatography (GC) in utility as a practical separations tool. Indeed, these techniques are very much complementary to one another. Yet, there are several specific instances where CE is clearly the method of choice. One of these areas is the study of single biological cells.

Single-cell studies can potentially benefit clinical diagnosis and treatment. In regular blood tests,

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hundreds of thousands of cells are homogenized to provide sufficient amounts of analytes for quantification. At the early stages of disease or carcinogenesis, only a few cells may carry the specific chemical or biochemical markers indicative of infection. Unfortunately, such markers are likely to be completely masked by the averaged contents of the overabundant healthy cells. On the other hand, if cells are examined individually, the chances of recognizing abnormal cells are substantially better. One will still need to analyze a large number of cells to provide a meaningful diagnosis. However, discrimination will always be better without first averaging the cell contents. In the treatment of diseases, it is also likely that the uptake of and the response to pharmaceuticals can be very different between healthy and disease-stricken cells. The understanding of such variability can lead to better drug design and control of side effects.

Chemical analysis of single cells has been achieved through a variety of techniques. The separation and direct observation of several hemoglobins was reported almost 30 years ago [1]. For large cells, miniaturized versions of thin-layer chromatography [2], mass spectrometry [3], LC [4] and enzymatic radiolabeling have been successfully applied. Examples of micromanipulation and sensitive voltammetric detection after separation include the analysis of single snail neurons [5] and individual bovine adrenal medullary cells [6]. Flow cytometry is a well established tool in cell biology [7]. Fluorescence microscopy [8], especially with the recent development of confocal imaging [9], is another broadly applied technique. The detection of individual ion-channel events has been demonstrated in patch-clamp experiments [10].

A general approach to characterize complex samples such as biological cells is to incorporate chemical separation before measurement, for example capillary LC [11], and CE [12]. In the case of CE, the cells must be transferred directly into the capillary column. This can be accomplished under the field of view of an optical microscope [13]. The injection end of the separation capillary is brought into the observation region with the aid of micromanipulators. The capillary opening can then be aligned with the selected cell. By pulling a vacuum through an air-tight syringe connected to the opposite end of the capillary, the selected cell is drawn into the column. The capillary is then removed from the micromanipulator and placed in the vial containing the running buffer. Lysing is achieved by various means. The intracellular fluid is then completely released, completing the sample injection process.

The study of single cells by CE benefits from many of the inherent features of its operation. These are:

(1) Small sample size: Mammalian cells are typically around 10 μm in diameter and 1 pl in volume. To process these in standard scale GC or LC will involve dilution factors of at least 10^6. Not even the best detectors can compensate for this degradation in concentration. 300 μm I.D. microcolumns for LC are commercially available. Still, there is a 100–1000-fold dilution compared to CE or open-tubular LC in 5 to 15 μm I.D. capillaries.

(2) High separation efficiency: Even without a great deal of effort, one can achieve $N=100\,000$ theoretical plates in typical CE separations. In dealing with a complex mixture as biological fluids, interferences to the measurement are abundant. Also problematic is the fact that many of the components are unknown. There are few hints as to how one should fine tune selectivity in those separations. The large number of available theoretical plates makes it much easier to get started.

(3) High separation speed: The large electric field gradient that can be applied has led to CE separations approaching 1 ms [14]. This opens up the possibility of following cellular events at that time scale. Even if temporal information is not needed, fast separation means high throughput, which is a major consideration for screening cell populations.

(4) Biocompatible environment: Water-based buffer systems are most friendly to biological cells and biological molecules. Although pH is one of the obvious parameters for altering selectivity in CE, very often extreme conditions are not needed to affect separations. So, with careful design, it is possible to use CE to explore the biological response of cells in vitro.

(5) Highly multiplexed operation: Although to date capillary arrays have been demonstrated only for DNA analysis [15–17], there is no inherent reason why simultaneous operation in 100 capillaries cannot be applied to any CE separation. If CE

analysis of single cells does become clinically useful, the need to analyze a large number of cells per individual, and a large number of individuals per medical laboratory, will make such parallel approaches a necessity. This is a feature that makes CE very different from GC or LC, where multiple complete systems are needed for simultaneous analyses.

(6) Low cost: The small scale intrinsic to CE means a low consumption of buffer, exotic additives, labeling reagents, etc. There is also less waste to dispose of at the end. The capillaries themselves, at least in the bare fused-silica version, are much cheaper than GC or LC columns. A variety of dynamic coating schemes have been developed so that rarely will one need special bonded coatings. Chip-based CE further promises to bring ''disposal'' columns for large-scale applications.

Detection of the separated components in CE, however, remains problematic, since most compounds of biological interest are present in very small amounts. The problem is even more severe in single-cell studies because of the small I.D. capillaries that are required. Standard absorption detectors simply do not couple well with 5–15 μm capillaries. Both the number of photons that can be focused into the liquid core (shot noise) and the increased stray light from the remaining photons reduce the concentration limit of detection (LOD). For a commercial UV detector for CE in 50 μm capillaries, the peak volume of 10 nl and a concentration LOD of 10^{-6} *M* translates to an absolute LOD of 10^{-14} mol. This is just barely at the level of the major catecholamines in single adrenal medullary cells [18].

CE has been used to analyze snail neurons by using a microinjector to sample the intracellular fluid [19] and by using electrochemical detection. Recently, a radiochemical detector has been developed [20] and applied to single cell detection [21]. Mass spectrometry [22] is also emerging as a detection technique for individual cells. Pre-column derivatization by monobromobimane (mBBr) has been applied to the determination of glutathione (GSH) in human erythrocytes [13], in which the intact cell served as the reaction vessel. For pre-column derivatization, naphthalene dicarboxaldehyde (NDA) has been used with single bovine adrenal medullary cells [23] and *Helix aspersa* neurons [24], while fluorescamine has been used with *Aplysia californica* neurons [25]. More recently, an on-column derivatization method has been used to analyze rat pheochromocytoma (PC12) cells for NDA-amino acids [26]. In addition, a post-column reactor has been developed [27] in which *o*-phthaldialdehyde (OPA) derivatized proteins in human red blood cells have been detected.

Indirect laser-induced fluoresence (LIF) detection, in which the background electrolyte fluoresces and not the analyte, has been applied to the separations of Na^+ from K^+ [13,28] and lactate from pyruvate [29] in human erythrocytes. More unique detection schemes include an enzyme assay protocol used to detect lactate dehydrogenase (LDH) isoenzymes by monitoring the NADH production from the enzymatic reaction. LDH was quantified in erythrocytes [30], and lymphocytes and lymphoblastic leukemia cells [31] by this method. Glucose-6-phosphate dehydrogenase (G6PDH) was also studied by an enzyme assay detection scheme in combination with particle agglutination [32].

An interesting detection mode for CE is the use of the native fluorescence of biological molecules [33]. Not all biologically important species exhibit native fluorescence, but a large group of them do. These include peptides and proteins whenever aromatic amino acid residues are present, and signal transduction chemicals such as catecholamines. The absorption cross-sections and the fluorescence efficiencies of these molecules are quite low. That is why fluorescence derivatization is often employed in CE or LC for the sensitive detection of these species.

The main point is that laser-excited fluorescence is such a sensitive detection mode that even if one gives away several orders of magnitude in performance, the LOD is still within a useful range for studying the major intracellular components. Continuous-wave laser emission at 275 nm is available and is preferable to pulsed-laser systems because of the better focusing properties and because damage to the capillary columns is not a problem. Frequency doubling of visible argon-ion laser lines is also useful [33]. The availability of commercial versions of these recently has greatly facilitated applications [18,34]. Fused-silica capillaries tend to fluoresce when excited in the deep UV, presumably due to the presence of rare-earth inclusions. Quartz optics is a must throughout the system. Even distilled and

deionized water contributes to background fluorescence, since trace organic contaminants are always present. However, by selecting the appropriate pH to maximize the fluorescence efficiency and to avoid band broadening, one can achieve LOD in the 10^{-10} *M* or 10^{-19} mol range [35].

These are good reasons to develop native fluorescence as a sensitive detection mode for CE. In fluorescence derivatization of proteins, often multiple sites are involved and a host of products are produced. This, together with the unreacted reagent, puts extra constraints to the separation performance. Derivatization at concentrations below 10^{-8} *M* is also unfavorable because of kinetics. Even if derivatization is possible, quantitative information is difficult to obtain because complete reaction cannot be guaranteed. Derivatization also takes time. Following fast biological events will not be possible. Native fluorescence detection can alleviate all these problems. In addition, its non-invasive nature (provided one is careful not to work with high excitation powers) is highly desirable for certain applications, such as functioning as the first of many detection modes in a series.

In this article, we will review recent results in using native fluorescence detection in CE analysis of single cells. Because of the requirement of a state-of-the-art laser system, the approach has not yet attracted many practitioners. However, as will be shown below, this combination has already provided us with biological insights that are otherwise inaccessible. Studies in four different cell types are described. These are erythrocytes, pancreatic β-cells, adrenal chromaffin cells and mast cells.

2. Proteins in erythrocytes

The small quantities of proteins in single cells presents a challenge not only to detection but also to separation. Adsorption of proteins on bare fused-silica surfaces is well known. We have shown [36] that the injection of 1 amol of a model protein under proper conditions results in a fluorescence peak that represents 90–95% recovery of the injected material. Application of native protein fluorescence to single-cell studies is therefore feasible. A representative electropherogram of the proteins in a single erythrocyte is shown in Fig. 1 [37]. Signal-to-noise as well as the separation efficiency are very good. The major proteins, hemoglobin A_0, methemoglobin and carbonic anhydrase, are clearly depicted. We found that there were large variations from cell to cell in the amounts and the relative abundances of each protein. Since these cells have been stored for up to three days, oxidation of hemoglobin to methemoglobin explains most of these variations. The rate of change on storage is apparently not uniform within the cell population.

Human hemoglobin has an approximate molecular mass of 65 500. The main adult component, HbA_0, consists of two αβ dimers ($\alpha_2\beta_2$) and comprises about 90% of the total hemoglobin content [38]. While genetic variants involve one or more differing amino acids in the protein sequence of one or both

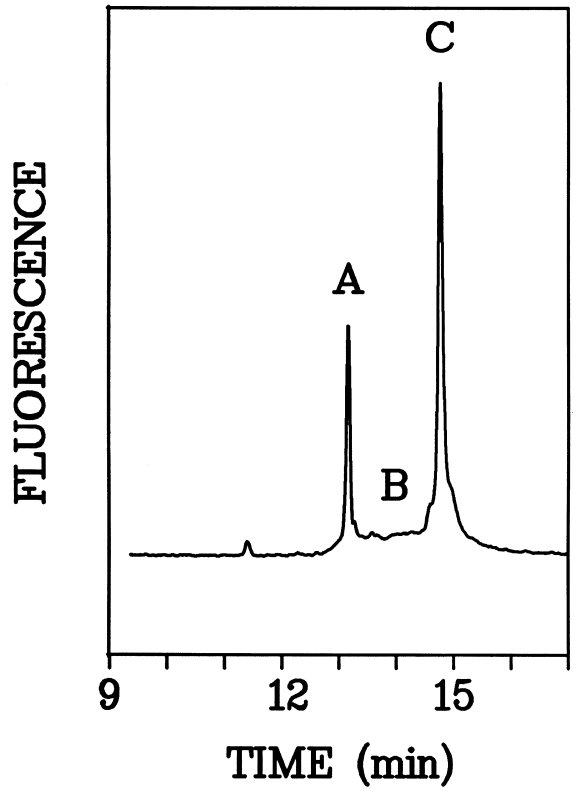

Fig. 1. Electropherogram of major proteins in a single erythrocyte. Peaks A, B and C are carbonic anhydrase (~7 amol), methemoglobin (~5 amol), and hemoglobin A_0 (~450 amol), as identified from migration times relative to standards. Reproduced with permission from Ref. [37].

types of globin chains, other variants, such as HbA_{1c}, arise from a post-translational modification [38]. In fact, HbA_{1c}, which constitutes roughly 5% of the total hemoglobin in normal erythrocytes, only differs from HbA_0 by one glucose molecule attached to the N-terminal valine of each β-chain [38]. There are many hemoglobinopathies for which diagnosis is based on the detection of variants. Examples include the presence of HbS in sickle cell anemia [39] or the increased amount of HbA_2 in β-thalassemia [40]. HbA_{1c} is known to be elevated two- to three-fold in untreated diabetes mellitus, and its monitoring serves to give a measure of long-term blood glucose control [41]. Additionally, HbA_{1c} may also be used to assess relative cell age.

We demonstrated a separation scheme based on free-zone electrophoresis for the separation of hemoglobin variants in single human erythrocytes [42]. A hydrophobic fluorocarbon coating is used, in which protein adsorption to the capillary wall is minimized. This is evident by the extremely narrow peak widths (Fig. 2). The half-widths of the β- and α-chains are 0.09 and 0.06 min, respectively. A fluorocarbon (FC) surfactant is also added to the acidic (pH 2.7±0.1) running buffer. Upon contact with the lower ionic strength running buffer, in which a surfactant is also present, the cell is easily lysed, and the hemoglobin molecules released. The protein is denatured and their constituent polypeptide chains are separated. The running buffer also serves to oxidize the heme groups from the ferrous to the ferric state.

Fig. 3 shows the separation of Hb in a single adult erythrocyte containing an elevated amount of fast Hb ($A_1=A_{1a}+A_{1b}+A_{1c}$). The fact that peaks 2 and 4 are substantially larger in Fig. 3 compared to Fig. 2 is positive confirmation of our peak assignments. From our single-cell determinations, the relative β- and α-glycated fractions were found to be 30% and 12%, respectively.

Capillary isoelectric focusing (cIEF) is a high-

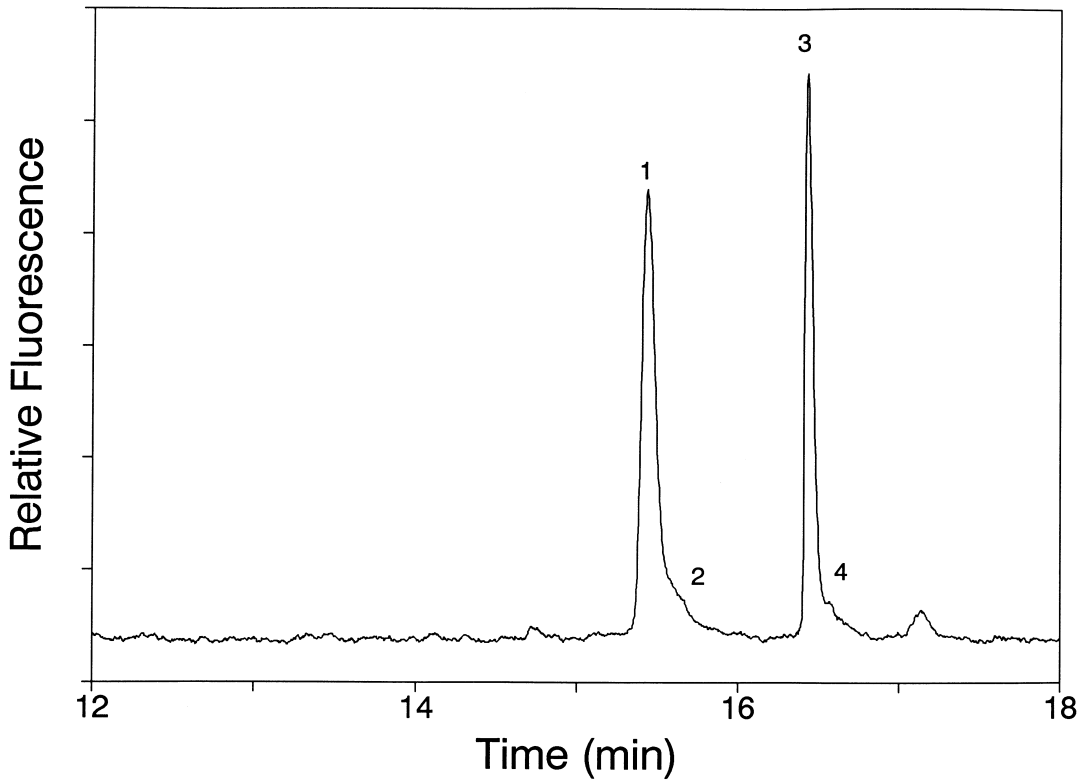

Fig. 2. Electropherogram of a single red blood cell (normal adult). Peaks: 1=β-, 2=β-glycated-, 3=α- and 4=α-glycated-chains. Reproduced with permission from Ref. [42].

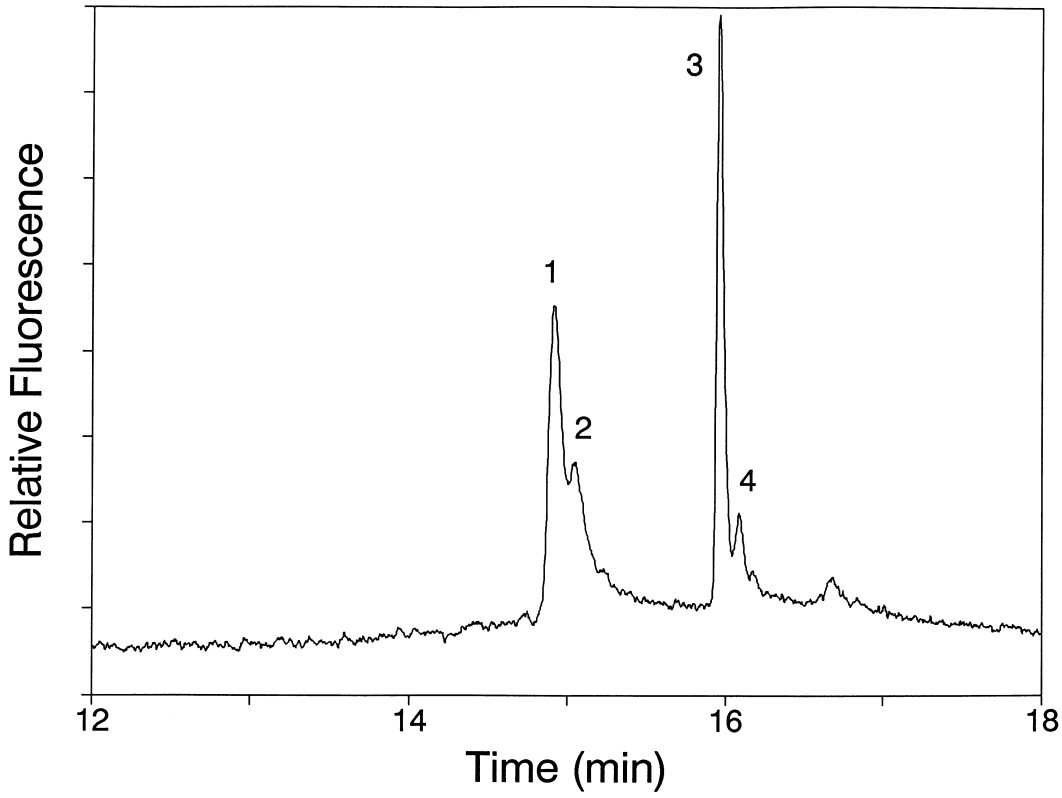

Fig. 3. Electropherogram of a single red blood cell (diabetic adult; i.e., elevated HbA_1). Peaks as in Fig. 2. Reproduced with permission from Ref. [42].

resolution mode of CE, in which amphoteric species (e.g., proteins) are separated according to their isoelectric points (p*I*). The high efficiency of this technique is well suited for the determination of hemoglobin (Hb) variants. Due to the difficulty in separating Hb by CE, most high resolution Hb separations are done by cIEF.

A modified version of cIEF was developed [43] to separate hemoglobin variants contained within single human erythrocytes. Laser-induced native fluorescence was used to detect the separated hemoglobins. In this method, baseline fluctuations were minimized and detection sensitivity was improved by using dilute solutions of anolyte, catholyte, and carrier ampholytes (with methylcellulose). Since electroosmotic flow (EOF) was used for mobilization of the focused bands, separation and detection were integrated into a single step. The capillary was first filled with only ampholyte solution, and the cell (or standard) was injected as in capillary zone electrophoresis. The ~90 fl injection volume for individual cells is $7 \cdot 10^4$-times lower than those previously reported. Adult (normal and elevated A_1), sickle (heterozygous), and fetal erythrocytes were analyzed, with the amounts of hemoglobins A_0, A_{1c}, S and F determined. The pH gradient for cIEF was linear ($r^2 = 0.9984$), which allowed tentative identification of HbF_{ac}. Variants differing by as little as 0.025 p*I* units were resolved. Fig. 4 shows the Hb separation from a single fetal (cord blood sample) erythrocyte in which HbF and A_0 (peaks 1 and 2, respectively) were identified.

3. Insulin in pancreatic cells

Insulin is an important hormone present in pancreatic β-cells for regulating glucose metabolism. An

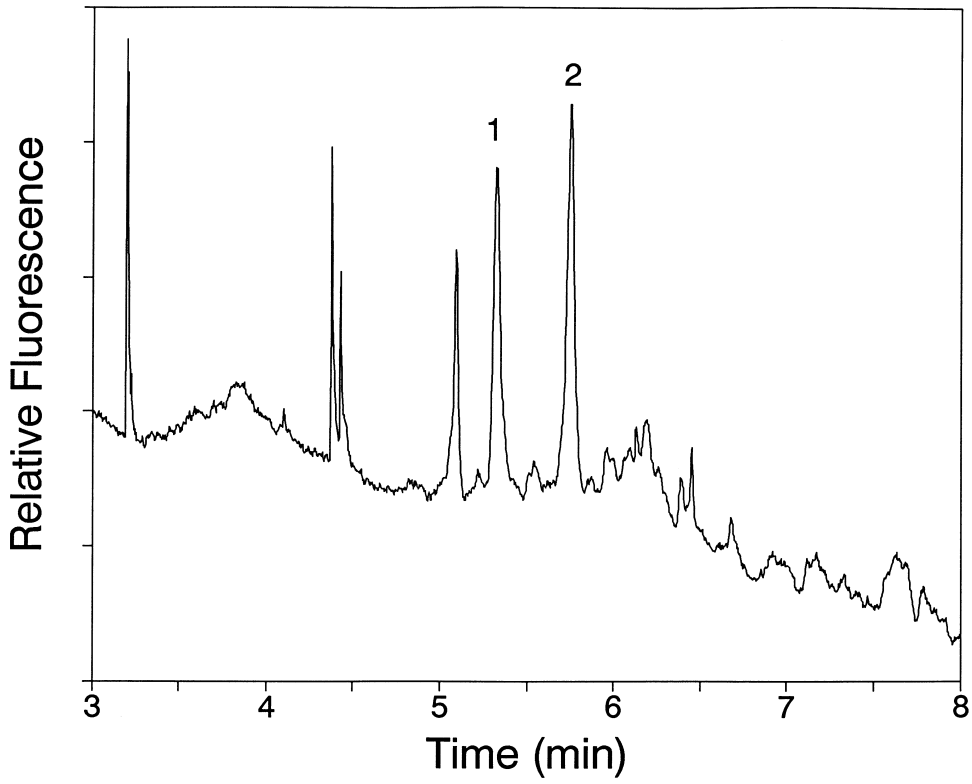

Fig. 4. cIEF of a single fetal red blood cell. Hb peaks: 1=F, 2=A_0, 3=F_{ac}. Reproduced with permission from Ref. [43].

increase in the blood glucose concentration will stimulate the secretion of insulin from the pancreatic β-cells. The secreted insulin is transported to the liver through blood circulation and stimulates the destruction of glucose by the liver to produce glycogen, so as to maintain glucose level within the normal range. Deficiency of insulin at the cellular level affects not only the glucose concentrations, but also influences the overall endocrine arena within which it is normally active [44]. Spontaneous diabetes is characterized by a nearly total deficiency of insulin (Type I) or improper insulin secretion (Type II) of the β-cells. The determination of insulin at the single-cell level may thus generate unique information about the variation of insulin contents and secretion behaviors among cells and the possible relationships between them. This will enhance our understanding of diabetes and possibly facilitate the development of better therapy. Insulin has been determined previously in islets of pancreatic β-cells [45], but the sensitivity there was not sufficient for single-cell analysis.

One of the major concerns in the separation of proteins by CE is the interactions with the inner surface of the fused-silica capillaries, which will cause peak broadening and change of EOF rate. We have recently demonstrated the application of non-bonded poly(ethylene oxide)-coated (PEO-coated) capillaries in protein separation [46]. A PEO-coated capillary is therefore tested for insulin separation [47]. A nice sharp peak is obtained in a reasonably short period of time. With the PEO-coated capillary, 73 amol of insulin can be detected with a good signal-to-noise ratio (S/N=10 peak-to-peak). The advantage of a PEO-coated capillary is that the EOF is very small. Also, there is less interference from negatively charged components, since they will move in the opposite direction and will not be detected.

Several problems arise when using PEO-coated

capillaries for single-cell analysis. First, unlike red blood cells, which are readily lysed by osmotic pressure in a few seconds, the induced tumor cells (RINm5F and βTC3) are very rugged and will not lyse in a reasonably short time in the running buffer to release all their cellular components. Second, the injected cell does not adhere well to the capillary wall, so that specialized lysing techniques like a tesla coil [31] or subsequent injection of lysing reagent cannot be easily applied. For these reasons, a bare capillary is employed for single-cell analysis even though the PEO-coated capillary is better for separation.

After cell injection, the capillary containing the cell is carefully removed from the microscope and placed in a reservoir containing 0.05% sodium dodecyl sulfate (SDS) and running buffer. SDS is electrophoretically injected into the capillary to lyse the cell. Then, the capillary is returned to the running buffer and the separation is initiated. Fig. 5 shows the electropherogram of the contents of a single βTC3 cell. A large peak due to injected SDS is evident around 2.7 min. However, that is well separated from the insulin peak.

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. It is therefore important 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.

We have developed an on-column protocol to

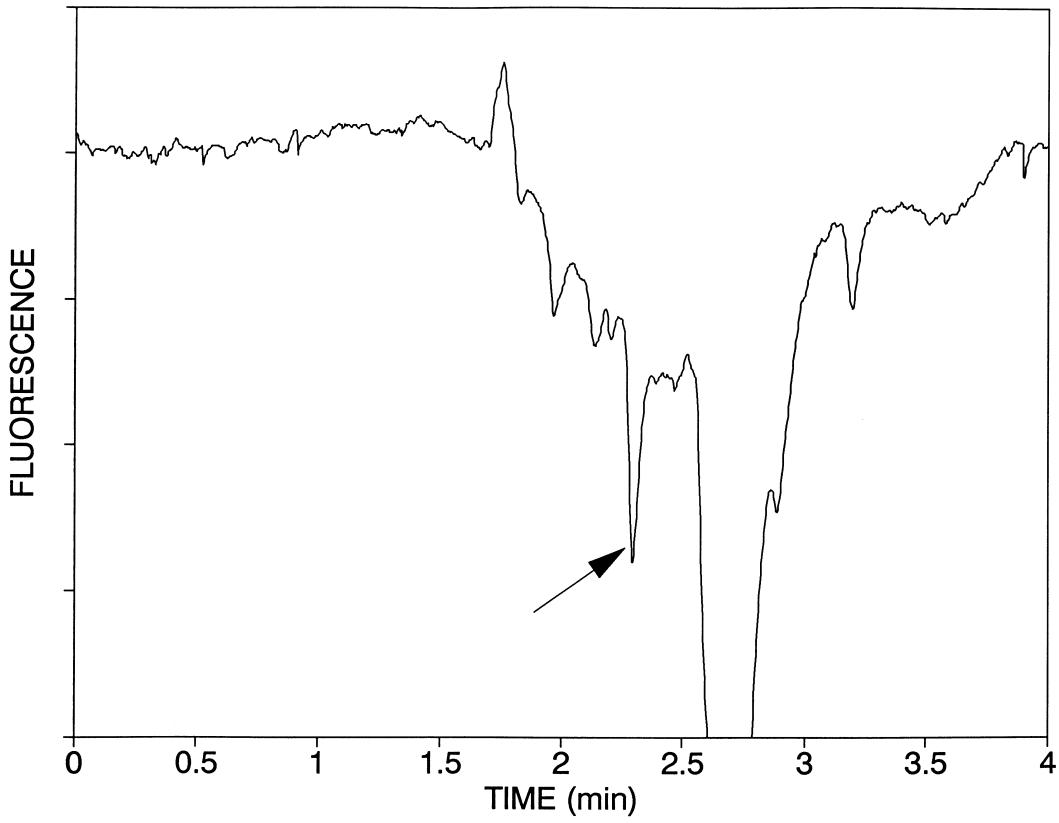

Fig. 5. Electropherogram of insulin (arrow) in a single βTC3 cell. Reproduced with permission from Ref. [47].

simultaneously monitor the release of insulin from a single βTC3 cell and the residual amount of insulin in the same cell [48]. 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 [49].

Hydrodynamic injection was used to inject a single cell into the end of the capillary. Once the cell adheres to the capillary wall (Fig. 6A), 20 μ*M* 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

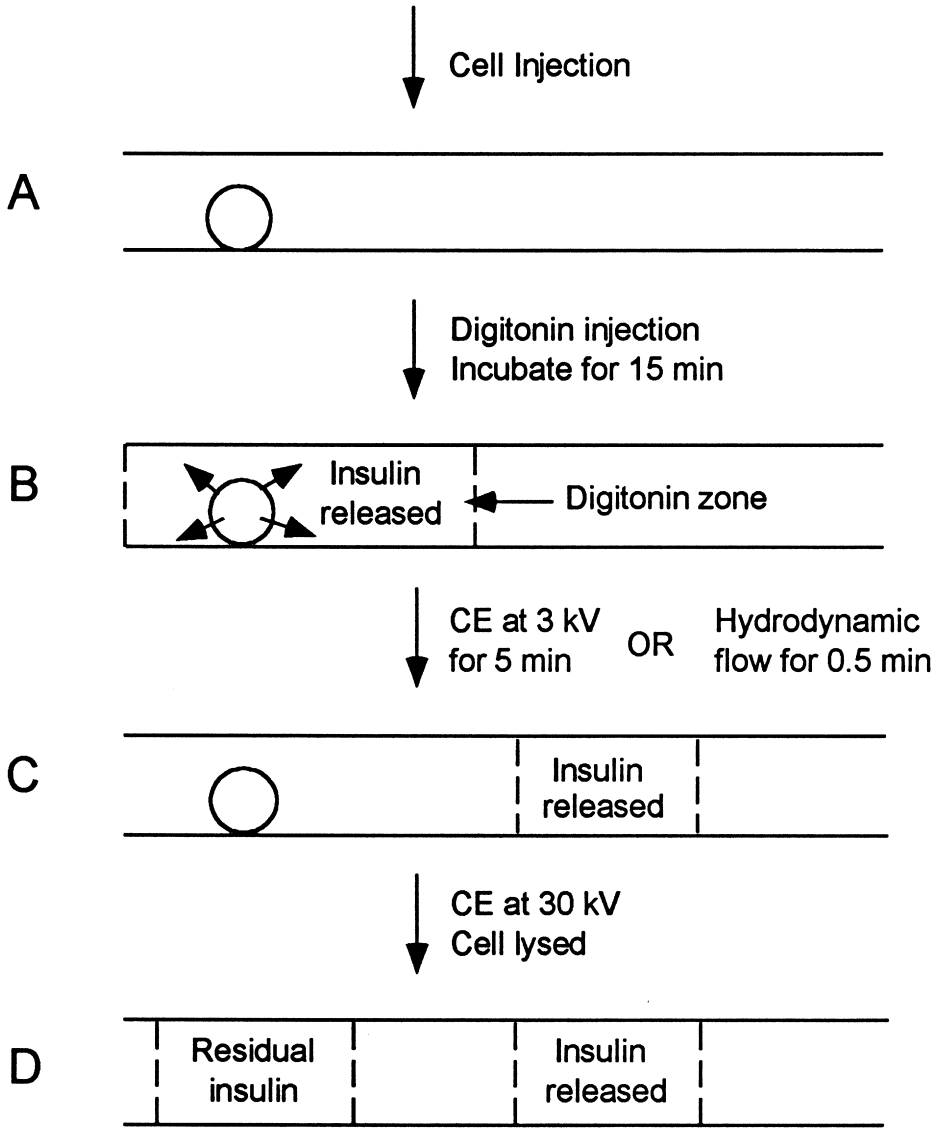

Fig. 6. Schematic diagram of on-column monitoring of insulin release from a single cell. Reproduced with permission from Ref. [48].

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. 6B). 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. Then, CE is run at 3 kV for 5 min to separate the released insulin zone from the cell (Fig. 6C). Alternatively, buffer solution (20 m*M* 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 EOF (Fig. 6D). Quantitation is achieved by measuring the peak areas (Fig. 7). Since this is a relative measurement, the percentage of insulin released by each cell can be calculated without additional calibration. This shows that dynamic events in a single cell can be monitored by CE.

4. Catecholamines in adrenal cells

Catecholamines (CA) are an important group of compounds [50]. Jorgenson and co-workers [6,51] used 15–20 μm open-tubular LC with electrochemical detection (ED) to determine the neurotransmitters and free amino acids in single neuron cells of the land snail, *Helix aspersia*. In addition, CE–ED was demonstrated for the determination of catecholamine and serotonin in a neuronal cell [52,53]. It is well known that chromaffin cells in the adrenal medulla synthesize and store the catecholamine hormones, epinephrine (E) and norepinephrine (NE), and secrete these in response to stimulation by secretagogue [54,55]. We used CE–LIF with an Ar-ion

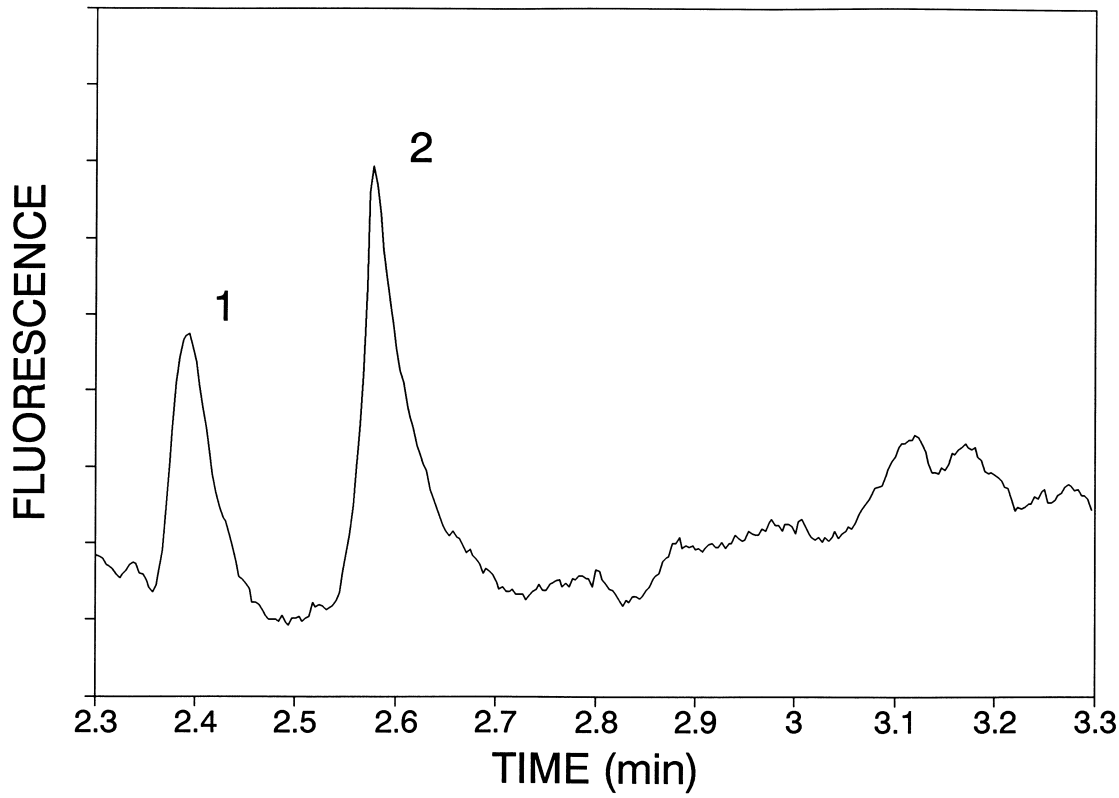

Fig. 7. Electropherogram of on-column release of insulin. Peaks: 1=Insulin released from the cell due to digitonin; 2=residual insulin, which is released due to lysis. The two peaks are separated electrophoretically after release. Reproduced with permission from Ref. [48].

laser at 275 nm to determine quantitatively the amounts of E and NE in single bovine adrenal medullary cells [18].

We studied the relative fluorescence efficiencies of catecholamines in the 320–350 nm region from pH 2 to 9 when excited at 275 nm. In general, the fluorescence intensity is higher at low pH. Even though these amines are expected to be fully protonated below pH of 7, solvent effects, presumably from the co-ions in the buffer solution, are probably responsible for the enhancement. Also, it is worth noting that under this condition amines are more stable.

It is possible to separate these amines based only on electrophoretic mobility at very low pH. pH 2.8 is favorable for detection. Separation under this condition provides an additional advantage in that there is less interference from acidic compounds and their metabolites, since only amines can migrate in this direction at such low pH values.

Fig. 8 shows the electropherogram of a single-cell analysis. Two major peaks corresponding to NE and E are depicted, with a good match of retention times with the standards. The signal-to-noise ratio is excellent, showing the power of native fluorescence detection.

After several runs, the retention times changed due to the adsorption of cell membranes and other species onto the capillary wall. Fortunately, this can be easily overcome by pressure flushing the capillary with running buffer and then re-equilibrating for 5 min. Since catecholamines are known to be secreted from cells even without any chemical or physical stimulation, a blank test after each run is necessary to guarantee there is an insignificant amount of catecholamine accumulated in the balanced salt solution.

The amounts of NE and E in single adrenal medulla cells from two different glands, which are from different cows, are significantly different. The

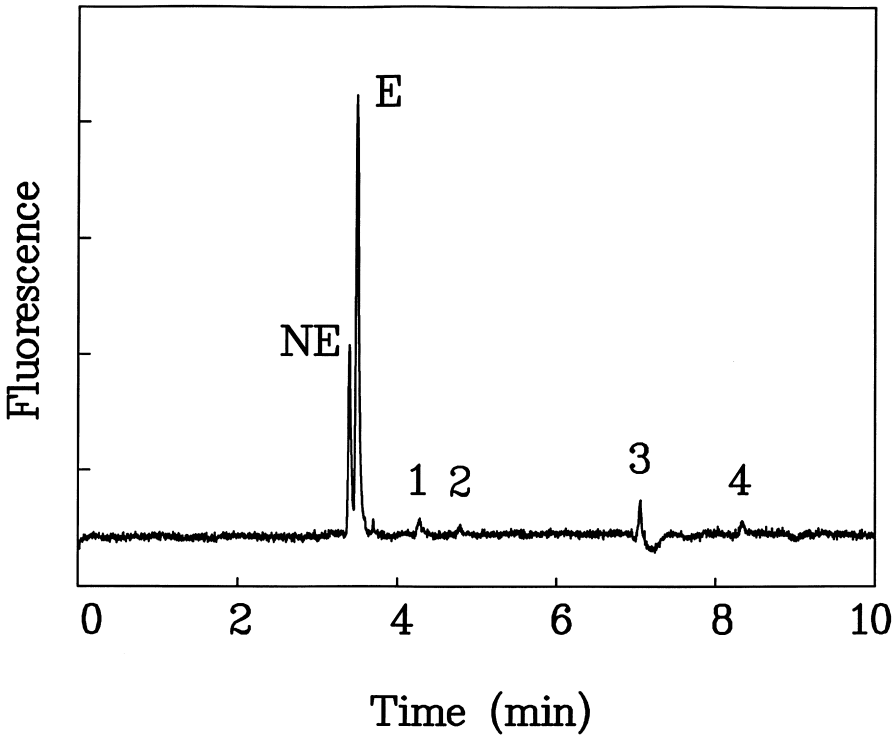

Fig. 8. Electrophoretic separation of intracellular components in a single bovine adrenal medullary cell. Buffer, 0.1 *M* citric acid at pH 2.3; capillary, 65 cm (effective length 45 cm)×16 μm I.D.; concentration, 20 μ*M* each. Peaks 1–4 are unknown components. Reproduced with permission from Ref. [18].

values measured are quite comparable to those measured by using ED [6]. Cell-to-cell differences in the amounts of NE and E are obvious. However, for each gland, the ratio is fairly constant. This shows that the variations for cells from the same gland are probably due to variations in cell size. The ratios of NE to E from the two glands are 2.7 and 5.6, which are close to the results found in the literature [56,57]. Since we did not fractionate [58] or culture the cells before analysis, we did not observe both NE-rich and E-rich cell types in the same gland among the limited number of runs. In a third gland (from a different cow), we did find two cells where the amounts of NE are larger than the amounts of E. Our results agree with Hochman's results [59] where the E content accounted for more than 95% of catecholamine. Also, the faster release of E compared to NE and the ease of NE conversion to E are reasons why in general the E content is higher than NE [60,61].

The secretion of catecholamines from individual bovine adrenal medullary cells was quantitatively monitored by capillary electrophoresis with laser-induced native fluorescence detection [62]. By using a physiological balanced-salt solution as the running buffer for CE, the amount of NE and E secreted by their physiological secretagogue, acetylcholine (ACh), and the amount remaining in a single cell can be simultaneously quantified. The secretion process was also monitored dynamically with this method by continuously passing acetylcholine over the cell during stimulation [62]. From the peak width and shape of the released material, one can estimate the time scale of the release process.

Although catecholamines have closely related structures, they can be well separated by CE under acidic conditions [18]. At higher pH, they cannot be resolved by CE [63]. We found that in physiological buffer treatment of the capillary is very important for catecholamine separation. The resolution of CAs improved dramatically when the capillary was pre-treated with 0.1 *M* HCl. Treatment with HCl presumably removes all potential ion-exchange sites on the capillary wall. In practice, 10 min of treatment with 0.1 *M* HCl followed by 10 min equilibration with the running buffer can make the capillary last for a few hours without significant loss of resolution.

As seen from the experiment above, it is very important for the cell to adhere to the capillary wall. This not only ensures the viability of the cell, but also helps to prevent the cell from further migration down the capillary so that the subsequent on-column procedures (introduction of secretagogue, migration of released zones and lysing of the cell) can be implemented. We have found that treating the capillary with NaOH can help cell adhesion to the capillary wall [48,64]. However, NaOH treatment deteriorates the resolution of CAs. This problem was solved by treating only a small length of the inlet end of the capillary with NaOH. In practice, a ~6 mm plug of 0.1 *M* NaOH was injected into the inlet end and the capillary was incubated for 5 min. The NaOH plug was then pushed out by applying pressure at the outlet end of the capillary.

Fig. 9 is a typical electropherogram obtained from static release experiments for single adrenal chromaffin cells. The first two peaks correspond to NE and E released during the 5 min incubation with ACh, respectively. These extra peaks were absent if ACh was not present or if the incubation time was very short (seconds). The last two peaks represent the residual NE and E in the cell after secretion (as a result of cell lysis).

In agreement with our bulk release studies and literature reports [61], on the average, a higher percentage of the total NE ($34\pm21\%$) was released than E ($25\pm23\%$) on the average when the cells are stimulated by ACh. This may be explained by the biosynthetic processes of NE and E. NE is synthesized from dopamine within intracellular secreting vesicles. The biosynthetic steps leading to NE occur in all the adrenal chromaffin cells. However, the conversion of NE to E by phenylethanolamine N-methyltransferase (PNMT) occurs in cytoplasm. Therefore, NE must return to the cytoplasm, the location of PNMT, to be converted to E, which is then transported by an energy-dependent process back to the vesicles to be ready for release [65,66]. As a result, NE is more readily released by exocytosis than E, and a higher percentage of the total NE is thus released. However, differential release of NE and E may also depend on the type of secretagogues used [60]. There are reports which concluded that all E is secreted from E-rich cells and that all NE is secreted from NE-rich cells [60,67]. It is interesting to note that in this study there seems to be no apparent relationship between the ratio of

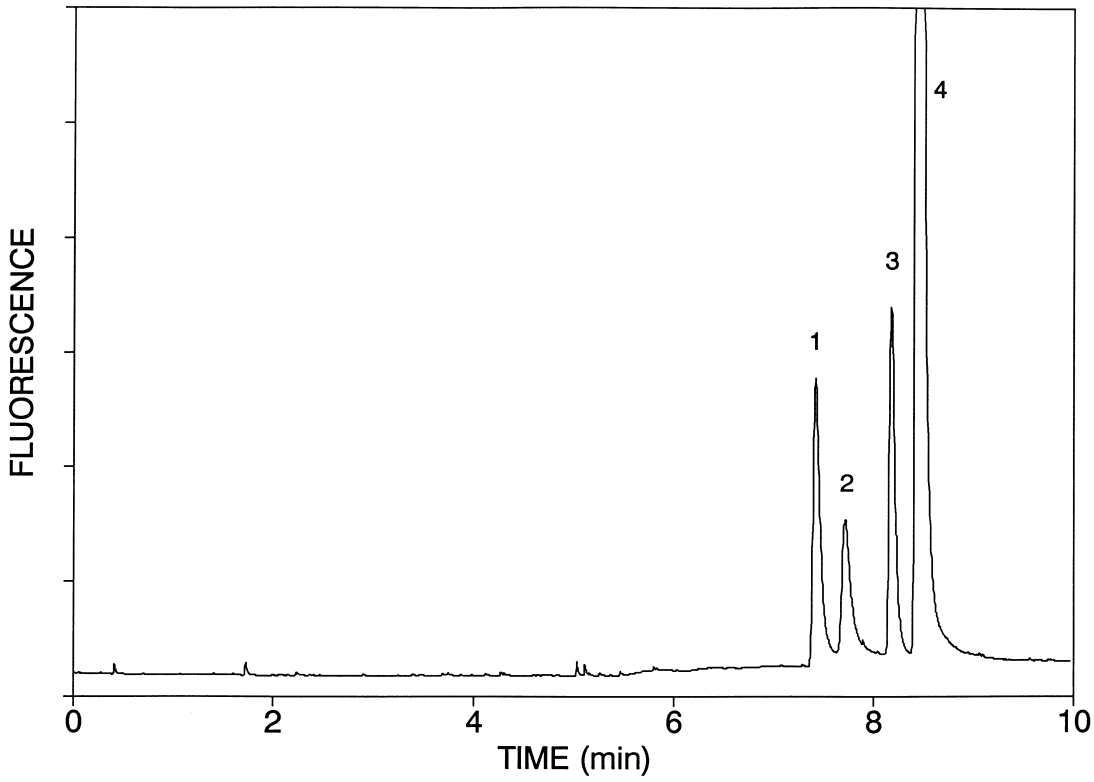

Fig. 9. Electropherogram from on-column static release from a single adrenal chromaffin cell. Peaks 1 and 2 correspond to the released NE and E, respectively. Peaks 3 and 4 represent the residual NE and E inside the cell after secretion. The ordinate has been expanded to highlight peaks 1–3. Reproduced with permission from Ref. [62].

NE/E released and the original ratio of NE/E in the cell, even for cells from the same adrenal gland.

To learn more about the time scale of the release process, a low voltage (3 kV or 6 kV) is used to drive the ACh continuously past the cell immobilized on the capillary wall. The released NE and E will migrate away from the cell immediately after release. Because release consists of exocytosis over a relatively long time, this will make the release peaks for NE and E even broader than those in the static experiments. Fig. 10 shows the electropherograms for dynamic release experiments for single chromaffin cells. As can be seen, at 6 kV the release peaks are broader relative to the release peaks at 3 kV. This is expected because the released CAs are spread further axially at 6 kV (0.035 cm s^{-1}) than at 3 kV (0.018 cm s^{-1}) for identical release times. The absolute migration times are not very reproducible from cell to cell due to capillary contamination as mentioned above. However, the peak separations between the released and residual species were reproducible. The theoretical peak separations between the release peak and the lysis peak are 1.67 min and 3.33 min for the application of 3 kV and 6 kV during stimulation, respectively. The experimental results are 1.57 ± 0.10 and 2.96 ± 0.28 min for two sets of 5 cells.

Close examination of Fig. 10 reveals that the release peaks are highly skewed rather than Gaussian-shaped. Indeed, the actual peak shape traces out the time course of release during dynamic stimulation at low voltage. It should be noted that since electrophoresis was run at 18 kV, the time axis in Fig. 10 should be scaled $6\times$ and $3\times$ for 3 and 6 kV stimulation, respectively. We can conclude that release occurs over the entire 10-min stimulation period, with the majority of the material being produced early on in a 90 s interval.

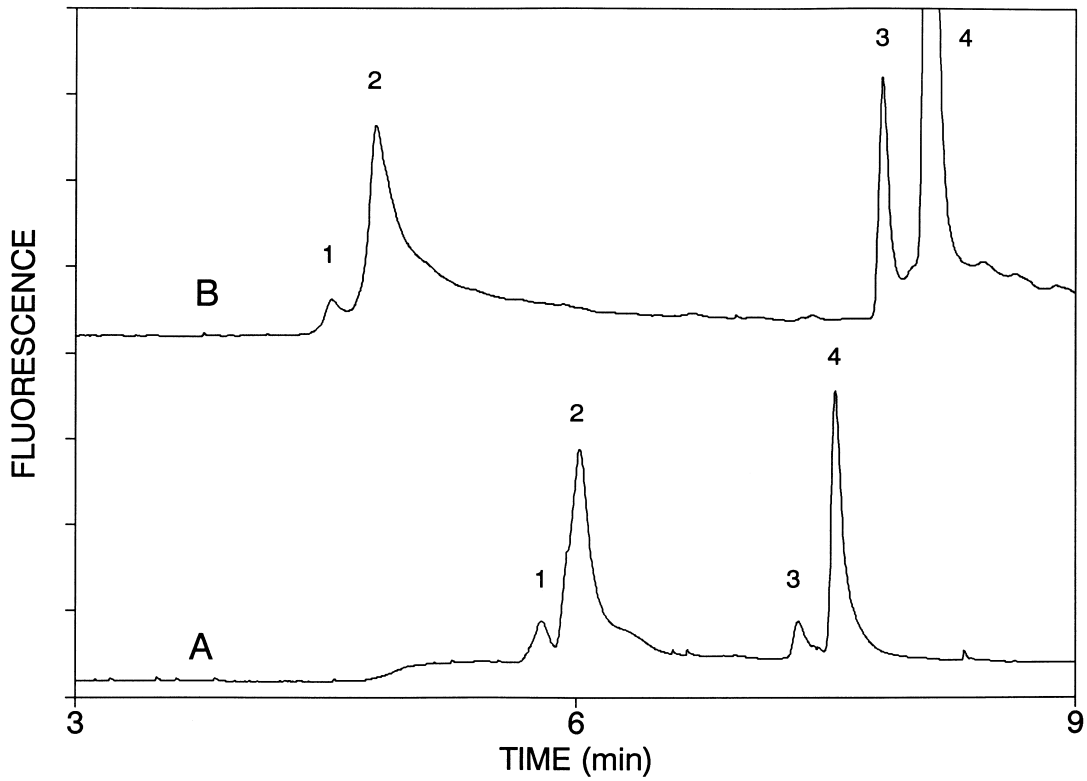

Fig. 10. Electropherograms from on-column dynamic release from single adrenal chromaffin cells. (A) 3 kV is applied when stimulated with ACh; (B) 6 kV is applied when stimulated with ACh. Peaks as in Fig. 9. The ordinate has been expanded to highlight peaks 1–3. Reproduced with permission from Ref. [62].

5. Secretion in mast cells

Serotonin (5-hydroxytryptamine) is an important biogenic amine in both mammalian and non-mammalian signal transduction systems. It has been reported that brain serotonin plays a role in psychiatric syndromes [68] including obsessive-compulsive disorder [69] and depression [70], as well as suicide [71]. In addition, its presence in peripheral tissues may point to its involvement in other disorders, such as those involving the gastrointestinal tract [72].

Functional release of intracellular constituents can occur via exocytosis [73], which is a very common cellular event. In this secretion process, membranes of secretory granules in the cytoplasm first fuse with the plasma membrane, followed by extrusion of the granules into the extracellular matrix. Mediators (e.g., histamine and serotonin) are then displaced from the granular core. Secretion from mast cells has been studied with microelectrodes [74]. We were able to follow on-column exocytotic release from individual rat peritoneal mast cells (RPMC) using CE–LIF [64]. In this novel application, we extend CE from its typical use as a separation device to one in which temporal monitoring of a biological event is achieved.

Fig. 11A shows a complete electropherogram of on-column exocytosis (and release of serotonin), followed by cell lysis, from a single RPMC. The serotonin peaks are seen around 3–5 min, whereas a band of protein peaks is evident around 8–12 min. The two major peaks correspond to release (3.8 min) and lysis (4.7 min) of the mast cell. In all experiments, there is 1.5 min of electrophoresis before the data are collected (i.e., data collection begins immediately following the lysis step).

Fig. 11B is the expanded plot of the region depicting the release and lysis peaks from individual

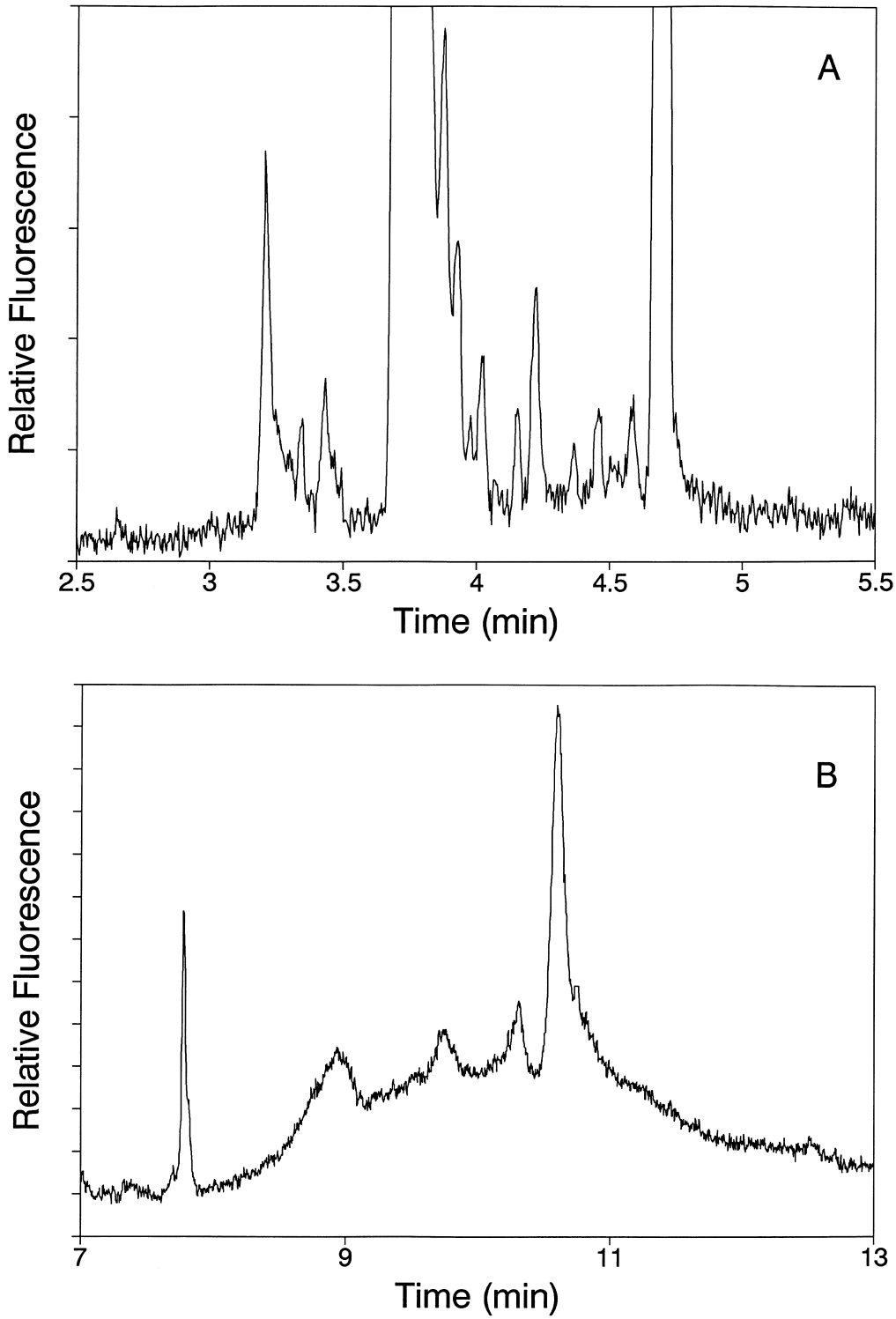

Fig. 11. Electropherogram of individual RPMC. (A) Expanded region between release and lysis events, and (B) protein region. Each mark on the ordinate in A and B corresponds to an identical signal intensity. Reproduced with permission from Ref. [64].

RPMCs. The general patterns of these runs are consistent with one another, yet exhibit features that are consistent with the type of intercellular differences in exocytosis observed by optical microscopy. In every case the main release peak is broader than the lysis peak, which indicates that the exocytosis process occurs more slowly than cell lysis by SDS. The time course of these events is consistent with observation using a microscope. The main release peak actually comprises many individual exocytotic events which, because of axial diffusion, are not resolved in the electropherograms. Even so, some features are clearly evident within each main peak.

Note that the main release peak is not necessarily the first feature in the electropherogram, since there is a delay between contact with polymycin (Pmx) and exocytosis. Also evident are small peaks which are indicative of single-granular events. These small peaks were not found for off-column release or for either on-column or off-column lysis. Again, intercellular variations are evident, with some cells showing more of these events than others. These data depict a time profile similar to that revealed by optical microscopy, i.e., an initial burst of degranulation followed by a substantially delayed release of a few more granules. It is significant that these small peaks are not found before the release event or after complete cell lysis. Vesicular membranes are clearly dissolved on contact with SDS, followed by displacement of all of the serotonin contained within the granules.

The mean diameter of RPMC granules has been determined by Helander and Bloom [75] using electron microscopy to be 0.777 ± 0.090 μm, which corresponds to a volume of 246 al. The serotonin amounts of 54 peaks were determined from the eight RPMCs that were analyzed. By comparing the granular areas with the area and injected amount of serotonin standard, corresponding granular amounts were calculated. Deconvolution allows peak areas to be determined even if there is overlap between the granule peaks. The average content of serotonin per event is consistent with published results [75]. These data show evidence of single granule detection following degranulation (via exocytosis) of individual RPMCs. Since these single granules elute slightly after the main peak, it is likely that they were released later than the initial burst. Similar patterns are observed optically. Even if these granules had remained tethered to the cell following exocytosis, exposure to the saline buffer should have caused the serotonin to be displaced without considerable delay. Another possibility is that some of these small peaks reveal serotonin released due to transient fusion [76]. In this process, the vesicle fuses with the plasma membrane briefly and releases only part of its contents before returning to the cytoplasm. In these experiments we cannot distinguish between transient and irreversible fusion. However, it is unlikely that transient fusion occurs (during the period between the main release peak and the lysis peak) without being accompanied by the primary cellular response of exocytosis.

In fact, we have here the ultimate cellular sampling probe-one that interrogates a 10-μm sized mammalian cell immobilized at a well-defined location. From the analysis of eight individual RPMCs, the average percentage of serotonin released was $28 \pm 14\%$. The features of the electropherograms that we have denoted as single exocytotic events reveal an average serotonin amount of 5.9 ± 3 amol per event. At 250 al each, these are the smallest ''samples'' ever studied by CE. In addition to the detection of serotonin, intracellular proteins originating from both exocytosis (i.e., from the granules) as well as from cell lysis were detected using native protein fluorescence.

We have recently improved the temporal resolution so that the primary exocytotic events can be resolved and quantified [77]. This way, there will not be any bias in interpreting the peaks as being derived from single granules. Fast CE has been demonstrated before, with peak widths in the low ms to sub-ms regime [14]. This is simply done by using very high applied potentials and short migration distances to limit axial diffusion of the analyte zones. In our on-column stimulation experiments, very short migration distances are not easily achieved due to the need to inject the cell under a microscope. With a moderate capillary length, the applied potential will have to be very high to produce the large field gradients needed for fast separations. We fixed the maximum applied voltage at 28 kV based on safety and arcing considerations.

The small quantities of material in each granule and the relatively low fluorescence efficiency provide

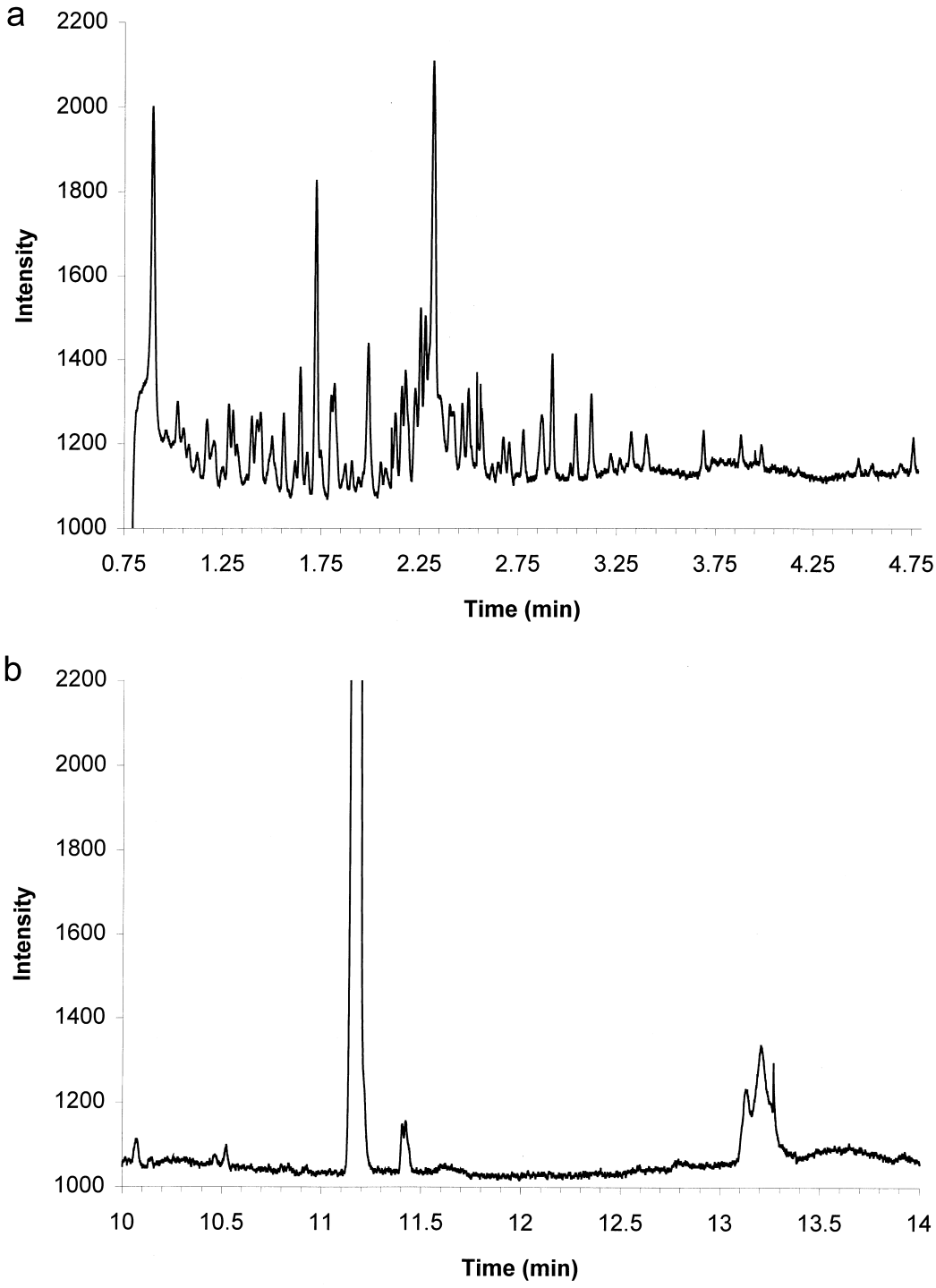

Fig. 12. Electropherogram for a typical RPMC. (a) Release region, (b) lysis region, and (c) converted electropherogram from (a). Conditions were −28 kV for Pmx (50 units/ml) injection for 40 s, −10 kV for running voltage. Capillary length was 30 cm (20 cm effective length). The release of serotonin from individual granules gives rise to distinct peaks in the electropherograms (a) and (c). Reproduced with permission from Ref. [77]. Continued overleaf.

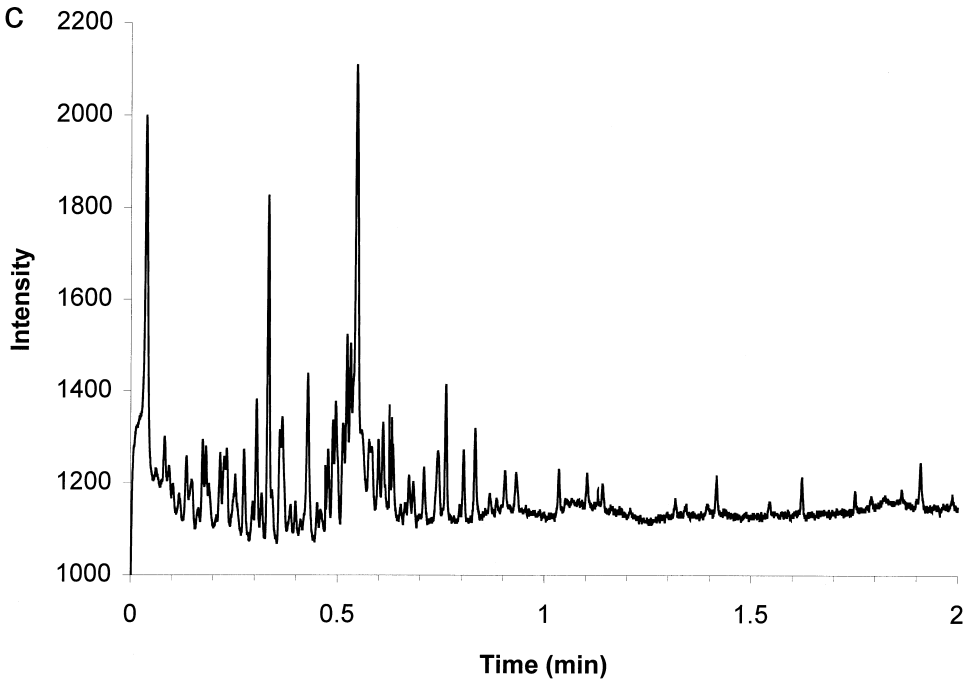

Fig. 12. (*continued*)

a challenge for detection. The S/N can be improved by using longer integration times. This however is at odds with our need to provide high temporal evolution for following the cellular events. We were able to resolve this conflict by introducing the secretagogue at a high linear velocity (applied potential) but switching to a lower velocity during detection. The effect of going from −28 kV to −10 kV is to increase the temporal resolution three-fold. The S/N was increased by more than the standard factor because at the slower speed one can use an integrating A/D converter rather than a sample-and-hold A/D converter. An effective time resolution of 0.002 min and a detection limit of 0.9 amol ($S/N=3$, rms) were achieved.

Pmx was migrated over the cell at a larger negative voltage (−28 kV) than the running voltage (−10 kV). Since electropherogram data were collected at the lower negative voltage, a conversion was applied so that the observed electropherograms and related calculations show events occurring at the larger negative voltage, i.e., in real time. Typical observed and converted electropherograms are depicted in Fig. 12a and c, respectively. Serotonin is stored in granules that are $2.5 \cdot 10^{-16}$ l in volume. Therefore, each peak in Fig. 12a and c corresponds to amol amounts of serotonin inside single granules released from the cell. Binding proteins are also released with each granule. However, these are not observed because they are in much lower amounts and migrate much slower than serotonin. Also in Fig. 12, we see that the abundance of peaks occur during the initial stages of stimulation and the small peaks disappear after the cell lysis event. Furthermore, no peaks were observed if the stimulant Pmx was absent. This indicates that we are observing exocytosis events. A broad peak was observed at 13.2 min. This corresponds to proteins dissolved during cell lysis, identical to observations in our earlier studies [64].

6. Conclusions

The analysis of intact cells by CE, either in their entirety or as subcellular compartments, has been

very successful. However, to become clinically useful, great strides must be taken to increase throughput, i.e., the number of cells that can be analyzed in a given time. The separation step consumes most of the analysis time, but it is not desirable to increase separation speed at the risk of sacrificing separation efficiency.

One potential improvement to cell throughput is that of simultaneously monitoring many capillaries or separation channels. Plausible multiplexed instrumentation has been developed for the purpose of DNA sequencing [15–17,78], however, to apply these to the analysis of individual cells, additional factors remain to be addressed. A standard must be used to normalize the migration times, since each capillary behaves slightly differently [17]. Improving the speed and ease with which individual cells are introduced into the capillary is necessary, especially if multiplexing is desired. Even an experienced experimenter is inherently limited by the nature of present injection methods, which at best take about 10 s and at worst over 1 min for the complete cell injection process. A suitable interface to a flow cytometer may be a viable approach. Additionally, modifications to the detection apparatus that would permit simultaneous sensitive detection in capillaries having an I.D. of approximately 20 μm are necessary.

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