Novel integrated microdialysis–amperometric system for in vitro detection of dopamine secreted from PC12 cells: Design, construction, and validation

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ABSTRACT

A novel dual channel in vitro apparatus, derived from a previously described design, has been coupled with dopamine (DA) microsensors for the flow-through detection of DA secreted from PC12 cells. The device, including two independent microdialysis capillaries, was loaded with a solution containing PC12 cells while a constant phosphate-buffered saline (PBS) medium perfusion was carried out using a dual channel miniaturized peristaltic pump. One capillary was perfused with normal PBS, whereas extracellular calcium was removed from extracellular fluid of the second capillary. After a first period of stabilization and DA baseline recording, KCl (75 mM) was added to the perfusion fluid of both capillaries. In this manner, a simultaneous "treatment–control" experimental design was performed to detect K^+-evoked calcium-dependent DA secretion. For this purpose, self-referencing DA microsensors were developed, and procedures for making, testing, and calibrating them are described in detail. The electronic circuitry was derived from previously published schematics and optimized for dual sensor constant potential amperometry applications. The microdialysis system was tested and validated in vitro under different experimental conditions, and DA secretion was confirmed by high-performance liquid chromatography with electrochemical detection (HPLC–EC). PC12 cell viability was quantified before and after each experiment. The proposed apparatus serves as a reliable model for studying the effects of different drugs on DA secretion through the direct comparison of extracellular DA increase in treatment–control experiments performed on the same initial PC12 cell population.

Dopamine (DA)[1] is a catechol-like neurotransmitter that has been implicated in cognitive functions [1], in reward pathways [2], and in Parkinson's disease [3,4]. DA biochemical dynamics have been studied extensively both in vivo and in vitro using different techniques, including microdialysis [5–8] and voltammetry [9]. Indeed, oxidation of DA to the corresponding orthoquinone (DA–OQ) is pos-sible on Nafion-coated carbon electrodes with a good selectivity against electrochemically oxidizable anions [10,11]:

$$DA\ (Ox) \rightarrow DA{-}OQ + 2e^- + 2H^+$$
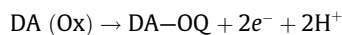
A wide variety of techniques have been used for studying in vitro DA secretion/release, and most of them use PC12 cells be-cause they are capable of synthesizing, secreting, and metabolizing DA. The PC12 cell line, obtained from rat pheochromocytoma of the adrenal medulla, is also used as a tool to understand the biochem-ical mechanisms underlying the physiology and degeneration of central dopamine neurons [12–14]. In previous studies, we suc-cessfully used PC12 cells to investigate the mechanism of nitric oxide (NO) donor-induced DA secretion [7] and DA oxidative homeostasis in vitro [6,8]. In such studies, DA was quantified in microdialysates by high-performance liquid chromatography with electrochemical detection (HPLC–EC). The aim of the current re-search was to combine a modified device for in vitro microdialysis with a novel, dual channel amperometric system for the online detection of DA secreted from PC12 cells. The new electronics de-

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[1] *Abbreviations:* DA, dopamine; DA–OQ, dopamine–orthoquinone; NO, nitric oxide; HPLC–EC, high-performance liquid chromatography with electrochemical detection; 3-MT, 3-methoxytyramine; DOPAC, dihydroxyphenylacetic acid; HVA, homovanillic acid; UA, uric acid; AA, ascorbic acid; PBS, phosphate-buffered saline; EGTA, ethyleneglycoltetraacetic acid; DCP, dual channel potentiostat; ADC, analog-to-digital converter; DAC, digital-to-analog converter; IC, integrated circuit; OPA, operational amplifier; WE, working electrode (DA microsensor); RE, reference electrode; AE, auxiliary electrode; USB, Universal Serial Bus; AUX–CE, auxiliary counter electrode; F-TEC, flow-through electrochemical cell; DR, drug reservoir; FM, fluid mixer; DMEM, Dulbecco's modified Eagle's medium; EDTA, ethylenediaminetetraacetic acid; COMT, catechol-*O*-methyl-transferase; DAT, dopamine transporter.

sign, derived from a previously published apparatus [15,16], allows simultaneous "treatment–control" experiments on the same initial cell suspension so as to detect drug-related DA secretion. Instead of measuring a single exocytotic event [13] or single-cell DA secretion [14], the proposed apparatus serves as a rapid and reliable model for studying the effects of different drugs on DA secretion from a large population ($\sim 10^6$) of PC12 cells and performing a direct comparison of extracellular DA increase in treatment–control paradigms. The further miniaturization of the electronics and the integration with ultra miniature peristaltic pumps could permit the realization of a low-cost miniaturized device for rapid drug screenings in vitro.

Materials and methods

Reagents and solutions

All chemicals were analytical reagent grade and were used as supplied and dissolved in bidistilled deionized water (Milli-Q). DA, 3-methoxytyramine (3-MT), dihydroxyphenylacetic acid (DO-PAC), homovanillic acid (HVA), uric acid (UA), and ascorbic acid (AA) were purchased from Sigma (Milano, Italy). Nafion perfluorinated resin solution (5% in aliphatic alcohols) was purchased from Aldrich (Milano, Italy). The phosphate-buffered saline (PBS) solution was made using NaCl (137 mM), KCl (2.7 mM), Na_2HPO_4 (8.1 mM), KH_2PO_4 (1.47 mM), $CaCl_2$ (1.19 mM), $MgCl_2$ (0.54 mM), and glucose (7.5 mM) from Sigma and then adjusted to pH 7.4. In calcium-free experiments, PBS solution was made without $CaCl_2$ and adding ethyleneglycoltetraacetic acid (EGTA, 1 mM). The stock solutions of DA and 3-MT (250 μM) were freshly prepared in water immediately before use. The concentrated solutions of DOPAC (250 μM), HVA (250 μM), and AA (1 mM) were prepared in 0.01 M HCl and stored in the freezer for up to 1 week before use. UA solutions (1 mM) were made in 2 M NaOH, stored at below 4 °C, and diluted in water before use.

Materials and electronic components

Electronic parts were obtained from the Farnell In One spa (Milano, Italy). The power section of the dual channel potentiostat (DCP) was stabilized using Linear voltage regulators (LM1086-5 and -3). The amperometric section of the DCP was built using six operational amplifiers (National LMC6042), whereas the differentiator–amplifiers were two AD626 units from Analog Devices. The 12-bit analog-to-digital converter (ADC)–digital-to-analog converter (DAC) system was built with integrated circuits from Maxim/Dallas and Analog Devices: MAX1270 (ADC), MAX5156 (DAC), and ADR370 (DAC–VRef). The MCU used in the DCP was the Propeller 32-bit multicore microcontroller (Parallax, Rocklin, CA, USA). The serial-to-USB converter was an FTDI FT232R controller (Future Technology Devices International, Glasgow, UK).

DCP design and construction

The DCP was built as described previously [15] and includes two distinct parts: the amperometric and digital sections. The two-channel amperometric section (Fig. 1) was developed around the integrated circuit (IC) LMC6042, a dual single-supply operational amplifier (OPA). The following description concerns a single analog channel. The DAC (MAX5156) produces the voltage necessary to polarize the DA microsensor (working electrode [WE]) at +250 mV versus Ag/AgCl reference electrode (RE). A two-OPA potentiostat controls the RE and the auxiliary electrode (AE). The current-to-voltage (I/V) converter is a single-supply adaptation of a classical transimpedance amplifier [16] with a

fixed gain depending on the value of Rf1-2 (10 MΩ). The AD626-based difference amplifier has the dual function of removing the potential applied to the WE [17] while amplifying the resulting signal 100 times (1 nA/V). The core of the digital section was a Propeller, a 32-bit multicore CMOS IC containing eight independent controllers called COGs. The MCU, working at 80 MHz, controlled the ADC, the DAC, and the serial transmission of data to the PC. MCU peripherals have independent data and control lines to prevent hardware collisions during parallel computing. A Universal Serial Bus (USB) provided the stabilized power supply to the DCP.

Firmware and software

The Propeller firmware is developed in Spin, a specialized interpreted language, and then transferred to the MCU by an in-circuit serial programmer. The DA microsensor applied potentials (+250 mV vs. Ag/AgCl) are generated using a two-channel DAC controlled by two COGs. The resulting sensor currents are converted to digital values using two more COGs interfaced to a multichannel ADC. The remaining COGs perform digital signal processing and generate serial data for the interfacing PC via USB. Completely customizable data acquisition software, allowing the PC to record, plot, and display the received data, was developed in Visual Basic.NET 2008 using ZedGraph charting class library.

DA microsensor and flow-through electrochemical cell

The Nafion-coated WEs (DA microsensors) were made using Teflon-insulated silver wire (30 mm in length, Ø = 250 μm, Advent Research Materials, Suffolk, UK). Approximately 1 mm of the wire was exposed and inserted in a glass micropipette filled partly with graphite-loaded (55%, w/w) epoxy resin (Araldite-M, Sigma–Aldrich). The 500-μm diameter carbon composite disk electrode (area = 0.0019 cm^2) was fabricated by mixing 850 mg of graphite (synthetic graphite powder, particle Ø < 20 μm, Sigma–Aldrich) with 500 mg of Araldite-M and 200 mg of harderer [18] and filling a precut (10 mm) 10-μl glass micropipette (microcaps, Drummond Scientific, Broomall, PA, USA) with the mixture. The silver wire guaranteed a good electrical contact. After 24 h at 40 °C, the disk was polished using sandpaper (1200 grain) and diamond powder and Nafion treatment was performed (three coats and 20 min drying at 120 °C). DA oxidation potential was experimentally obtained at +250 mV versus Ag/AgCl using cyclic voltammetry at 100 mV/s (CV37 voltammograph, Bioanalytical Systems, West Lafayette, IN, USA). DA microelectrodes were calibrated in quiescent conditions by adding known volumes of a standard DA solution in PBS. Only electrodes with DA detection limits ≤25 nM and high selectivity against AA (DA/AA > 1000:1) were used. No significant interference signals were observed on exposing sensors to other oxidizable anions (UA, DOPAC, and HVA) even at pharmacologically relevant concentrations (up to 0.5 μM). The pseudo-reference electrode (RE) and auxiliary counter electrode (AUX–CE), used in the flow-through electrochemical cell (F-TEC), were an AgCl-coated silver wire and a 2-mm platinum wire (Ø = 125 μm), respectively. RE was prepared by immersing 1 mm silver wire (Ø = 125 μm) in a saturated KCl solution and applying +500 mV for 1 min. The WE, RE, and CE were glued together using epoxy resin and soldered to a 2.54-mm PCB connector. The F-TEC was assembled using a portion of a 10-μl Hamilton micropipette tip (inlet section) and a 250-μl microcentrifuge tube (Alpha Laboratories, Eastleigh, UK) (outlet section). The inlet and outlet sections were connected to 25 mm polyethylene tubing (0.58 mm i.d., Portex, Hythe, UK). The final volume of the F-TEC was approximately 10 μl. A schematic diagram and picture of the F-TEC are shown in Fig. 2. Two
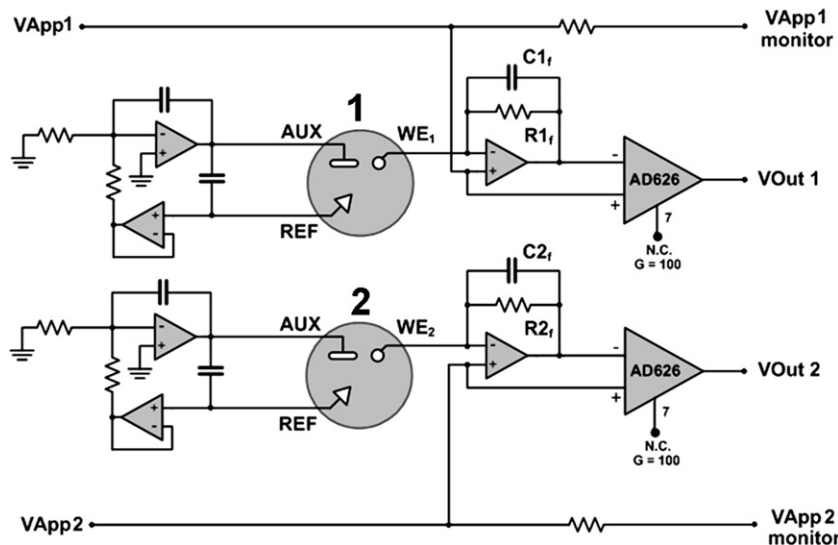
Fig. 1. Schematic representation of the DCP developed and used in this study. The device has two identical channels consisting of a two-OPA potentiostat, a current-to-voltage converter, and a differentiator/amplifier. Each channel controls a complete electrochemical cell (1,2) connected to one outlet of the microdialysis tube. $WE_{1,2}$, working electrode (DA microsensor); REF, reference electrode (RE); AUX, auxiliary electrode; $VApp_{1,2}$, applied voltage (+250 mV vs. RE); $VOut_{1,2}$, output voltage.
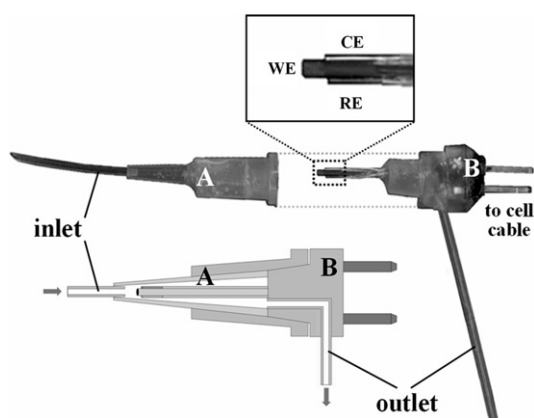

Fig. 2. Picture and schematic diagram of the F-TEC developed and used in this study. The cell is composed of an inlet section (A) and an outlet section (B) connected to one channel of the potentiostat through a cell cable. WE, working electrode (DA microsensor); RE, reference electrode; CE (AUX), counter (auxiliary) electrode.

Fig. 3. Picture and schematic diagram of three sections (1–3) of the dual channel microdialysis tube used in this study. $C^{1,2}$, microdialysis capillaries; $I^{1,2}$, inlet tubes; $O^{1,2}$, outlet tubes; $A^{1,2}$, aspiration tubes; $L^{1,2}$, loading tips.

F-TECs having similar DA responses were selected and used for each dual channel system.

Capillary tube construction for in vitro microdialysis

The capillary tube for microdialysis of PC12 cells (Fig. 3) is an adaptation of an in vitro device described previously [6–8]. The microdialysis probe was constructed using two sections of plastic-coated silica tubing (150 μm o.d., 75 μm i.d., Scientific Glass Engineering, Milton Keynes, UK), each placed in the center of a semipermeable polyacrylonitrile dialysis fiber (AN-69, Hospal Industrie, Meyzieu, France). Each semipermeable membrane had an active length of 40 mm. Then each section of plastic-coated silica tubing was positioned in the center of polyethylene tubing (0.58 mm i.d., 35 mm long, Portex). The two sections of silica tubing served as inlets. Dialysates from each polyacrylonitrile dialysis fiber were collected from polyethylene tubing, which served as outlets. Two more sections of plastic-coated silica tubing (40 mm long) were sealed outside the polyethylene tubing for loading the PC12 cell suspension in two separate dialysis chambers. All parts
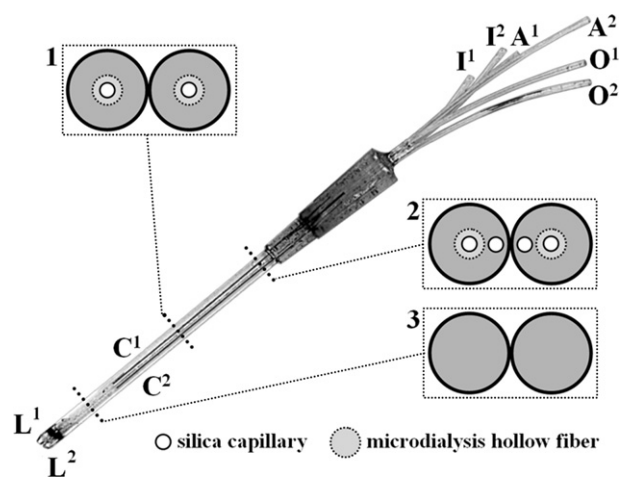
were coated with quick-drying epoxy glue. After that, microdialysis probes (semipermeable polyacrylonitrile dialysis fibers plus sealed plastic-coated silica tubing) were placed in two nonheparinized microhematocrit capillary tubes (7.5 mm long, 1.1 mm i.d., Chase Scientific Glass, Rockwood, IL, USA). The final volume of each microdialysis chamber was approximately 50 μl. Two distinct inlets and outlets allowed separate dialysate sample collection and separate pharmacological treatments. Two simultaneous and independent experiments could be performed at the same time using two samples of the same original PC12 cell suspension.

Drug reservoir and fluid mixer

A 50-μl drug reservoir (DR) (Fig. 4, bottom left schematic) was assembled using a portion of a 100-μl Hamilton micropipette tip and a 250-μl microcentrifuge tube (Alpha Laboratories). As shown in Fig. 4, the liquid normally flows in the DR using the main circuit (inlet 1 and outlet 3); an auxiliary circuit (inlet 2 and outlet 4) permits a change in the DR content by acting as injector. DR inlets and
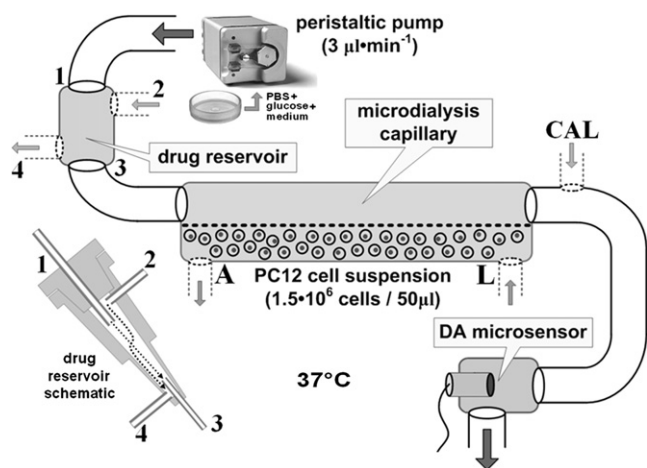
Fig. 4. Single channel schematic of the microfluidic system used in this study. A peristaltic micropump moves a PBS-based solution in the capillary tube passing through a drug reservoir (1→3) and carrying the drug loaded in it using an auxiliary circuit (2→4). PC12 cells were loaded (L) in the microdialysis chamber by aspiration (A). A complete electrochemical cell, including a DA microsensor, is connected at the end of the circuit for online monitoring of DA secreted from PC12 cells. Direct DA microsensor calibration was performed using the CAL input.

outlets were constructed by cutting four needles: three having an external diameter of 22 G (1, 2, and 4 in Fig. 4) and one having an external diameter of 27 G (3 in Fig. 4). This technical detail facilitates DR loading with a drug-containing solution and ensures minimal impact on microdialysis tube microfluidics. A fluid mixer (FM) (a and b in Fig. 5, top left schematic) was made using one 23-G needle (a) and 15 mm of plastic-coated silica tubing (b, CAL input in Fig. 4) (150 μm, o.d., 75 μm i.d., Scientific Glass Engineering). The needle was directly connected to the outlet of the microdialysis tube. Two DRs and two FMs were made for each dual channel system.

Fig. 5. Dynamic DA microsensor calibration performed bypassing the microdialysis tube. A microperistaltic pump and an injection pump, loading 1 μM DA in PBS, were directly connected to the F-TEC using a miniaturized fluid mixer (FM). The calibration was performed with nine DA injections lasting 2 min each. Before and between injections, only the microperistaltic pump was active, running at 3 μl/min. As shown in the top left inset graph, during DA injections (1–9, at arrows), a fixed flux of 3 μl/min (100%, FM, c) was maintained, decreasing the microperistaltic flux (−0.333 μl/min/injection, FM, a) and increasing the injection pump flux (+0.333 μl/min/injection, FM, b) and the DA concentration (+0.111 μM/injection). A total of 6 μl of DA-containing PBS reached the electrochemical cell after each 2-min injection (DA, 0–1 μM).

Cell culture

PC12 cells were maintained at 37 °C in 60-mm plastic culture plates in a humidified atmosphere of 5% CO_2/95% air as described previously [19]. The formulation of the culture medium was as follows: Dulbecco's modified Eagle's medium (DMEM)–F12 supplemented with 10% horse serum and 5% fetal calf serum. After short-term culture [20] (15–20 passages), the cells were washed twice using 2 ml of modified PBS and DMEM (10%), harvested by trituration, and centrifuged (94g for 5 min). Cells were resuspended in PBS–DMEM; thereafter, the number of cells/50 μl was assessed by trypan blue in a Burker chamber. The initial volume of the cell suspension was eventually adjusted to reach a final concentration of between 2.5×10^5 and $1.5 \times 10^6/50$ μl.

Experimental procedure

The experimental apparatus was assembled (Fig. 4), and currents were recorded until a stable baseline was obtained. DA microsensor calibration was performed before and after each experiment using the CAL inlet (Fig. 4) and following the procedure illustrated in Fig. 5. The cellular microdialysis tube was perfused with PBS–DMEM by means of a peristaltic microinfusion double channel pump (P720 peristaltic pump, Instech, Plymouth Meeting, PA, USA) at a flow rate of 3.0 μl/min. PBS–DMEM was kept in a 60-mm plastic culture plate in an IR incubator at 37 °C in a humidified atmosphere of 5%CO_2/95% air. The pump channels were connected to the inlets by a length of polyethylene tubing. Each of the two microdialysis chambers (C1 and C2 in Fig. 3) was then loaded with 50 μl of PC12 suspension. The cell aspiration was performed manually by means of two 1.0-ml syringes connected to the plastic-coated silica tubing sealed outside the polyethylene tubing (1 in Fig. 6A). Thereafter, the perfusion apparatus was kept at 37 °C and DA currents were recorded for the entire time of the experiment. The DRs were loaded with KCl 120 min after cell loading (2 in Fig. 6A).

Chromatographic analysis of dialysates from PC12 cell suspension

DA was quantified in dialysates of selected experiments (1.5×10^6 cells) by HPLC–EC, as described previously [6–8,19], using an Alltech 426 HPLC pump (Alltech, Sedriano, Italy) equipped with a Rheodyne injector (model 7725, Rohnert Park, CA, USA), a column (15 cm × 4.6 mm i.d., ODS80TM C18, Toso Haas, Stuttgart, Germany), an electrochemical detector ANTEC–Leyden EC controller (ANTEC, Zoeterwoude, The Netherlands), and a PC-based ADC system (Varian Star Chromatographic Workstation, Varian, Walnut Creek, CA, USA). The mobile phase was citric acid (0.1 M), ethylenediaminetetraacetic acid (EDTA, 1.0 mM), MeOH (8.7%), and sodium octylsulfate (48 mg/L), with a flow rate of 1.2 ml/min and pH 2.9.

Assessment of cell viability

Trypan blue exclusion cell viability was assessed twice in the same PC12 suspension: before filling the capillary apparatus and at the end of each experiment (6 h after PC12 cell loading into the microdialysis tube). For each experiment, the viability rate was given as the difference between final and initial percentages of nonviable cells and as the difference between nonviable cells in each microdialysis chamber.

Statistical analysis

DA oxidation currents were expressed in picoamps (pA) and given as means ± standard errors. Concentrations of DA were expressed in micromoles (μM). After quiescent DA microsensor
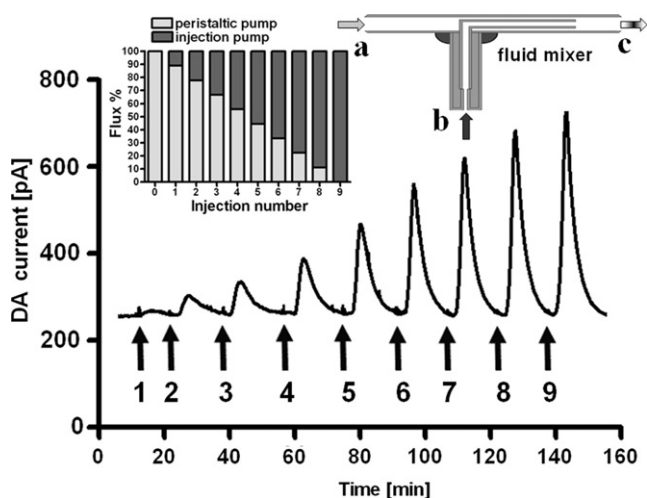
calibrations, linear regression was calculated between oxidation current and injected DA concentrations. Following dynamic DA microsensor calibrations and PC12 cell experiments, linear regression was performed using DA current values (peak height). Pearson's correlation coefficient was used to compare the correlation between the PC12-loading DA current and the KCl-induced DA current. The null hypothesis was rejected when $P < 0.05$.

Results

Quiescent and dynamic DA microsensor calibration

All in vitro calibrations of DA microsensors were performed at 37 °C starting 24 h after F-TEC manufacturing and were repeated daily for up to 7 consecutive days. The system response to DA was tested in quiescent conditions by connecting each channel of the DCP to the outlet section of F-TEC comprising a DA microsensor and reference and counter electrodes (Fig. 2B). A 25-ml Teflon beaker was adapted as described in a previous article [16] and was used for calibration. Before the calibration procedure, the F-TEC-B was placed in the PBS-containing beaker and a constant potential of +250 mV versus Ag/AgCl was applied. The calibration apparatus was placed on a stirrer until a stable baseline was reached. A 32-mm-diameter magnetic bar was used to mix the solution after each addition of dopamine, and the resulting oxidation current was recorded after stirring. DA calibration in quiescent conditions (data not shown) displayed excellent linearity with a slope of 643 ± 17 pA•μM^{-1} ($r^2 = 0.997$, $n = 6$). After 6 days, sensitivity decreased to 425 ± 56 pA•μM^{-1}, maintaining good linearity ($r^2 = 0.986$, $n = 6$). Dynamic calibration of DA microsensors (Fig. 5) was performed under stable flux conditions (3 μl/min) connecting the peristaltic pump and the microinjection pump to the FM. The linear regression was calculated using the DA peak height as current values (after each DA injection), with sensitivities of 553 ± 21 pA•μM^{-1} ($r^2 = 0.989$, $n = 6$) and 364 ± 46 pA•μM^{-1} ($r^2 = 0.997$, $n = 6$) being obtained on days 1 and 7, respectively. For a detailed description of the dynamic calibration procedure, refer to figure legend of Fig. 5.

Effect of different DA concentrations in the microdialysis chamber and perfusion flux changes on DA current

In a first series of experiments performed 1 day after F-TEC construction, PBS solutions, containing different concentrations of DA, were loaded in the microdialysis chamber simulating a PC12 cell aspiration. DA currents, after a sequence of three consecutive loadings (250, 500, and 1000 nM), changed as described in Fig. 7A. The linear regression was calculated using the DA peak heights as current values (after each DA aspiration) and obtaining sensitivity of 464 ± 44 pA•μM^{-1} ($r^2 = 0.982$, $n = 6$). In a second series of experiments, three consecutive fillings of the microdialysis chamber were performed with a PBS solution containing DA (250 nM) at different perfusion regimens: 6 μl/min, 3 μl/min, and 1.5 μl/min. The perfusion flux was changed before each DA aspiration, resulting in a variation of the DA oxidation peak height, as illustrated in Fig. 7B. The linear regression was calculated, and a decrease in DA sensitivity of 29 ± 9 pA ($r^2 = 0.906$, $n = 6$) was observed for every microliter/minute increase in perfusion flow rate. A flux of 3 μl/min was selected to perform experiments with PC12 cells.

Effect of PC12 cell number on DA secretion

After three DA loadings and linear regression analysis (as described in the previous paragraph), the microdialysis chamber was rinsed with bidistilled water and PC12 cells were loaded as described in Materials and Methods (1 in Fig. 6A). Several experiments were performed, loading different numbers of cells
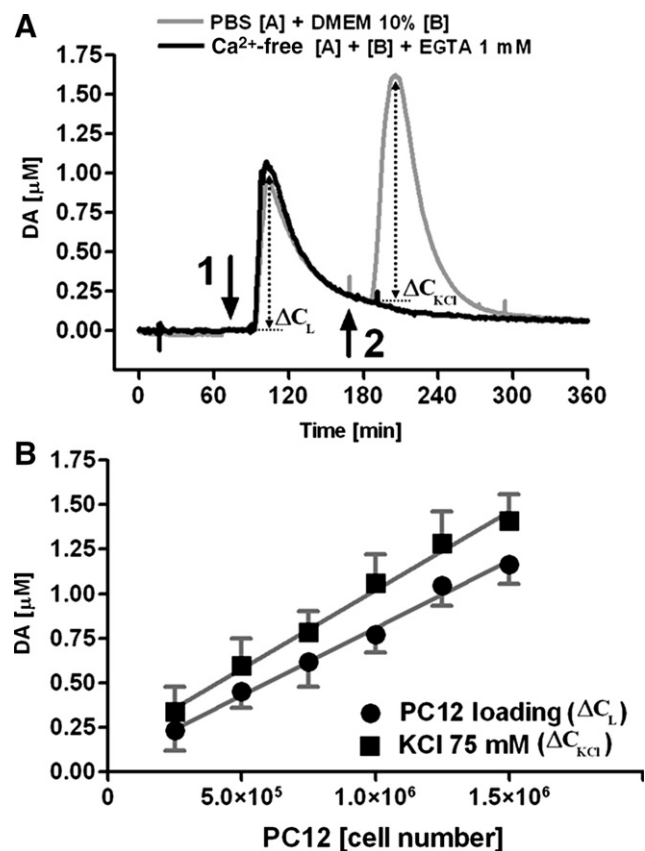

Fig. 6. (A) DA variations for 1.5×10^6 PC12 cell loading (ΔC_L, 1) and KCl (75 mM) injection (ΔC_{KCl}, 2) recorded simultaneously in normal and Ca^{2+}-free perfusion fluids (light and dark gray curves, respectively). (B) ΔC_L and ΔC_{KCl} plotted versus PC12 cell number ($n = 3$ for each cell number) (see text).

ranging from 2.5×10^5 to 1.5×10^6/50 μl. Then, 7 min after cell loading, a DA peak appeared (Fig. 6A) with a calculated concentration (ΔC_L) proportional to the number of PC12 cells in the suspension. ΔC_L was determined after each experiment, using the linear equation obtained from DA aspirations, and plotted versus PC12 cell number (Fig. 6B). Linear regression yielded sensitivity of 0.754 ± 0.033 pM (5.76 ± 0.25 fg) per single PC12 cell loaded in the microdialysis chamber ($r^2 = 0.992$, $n = 6$). As illustrated in Fig. 6A, no differences in ΔC_L were observed in either the presence or absence of Ca^{2+} in the perfusion fluid. Chromatographic analysis of dialysates showed a maximal initial DA concentration of 1.188 ± 0.037 μM/1.5×10^6 cells and 0.136 ± 0.023 μM/1.5×10^6 cells 120 min after PC12 loading, confirming previously reported data [7].

Effect of KCl on DA secreted from PC12 cells in either the presence or absence of extracellular Ca^{2+}

The dual channel microdialysis apparatus was perfused using modified PBS in either the presence (channel 1) or absence (channel 2) of CaCl$_2$. KCl (75 mM) was injected in both DRs (2 in Fig. 6A) 90 min after PC12 loading, and a DA secretion peak appeared 8 min later only in the presence of extracellular Ca^{2+} (Fig. 6A). The DA peak had a calculated concentration (ΔC_{KCl}) proportional to PC12 cell number and extracellular KCl and CaCl$_2$ (data not shown). ΔC_{KCl} was plotted versus PC12 cell number (Fig. 6B), and linear regression was calculated with a resulting DA concentration of 0.88 ± 0.04 pM (6.7 ± 0.3 fg) per single PC12 cell in the microdialysis chamber ($r^2 = 0.991$, $n = 3$). HPLC analysis of dialysates con-
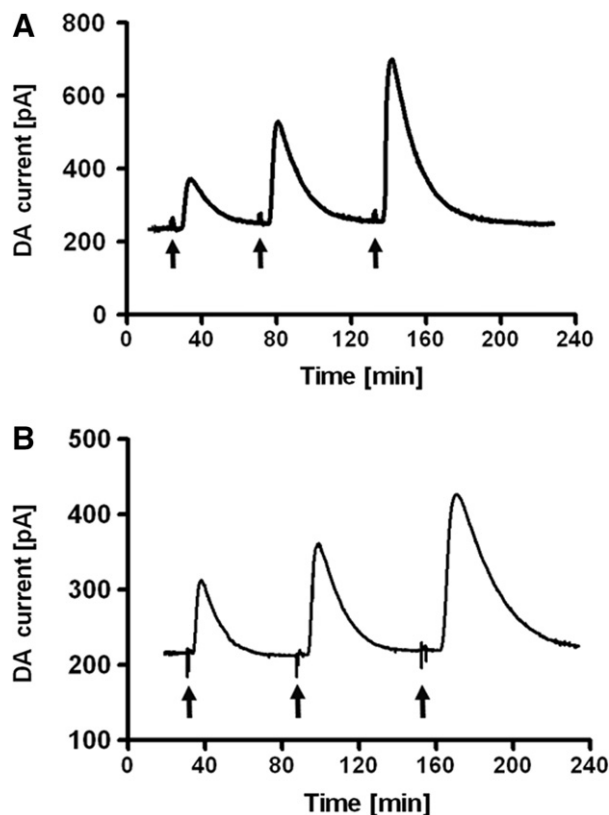
Fig. 7. DA current after a series of three consecutive loadings (at arrows) of PBS solution containing DA at different concentrations (250, 500, and 1000 nM) and a fixed perfusion flow rate (3 µl/min) (A) or containing a fixed DA concentration (250 nM) and different perfusion flow rates (6, 3, and 1.5 µl/min) (B). Aspirations in the microdialysis tube were performed as described for PC12 cell suspension loading (see text) using a 1-ml Hamilton syringe.

Fig. 8. Microphotographs of PC12 cells before (A) and 6 h after (B) loading in the microdialysis tube (75 mM KCl infusion). PC12 cells were gently aspirated in the tube as small clusters or single cells (A) using a 1-ml Hamilton syringe. At the end of the experiments, PC12 cells were found to aggregate in big clusters. However, no significant decrease of cell viability (>96%) was observed.

firmed a maximum DA concentration increase of $1.269 \pm 0.044\ \mu M/1.5 \times 10^6$ cells 40 min after KCl injection [7]. Using Pearson's correlation coefficient, a significant correlation was found between ΔC_L and ΔC_{KCl} mean values ($r = 0.9953$, $r^2 = 0.991$, $P < 0.0001$, xy pairs = 6). No K^+-evoked DA secretion was observed in the absence of extracellular Ca^{2+} (Fig. 6A).

PC12 cell viability before and after microdialysis experiments

The assessment of cell viability was performed as described in Materials and Methods. After an initial preparation, the number of viable cells was assessed by trypan blue in a Burker chamber (initial viability was $97.5 \pm 2.7\%$, $n = 36$). PC12 cells were gently aspirated in the microdialysis tube as small clusters or single cells (Fig. 8A). At the end of microdialysis experiments, PC12 cells were found to aggregate in big clusters (Fig. 8B). However, no significant decrease of cell viability was observed (final viability was $96.2 \pm 3.4\%$, $P < 0.05$, $n = 32$).

Discussion

Microelectronics and DA microsensor performance

The amperometric module of the DCP was optimized for single-supply, low-voltage operation by distributing the gain between the first and second stages of amplification. Compared with our previous device [17], the gain was distributed between the I/V converter and differentiator. As shown in Fig. 1, fixing Rf to 10 MΩ, a precision differentiator/amplifier (AD626) controlled the amplification factor (1 nA/V). In this manner, we successfully overcame the lim-
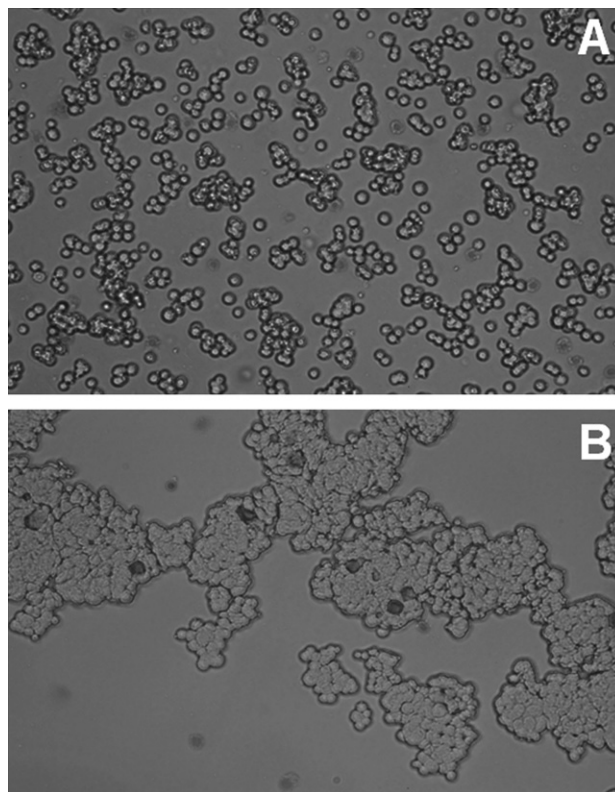
itations of the previous DCP design [17] related to variations in the resistor values, mainly in the difference circuit. The result is that the module is characterized by gain precision, stability, and an excellent linear response. The system can operate only in oxidation mode, and it is particularly suited to work with direct oxidation sensors such as DA microsensors and biosensors with H_2O_2 detection [21]. DA microsensor characteristics were evaluated for up to 3 weeks in vitro, showing good accuracy and precision. DA secreted from PC12 cells is metabolized by catechol-*O*-methyl-transferase (COMT) to 3-MT [7]; however, no significant interferences were observed on exposing DA sensors to 3-MT. Indeed, this O-methylated metabolite is oxidized at a potential of +625 mV versus Ag/AgCl [15], much higher than DA. A decrease in sensitivity (44% under quiescent conditions and 45% under dynamic conditions) compared with initial values (day 1) was measured at day 7 and was attributed to microsensor aging. As a result, the symmetry between the two microsensors used in each experiment was maintained for the entire period of testing (7 days). Small baseline differences between sensors were further corrected by software (offset) 60 min before each experiment. F-TECs can be used for 1 week without problems, performing calibrations daily, immediately before experiments, as shown in Results. After 1 month of continuous operation, we observed a reduction in sensitivity (67% under dynamic conditions vs. day 1) and an increase, sometimes unpredictable, in the asymmetry between the F-TECs, most likely related to RE decline or WE Nafion coating damage.

DA secretion from PC12 cells

As shown in Materials and Methods as well as in Results, dynamic DA calibration, performed after aspiration of different con-

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centrations of DA in the microdialysis chamber, was used to estimate DA secreted from PC12 cells into the extracellular space. An initial overflow of DA was observed after PC12 cell loading, with a maximal concentration above $1 \mu M/1.5 \times 10^6$ cells using a perfusion flux of $3 \mu l/min$. DA concentrations declined progressively from both outlets during the first 80 to 120 min of PBS–DMEM perfusion. This initial DA peak is related mainly to cell manipulations before loading. Indeed, mechanical stress of PC12 during preparation procedures resulted in an increase in DA overflow. DA clearance from the extracellular fluid has been linked to perfusion flux or rapid metabolism to 3-MT by COMT [6–8] rather than PC12 uptake. Perfusion with the dopamine transporter (DAT) selective inhibitor GBR-12783 did not affect DA clearance time (data not shown). DA then reached a steady-state level with a concentration below $0.15 \mu M/1.5 \times 10^6$ cells. KCl exposure, in either the presence or absence of extracellular Ca^{2+}, induced DA secretion proportional to extracellular K^+ and Ca^{2+} but also PC12 cell number. Because of the above-mentioned observations, ΔC_L and ΔC_{KCl} could be used to roughly calculate the number of PC12 cells loaded in the microdialysis chamber. These results confirm previous HPLC-based in vitro findings [7] in terms of both DA concentration and secretion time course. Microdialysis tubing can be used continuously over 24 h without reduction in extraction properties. Unfortunately, PC12 loading can be performed only once because of initial cell adhesion and dialysis membrane fouling. This necessitates the use of a new microdialysis tube for each experiment, whereas the same F-TECs can be used for up to 1 week. Differences in microdialysis tube performance, related to construction procedures, were eliminated by performing calibrations daily, immediately before experiments, as outlined in Materials and Methods. A limitation of the system, related to the use of the microdialysis technique, is that temporal dynamics of DA secretion appear to be slower than that resulting from carbon fiber microsensors placed adjacent to individual PC12 cells [13,14]. However, in comparison with a previous in vitro microdialysis study [5], the small volume of the dialysis chambers and the high number of cells contained in them have the effects of reducing the volume of the extracellular compartment, increasing the concentration gradient of DA through the microdialysis membranes, and increasing the system response time to DA secretion.

PC12 cell viability

No significant decrease in cell viability was observed at the end of the experiments, as reported in Results. In selected control experiments, PC12 cells were left inside the microdialysis chamber for up to 12 h in standard perfusion conditions ($3 \mu l/min$) without a significant decrease in cell viability. Only after 24 h was the viability reduced to less than 95%.

Conclusions

We have presented a novel dual microdialysis in vitro system coupled with DA microsensors for the flow-through detection of DA secreted from PC12 cells. A simultaneous treatment–control experimental protocol was performed to detect K^+-evoked DA secretion. The microdialysis system was calibrated and tested under different experimental conditions. This novel apparatus serves as a rapid and reliable model for studying the effects of different drugs on PC12 cell DA secretion. Different cell types might be studied, and DA microsensor could be easily substituted with different microsensors or biosensors, allowing the in vitro detection of other interesting molecules such as nitric oxide, glutamate, glucose, and lactate. The further miniaturization of the electronics and the integration with ultra miniature peristaltic pumps could permit the realization of a low-cost miniaturized device for rapid drug screenings in vitro.

Acknowledgments

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