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Cell-to-Cell Scanning in Capillary Electrophoresis

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A widespread limitation in using cell-based biosensors for repetitive chemical analysis is loss of agonist-induced response caused by receptor desensitization. We overcome this problem by scanning an array of immobilized cells underneath a capillary electrophoresis column outlet. In this way, electrophoretically fractionated components that exit the separation capillary are always directed onto cells previously unexposed to receptor agonists. To demonstrate this concept of response recovery using a scanning format, we have chosen the bradykinin B_2 receptor system in the NG108-15 cell line, which is known to undergo desensitization. Whereas four subsequent injections of 250 μM bradykinin separated by 120 s are found to reduce the NG108-15 cell response markedly, scanning to new cells can fully restore the response during the separation. Furthermore, by pretesting individual NG108-15 cells for an agonist response and then later scanning back to the same cell, we achieved a 100% success rate in detecting bradykinin in subsequent electrophoretic separations.

Membrane-bound receptors enable living cells to detect ligands in complex biological environments with a high degree of molecular specificity. The selective binding of the ligand to the receptor can trigger a large biochemical amplification by the opening of ion channels or the generation of second messengers in G-protein cascades.[1] Cells in the olfactory system, for instance, can respond selectively to a single odorant molecule,[2] and the binding of one neurotransmitter molecule can open a single receptor ion channel.[1] The combination of molecular recognition with high sensitivity has led to the development of many receptor-based biosensors,[3] including systems based on membrane receptors,[4−9] intact cells, tissues, and organs.[10−12]

An important advantage for many of these biosensor systems is the ability to detect chemical species that are often difficult or impossible to detect with conventional schemes. Unfortunately, the ligand specificity of a receptor is not absolutely complete; often, structurally related endogenous compounds activate a single receptor, and nonspecific chemical interactions can trigger a receptor-like response. To overcome problems with multiple agonist responses and nonspecific interactions, we developed a capillary electrophoresis single-cell biosensor (CE/SCB) system that combines a microcolumn chemical separation with a cell-based detector.[13] The benefits of using a chemical separation in combination with a cell biosensor include identifying the ligand on the basis of electrophoretic migration times[13] and obtaining information about the activated receptor subtype by using a selective receptor antagonist.[14]

A widespread limitation of many biosensor systems based on ligand-activated receptors is that they often suffer from receptor desensitization. Loss of receptor response is a general challenge regardless of whether the biosensor uses the receptor in the format of an intact cell or reconstituted into an artificial membrane.[15] A diminished cellular response, which we refer to as desensitization, can result from several possible mechanisms that include receptor down-regulation through internalization, modification of the functional properties of the receptor itself (by protein kinases and conformational changes and states), and inhibition of transduction pathways (for example, calcium pools and other second messengers).[16,17] The latter is a mechanism for heterologous desensitization in which the activation of one receptor can diminish the response for another receptor. The degree of desensitization depends on the concentration of the ligand and the exposure time of the ligand with the receptor.

To overcome receptor desensitization, a possible solution is to genetically engineer receptors that are not susceptible to deactivation or desensitization. Receptors of this type have been generated recently by Kobilka et al.,[18] but their utility in a cell detector has not yet been investigated. A more general solution is to replenish the biosensors so that fresh receptors are used for each analysis. In many of the receptor-based biosensor

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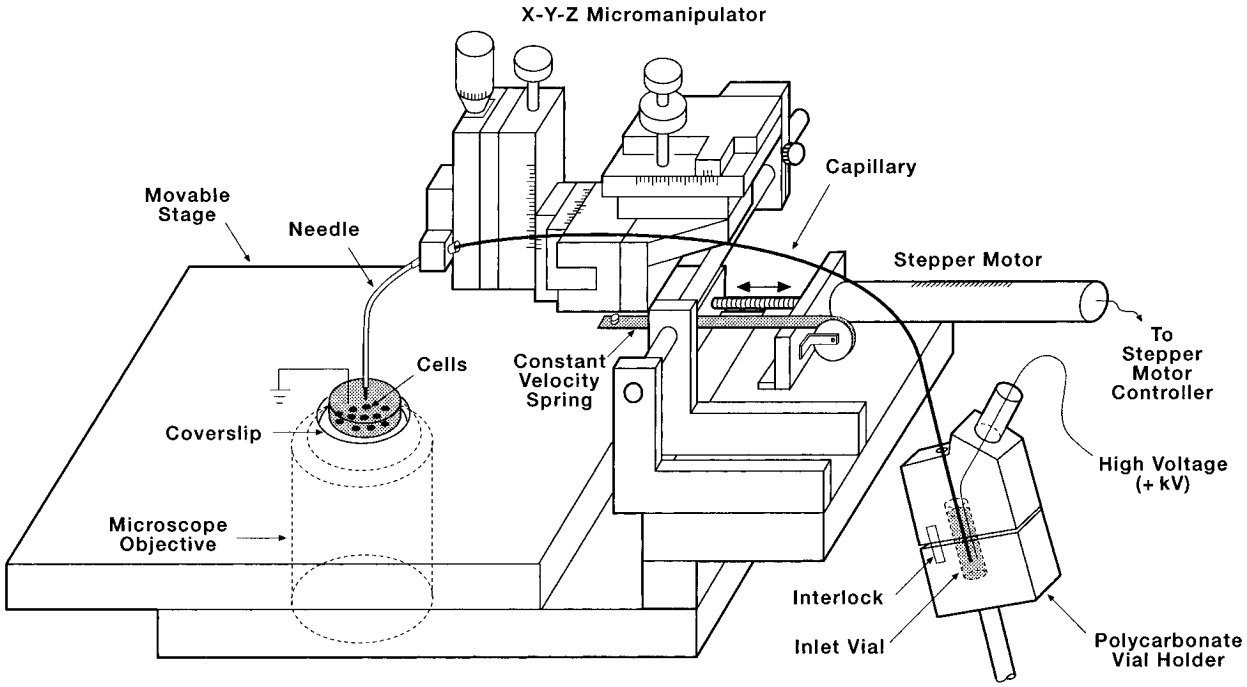

Figure 1. Schematic diagram of the capillary electrophoresis single-cell biosensor system with motorized stage.

formats, particularly those using living cells or whole organisms, replacing or refreshing the receptor membrane is not feasible.

With cell-based detectors, additional challenges of cell responsivity are also present. In addition to cell necrosy, some cells may not express the receptor or may have biochemical differences that prevent efficient signal transduction pathways that couple the receptor to a cellular response. For example, in one study using the NG108-15 cell line, it was found that 40% of the cells responded to opioid peptides, while 91% of the same cells responded to bradykinin.[19]

In this paper, we combine a capillary electrophoresis single-cell biosensor system with a cell-to-cell scanning stage to overcome some of these generalized problems of (1) receptor desensitization and (2) cell unresponsiveness. By scanning immobilized cells underneath the capillary outlet, a new cell (or group of cells) is used to assay each fractionated component for a response. Because the CE column delivers subnanoliter volumes onto the cells that are contained in 500 μL of buffer, the fractionated sample undergoes significant dilution. In this way, the sample affects only those cells located directly underneath or immediately adjacent to the capillary outlet. With numerous cultured cells on each coverslip, the capillary can scan to a new cell detector after each peak. Finally, we show that individual cells pretested for an agonist response can be used several times by scanning back to their position after allowing a period for recovery of response.

EXPERIMENTAL SECTION

Single-Cell Biosensor Capillary Electrophoresis System. The SCB/CE system is identical to that described previously[13] but with modifications to scan the stage. As shown in Figure 1, a micromanipulator positions a fused-silica capillary column above a single cultured cell or group of cells. To enable the stage to scan underneath the capillary, the movable plate on the microscope stage was attached to the microscope with a constant velocity spring, as shown in Figure 1. The end of a linear translation motor (Model 860SA, Newport Research Corp., Irvine, CA) was mounted against the side of the movable stage so that the spring applied a constant force against the motor. The motor was controlled by a Newport Model 860CM2 controller. The velocity of the motor was preset by the controller, and stage movement was initiated manually without further control. The velocity of the stage was determined by scanning over a micrometer ruler and recording the time required to travel a set distance.

Mouse neuroblastoma × rat glioma hybrid cells (NG108-15 cells) were used as detectors. Fractionated components are directed onto the surface of one or more cells, where they bind to receptors and subsequently elicit an increase in the cytosolic free calcium ion concentration ($[Ca^{2+}]_i$). The latter is detected by measuring the fluorescence from the intracellular calcium indicator, fluo-3 acetoxymethyl (fluo-3 AM) ester.[20,21] For all CE experiments, 25−35-cm-long capillaries (Polymicro, Phoenix, AZ) with 15-, 20-, or 25-μm inner diameters and 360-μm outer diameters were used. The outlet end of the capillary that is positioned above the cells was polished by Polymicro to provide a flat surface. In some experiments, the capillary outlet was electrically isolated from the SCB as previously described.[13] A narrow crack connecting the capillary channel to a grounded electrolyte reservoir was made ~5 cm upstream from the capillary outlet using the technique described by Linhares and Kissinger.[22] The use of these electrically isolated capillaries (15-μm i.d., 360-μm o.d.) prevented a transient calcium increase upon the application of an 18-kV (+600 V/cm) separation voltage. Separations were carried out in a standard cell buffer (HEPES buffer saline, HBS), which

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consisted of 135 mM NaCl, 5 mM KCl, 10 mM glucose, 2 mM $MgCl_2$, 2 mM $CaCl_2$, and 10 mM HEPES (pH 7.2). During the measurement, the cells were maintained in the same HBS buffer used for the CE separation. Separation voltages were applied to the injection end using a high-voltage power supply (Model CZE100R, Spellman, Plainview, NY); they ranged from 7 to 15 kV. The high-voltage lead was contained within a carbonate vial holder with a 1-in. wall thickness and equipped with an interlock for safety precautions against shock. The solution containing the cells was grounded. Injection volumes of 50−100 pL were made by gravity (diffusive and convective mixing and spontaneous fluid displacement[23,24] significantly contributed to this volume).

Cell Culture. The NG108-15 cells were grown in Dulbecco's Modified Eagle Medium (Gibco BRL/Life Technologies, Grand Island, NY), supplemented with 10% (v/v) fetal calf serum (Gibco), 5% (v/v) penicillin-streptomycin (Gibco), 5% (v/v) 200 mM L-glutamine, and 5% (v/v) solution of HAT (10 μM sodium hypoxanthine, 40 μM aminopterin, 1.6 mM thymidine; Gibco) in a humidified atmosphere of 93% air and 7% CO_2. Cells from the stock cultures were plated onto no. 1 coverslips several hours before fluorescence measurements. We took care to ensure that all conditions for cell handling and cell culture were as nearly identical as possible and that a new stock of cells was used after passing the cells eight times.

Calcium Measurements from Fluo-3 Fluorescence. Cells were loaded with fluo-3 AM ester (Molecular Probes, Eugene, OR) at 20 °C for ∼0.5 h. The loading medium contained 18 μM fluo-3 AM ester prepared in an HBS buffer solution. After being loaded, the cells were placed in the HBS buffer for an additional 0.5 h at the same temperature before the experiments. Cells were maintained in a 6 μM fluo-3 AM ester HBS solution during the fluorescence experiments to replenish dye that the cells extrude. After the dye loading, the cells were transferred to the stage of an inverted fluorescence microscope (Diaphot-TMD-EF, Nikon), where they were maintained at 37 °C by heating the 100× (0.8 numerical aperture) oil-immersion lens used for illumination and fluorescence collection. Cells were illuminated with 470−490-nm light (Nikon filter block, B-1A), and fluorescence (λ > 520 nm) was imaged onto a photomultiplier tube (PMT, Model R928, Hamamatsu Corp., Bridgewater, NJ) with a photomicrographic attachment (Microflex PFX, Nikon). An adjustable aperture on the photomicrographic attachment controlled the size of the microscope field, which effectively determined the number of cells imaged onto the PMT. The current from the PMT was converted to a voltage and amplified. High-frequency noise was removed by a low-pass filter with an RC time constant of 1 s. The voltage signal was digitized and displayed on an IBM personal computer. Drift or slope in the baselines of the electropherograms was removed by subtraction using a linear fit. Experiments used 1−10 cells for detection purposes. New cells were used for each electropherogram. Cells from the same culture dish were used for ∼3 h.

Chemicals and Materials. Fluo-3 AM ester was dissolved in DMSO and 5% (v/v) Pluronic F-127 (P-1572, Trademark of BASF Wyandotte; Molecular Probes). All chemicals used for buffer solutions are analytical grade from Sigma (St. Louis, MO). BK and Lys-BK (kallidin) (Sigma) were reconstituted in the HBS buffer solution, divided into aliquots, and stored at −20 °C until the day of experiment.

CE Absorbance Detection. Some experiments were performed using a home-built CE system equipped with an absorbance detector (Isco Model CV4, Lincoln, NE). The detector wavelength was held at 200 nm, with a time constant of 0.8 s. A 25-μm-i.d. capillary with a total length of 58 cm (33 cm to detector) was used. For all the CE experiments using the absorbance detector, injections were made as described for the CE/SCB system above.

Comparison of Cell Response with Electrophoretic Peak Shape. In the context of scanning the capillary during an electrophoretic separation, an important consideration is how closely the cell responds to the physical presence of a band eluting from the outlet of the column. Variability in the onset of a response or a long-lived response would make peak identification particularly complicated in the scanning format. Ionotropic receptors, for instance, typically give rise to a short-lived response (<1 s), whereas the response from a metabotropic receptor can last for seconds to minutes.[1] Other receptors may exhibit a long delay before the onset of a cellular response. Moreover, the onset of the response may be a function of the concentration.

With conventional physical detectors in CE, the time of response to the presence of analyte is fast, and the temporal width of the CE peak is roughly proportional to the time the analyte band remains in the detection zone. In contrast, the observed peak shape and time response of the fluorescence observed from fluo-3 chelated with calcium in our cell detector are entirely functions of the calcium signaling mechanism and may have no relation to the actual temporal width of the band impinging on the cell.

To characterize the peak response in our SCB system, we compared a separation using SCB detection (NG108-15 cell detectors) to the same separation using a conventional absorbance detector (Figure 2). Shown is a CE separation of BK and Lys-BK with SCB (Figure 2a) and absorbance (Figure 2b) detection. In each separation, field strength, buffer composition, separation distance to the detector, injection length, and column inner diameter were maintained nearly the same, but different capillary columns were used. The only other difference was in the concentrations of the components. The concentrations of Lys-BK and BK in the absorbance system were both 900 μM, and in the cell detector they were 0.45 and 120 μM, respectively. The higher concentrations used in the absorbance detector were necessary because of the small detection path length. Because Lys-BK is a more potent BK B_2 receptor agonist[25] than BK and the separation time between them is short, BK had to be at a higher concentration than Lys-BK to compensate for desensitization. Assuming differences in the electroosmotic flow for each column, the cell detector response produces comparable migration times. One source of the band broadening observed with the cell detector is the end-column detection format.[26] In separate experiments, however, longer injection bands of BK were introduced into the column for separation. The width of the cellular response did not appear to correlate with a longer injection band. These results suggest that the cell is initially activated by the onset of the BK band, but peak duration and shape depend on the

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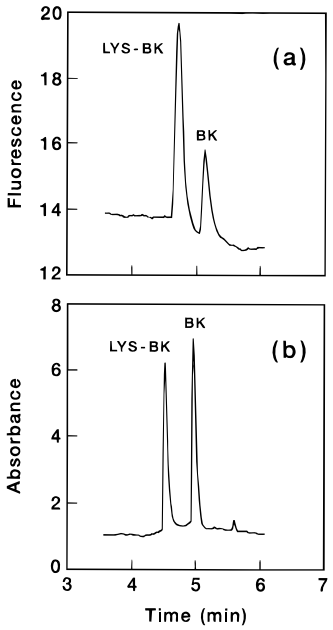

Figure 2. Comparison between electrophoretic separations of Lys-BK (first peak) and BK (second peak) using (a) a SCB detector and (b) an absorbance detector. Separation conditions for the SCB detection: 33-cm-long capillary, 25-μm i.d., 9.2-kV voltage, 72-μA current. Separation conditions for the absorbance detection: 59-cm-long capillary (33 cm to detector), 25-μm i.d., 16.6-kV voltage, 72-μA current.

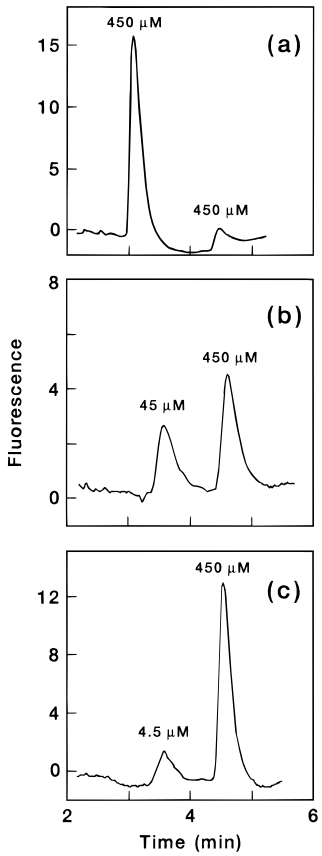

Figure 3. Electropherograms of consecutive BK injections at different concentrations spaced by 70 s. The concentrations of the first and second BK injections were: (a) 450 and 450 μM, (b) 45 and 450 μM, and (c) 4.5 and 450 μM. Each electropherogram is representative of three experiments for each of these three concentrations. An identical trend in detector response was observed for each. Separation conditions: 30-cm-long capillary, 20-μm i.d., 15-kV voltage, 48-μA current. The capillary outlet is positioned 30 μm above 5−10 NG108-15 cells.

mechanism for calcium spiking and not on the actual electrophoretic peak shape.

RESULTS AND DISCUSSION

Cellular Desensitization. Figure 3 illustrates the effects of cellular desensitization. Shown are electropherograms of two consecutive injections of BK spaced 70 s apart. In panels a−c, the concentrations of the first BK band were 450, 45, and 4.5 μM, whereas the second BK band was held constant at 450 μM. The degree of desensitization decreases as the concentration of the first BK band is reduced in relation to the second band. In another experiment (not shown), the second band showed a decreased response similar to that shown in Figure 3a when two consecutive injections of either 4.5 μM or 600 nM BK were made.

These experiments show that NG108-15 SCBs can undergo desensitization when fractionated components act on the same receptor system, but that the degree of signal loss is dependent on the concentration of the species. The extent of desensitization is also dependent on the time between successive peaks. With long enough intervals (25 min) between exposure, complete recovery of the response from NG108-15 cells to BK has been shown in separate studies.[17] Desensitization of BK receptors has been attributed to internalization of the receptor,[27] phosphorylation of the cytoplasmic domain of the receptor protein by serine and threonine kinases,[28] and depletion of the calcium pool.[17] While the properties of the desensitization evoked by BK on NG108-15 cells are specific to this cell line, other ligand−receptor systems and cell types will have their own characteristic time and concentration dependencies for desensitization.

Cell-to-Cell Scanning. Figure 4a shows an electropherogram in which four successive bands of BK (250 μM), spaced at 120-s intervals, desensitize a group of NG108-15 cells. The BK response is desensitized with each successive band; by the fourth injection, a complete loss of signal is observed. In Figure 4b, four equivalent injections of BK (250 μM) are similarly spaced at 120-s intervals. During this run, however, the stage is manually scanned a distance of 500−1000 μm after each band to direct the effluent onto a fresh group of cells instead of onto the same groups of cells for the entire electrophoresis separation. As is clearly observed, scanning the stage underneath the outlet of the fixed capillary renews the response of the CE/SCB system. During these scans (panels a and b), the electric field was applied continually to preserve the actual time course of the injections in the electrophoresis run, thus making comparisons straightforward.

In contrast to Figure 4a, cell-to-cell scanning in Figure 4b greatly recovers the signal for each peak. In seven out of seven experiments in which the capillary scanned to new places for each of the four BK injections (Figure 4b), we obtained a refreshed peak from the cell field (peak heights within a factor of 2 of the initial peak). The third and fourth peaks from six of six separations without scanning were 20-fold lower than the initial peak. The variations in peak height in the different cell fields

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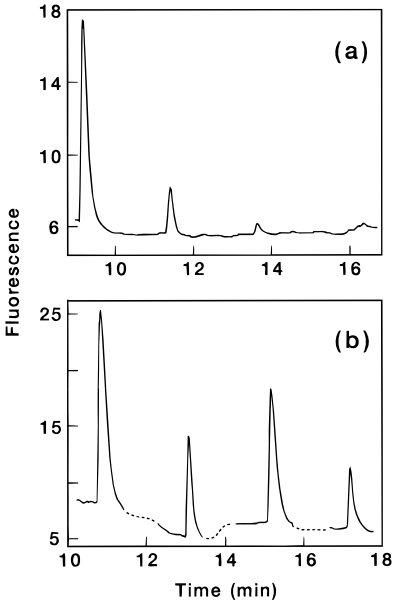

Figure 4. Cell-to-cell scanning. Shown are electropherograms obtained by making four consecutive injections of 250 μM bradykinin (a) separated by 120-s intervals with no scanning and (b) separated by 120-s intervals with manual cell-to-cell scanning after each peak. The dotted lines show the time during which the cells were scanned and the baseline was disrupted. In these manual scan separations, the voltage is maintained during the entire run (including scan time), and data acquisition need not be stopped. Separation conditions: 26-cm-long capillary, 20-μm i.d., 7-kV voltage, 24-μA current. The capillary outlet is positioned 30 μm above 5−10 NG108-15 cells.

likely result from differences in the number of the cells per imaging field, the size and spatial distribution of the cells, and the amount of the dye in each cell. Other factors such as the number of receptors per cell,[29] viability of the new cells, or differences in the efficiency of the signal transduction pathways may also be of importance. Procedures for normalizing many but not all of these cell-to-cell variations are routine in fluorescence microscopy.[30] A common solution is to use dual-wavelength ratiometric dyes such as fura-2 or indo-1. Fura-2 is frequently used to obtain quantitative dose−response curves in some cell types.[30] In our current single-wavelength system, however, we are unable to implement this procedure.

Semiautomated Scanning. Ideally, a motorized microscope stage should be used for cell-to-cell scanning. Figure 5 demonstrates the use of a semiautomated motorized scan to preselect the cell detector field. To program the scan speed, we equipped our microscope stage with a stepper motor that translates the stage in one dimension underneath the CE column (Figure 1). Two individual NG108-15 cells that were 121 μm apart were chosen as the cell detectors, and the motorized stage was preset to scan at 11 μm/s. Two injections of 120 μM BK were spaced 120 s apart. After the first BK peak appeared, the stage was scanned for 11 s (121 μm) at the preset speed, thus producing a robust response for the second peak.

Pretested Cell Response. The feasibility of testing a cell for a response to bradykinin and then scanning back for additional electrophoretic runs was performed. Shown in Figure 6 is a

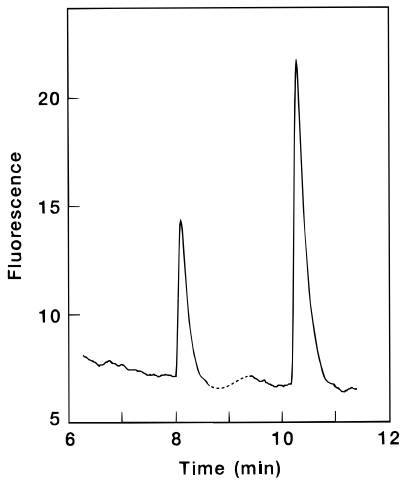

Figure 5. Electropherogram showing semiautomated scanning to preselected cells. Two consecutive injections of 120 μM BK are spaced by 120 s. After the first peak, the stage is scanned at the preselected speed of 11 μm/s for 11 s to correspond to the distance between two cells chosen before the electrophoresis separation. For comparison purposes, the baseline from the second peak was lowered to the same level as the first peak. All experimental conditions are otherwise the same as in Figure 4.

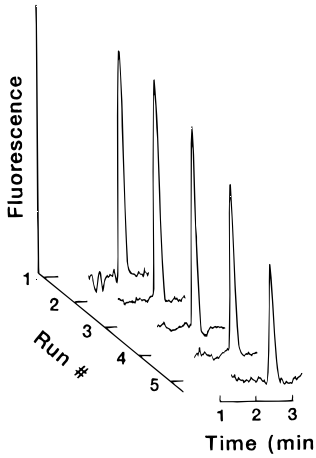

Figure 6. Multiple electropherograms performed on an individual NG108-15 SCB. The concentrations of BK were 600 nM (run 1), 25 μM (run 2), and 100 μM (runs 3−5). Experimental conditions: 30-cm-long capillary with 15-μm i.d., 360-μm o.d., capillary outlet electrically isolated (as described in text) and positioned 30−35 μm above the cell, 18−20-kV voltage, 24−33-μA current, and 10-cm gravity injection for 15 s.

sequence of repeated electropherograms obtained from the use of an individual SCB. The procedure for obtaining these electropherograms included pretesting the cell for a BK response, manually scanning the capillary ~300 μm away from the cell, waiting for 5 min, and repositioning the capillary above the cell, which was not moved during this time interval. To ensure that the separation voltage did not affect the long-term viability of the cells, we used a capillary that was grounded 5 cm from the outlet end, thus isolating the electric field from the cells. The concentration of BK in the pretest electropherogram (run 1) was 600 nM and was subsequently raised to 25 (run 2) and 100 μM (runs 3−5) to compensate for effects of desensitization. Using this procedure with seven entirely different SCBs that responded to BK in a pretest, each gave a BK response during a second electrophoretic separation. In total, these seven cells successfully detected BK in 24 separate electropherograms.

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CONCLUSION

We have shown that cell-to-cell scanning using a motorized microscope stage is a viable way to overcome effects from receptor desensitization in a CE/SCB system. Moreover, we illustrate the feasibility of using pretested cells in a cell-to-cell scanning format to ensure that cells being used in the analysis provide a viable agonist response. These results suggest that reusing pretested cells in combination with a programmable scanning stage system should greatly improve the reliability of cell-to-cell scanning procedures. In addition to renewing the response, cell-to-cell scanning could be implemented in a large-array format for screening components using a variety of primary cell cultures or cell lines engineered to express a specific receptor. The ability to scan multiple cell lines underneath the capillary outlet opens possibilities for screening ligands in complex mixtures and identifying new ligands for receptors.

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