Real-time measurement of PMA-induced cellular alterations by microelectrode array-based impedance spectroscopy

Andrée Rothermel, Matthias Nieber, Jana Müller, Peter Wolf, Maik Schmidt, and Andrea A. Robitzki

University of Leipzig, Leipzig, Germany

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For a feasible and cost-effective impedance measurement of cellular alterations in real-time, we combined commercially available microelectrode arrays (MEAs), consisting of 60 microelectrodes, with a conventional impedance analyzer. For proof of principle, a breast carcinoma cell line (MCF-7) was cultured on MEAs, and cellular alterations were measured by impedance spectroscopy at a frequency ranging from 10 Hz to 1 MHz. Cells were stimulated with phorbol 12-myristate 13-acetate (PMA) at different concentrations to activate protein kinase C (PKC)-mediated extra- and intracellular changes. By addition of 0.03 μ M PMA, an increase of the relative impedance (Z_{rel}) was observed after 10 min with a maximum at 1 kHz. Moreover, a gradual elevation of the impedance was measured 60 min after stimulation with PMA. If 0.3 μ M PMA was applied, the maximal amplitude of the relative impedance after 60 min shifted from 1 kHz (0.03 μ M PMA) to 150 Hz. Subsequently, the impedance was further increased up to 90 min after PMA application, after which the impedance reduced after 240 min. Since we could use MEAs for at least 10 times without affecting the sensitivity, our study revealed that commercially available MEAs comprising nanocolumnar titanium nitride electrodes are suitable microstructures for a highly reproducible and cost-effective multisite measurement of intracellular processes by impedance spectroscopy.

INTRODUCTION

One of the most exciting challenges for basic and applied sciences is measuring cellular properties under noninvasive conditions in real-time. In this respect, impedance spectroscopy represents a promising tool, since this technique fulfills the requirements for a nondestructive, real-time measurement of almost all living cells and tissues (1). For impedimetric recordings, an alternating current can be applied to single cells or complex tissues. The current flows from a working electrode through the medium—cell and/or tissue—to a counter electrode with which the cell itself behaves as a frequency-dependent resistor and capacitor. Depending on the applied frequency, alterations of intra- and extracellular properties or processes (e.g., apoptosis, necrosis, proliferation, and migration of cells) can be measured via the changed cellular impedance (2–5). However, the

bottleneck for applying this technique to a broad scientific and pharmaceutical community is the limited commercial availability of cost-effective, feasible, and reliable chip-based sensors with embedded microelectrodes for impedimetric measurement of living cellular systems. To overcome this problem, we used commercially available multi-electrode arrays (MEAs) and combined these with an already existing impedance analyzer. In principle, MEAs are composed of 60 substrate-embedded microelectrodes, predominantly consisting of noble metals such as gold, titanium, or platinum (i.e., materials that have been commonly used for traditional impedance spectroscopy). Since MEAs have been originally developed for extracellular recording of electrically active cells, all components of the chip are tested for biocompatibility; therefore cytotoxic effects can be excluded (6,7). To test whether MEAs can be used for impedance measurement of cellular

alteration, MCF-7 cells derived from human breast cancer cells were cultured as a confluent monolayer on the microelectrodes. MCF-7 cells were chosen since they (i) have already been used for impedance measurements (8) and (ii) represent a well-suited model system for inducing various morphological alterations caused by activation of intracellular signaling pathways, especially in terms of phorbol 12-myristate 13-acetate (PMA)-induced protein kinase C (PKC) signaling pathways (9–13). Here, we demonstrated the successful use of commercially available multimicroelectrode arrays for feasible, reliable, and cost-effective impedimetric measurements of PMA-induced intracellular changes.

MATERIALS AND METHODS

Design of Multielectrode Arrays

Each MEA (purchased from Multi Channel Systems, Reutlingen, Germany) consists of 60 planar round nanocolumnar TiN microelectrodes with a diameter of 30 μ m and an electrode distance of 200 μ m (Figure 1, A–C). The microelectrodes are arranged in an 8 $\times$ 8 array on a glass substrate and have been passivated with silicon nitride. A large electrode on the edge of the array serves as counter electrode. To maintain stable culture conditions during measurement, the chip containing the MEA adapter was placed into a CO₂ incubator (Figure 1D).

Cell Culture on the Chip

For impedance measurement, 5×10^5 MCF-7 cells were resuspended in 1000 μ L culture medium [RPMI, 10% fetal calf serum (FCS), 1% glutamax, 1% penicillin/streptomycin, 1 mM sodium pyruvate, and 10 μ g/mL insulin] and transferred into the chip reservoir. Cells were maintained under standard culture conditions (37°C, 5% CO₂, and 95% air). Attachment and growth of cells were monitored by light microscopy and impedance spectroscopy.

Experiments were not started until cultures reached a confluent stage to

Figure 1. Multielectrode arrays (MEAs) and measuring setup. (A) The microelectrodes in the recording field are arranged in the middle of the culture reservoir. (B) Phase contrast image of cultured MCF-7 cells on a planar multielectrode array with 60 substrate integrated microelectrodes, each with a diameter of 30 µm and an interelectrode distance of 200 µm. (C) Magnification of panel B shows the nanocolumnar structure of TiN electrodes (insert shows a SEM of nanocolumnar structured electrodes; scale bar = 500 µm) and electrodes covered by confluent cells. (D) Measuring setup of for impedance spectroscopy. The MEA chip is placed in the MEA-adaptor to allow the triggering of all electrodes by the biological interface, impedance analyzer, and computer. To ensure stable condition during impedance measurement, the complete MEA adaptor including the chip is located in a CO₂ incubator at 37°C. Inset of panel C by courtesy of Thoams Meyer, Multi Channel Systems, Reutlingen, Germany.

ensure that almost all 59 electrodes—with the exception of the counter electrode—were covered by cells. Medium was changed every 2 days. Twenty-four hours prior to measurements, medium was replaced by serum-free medium (RPMI, N2 supplement, 1% glutamax, 1% penicillin/streptomycin, 1 mM sodium pyruvate, and 10 µg/mL insulin) to avoid nonspecific effects caused by unknown serum components. For repeated use, cells were removed from the chip by tryptic digestion (0.25% trypsin/0.04% EDTA) overnight and the MEA was sonicated for 10 min in a 2% ultrasonol solution, pH 7.0. Thereafter, MEAs were extensively rinsed with distilled water and sterilized by autoclaving at 121°C for 20 min. Before MCF-7 cells were cultured

on MEAs, the chip surface was coated with laminin [5 µg/mL phosphate-buffered saline (PBS)] for at least 2 h at 37°C or overnight, respectively.

Impedance Measurement and Data Acquisition of Living Cells

Impedance spectroscopy was carried out by using a Solartron 1260A impedance analyzer and a Solartron 1294 biomedical interface (Solartron Analytical, Hampshire, UK), as well as a MEA adapter board (self-modified electronic board originally purchased from Multi Channel Systems). The latter is used to connect all 60 electrodes of the MEA chip with the impedance analyzer (Figure 1). For impedance analysis of MCF-7 cells, an alternating current of 10 mV was applied to the electrodes. The voltage drop between the detection electrode and the counter electrode was measured at 30–40 different sites within a frequency range of 10 Hz to 1 MHz. For PMA experiments, approximately 24 h before applying PMA, the culture medium was replaced by serum free medium (RPMI, N2 supplement, 1% glutamax, 1% penicillin/streptomycin, 1 mM sodium pyruvate, and 10 µg/mL insulin). The next day, impedance of MCF-7 cells was monitored, and PMA was not added until cultures showed a stable impedance over 3 h. Thereafter, 10 µL dimethyl sulfoxide (DMSO) in a final concentration of 10⁻⁸ M were added to the MCF-7 cultures to exclude nonspecific changes due to the solvent of PMA. PMA was added at various concentrations, and impedance was measured for up to 4 h. Scanning of the complete frequency range took 60–90 s. For data acquisition and data processing, the impedance analysis software SMaRT 2.3 (Solartron Analytical) was used. To point out alterations after application of PMA, the measured impedance $|Z|$ was normalized and expressed as relative impedance $|Z|_{rel}$, where $|Z|_b$ is the impedance value before and $|Z|_a$ after the stimulation with PMA.

$$|Z|_{rel} [\%] = \frac{|Z|_a - |Z|_b}{|Z|} \times 100$$

Statistical Analysis

Standard deviation was determined by a two-tailed Student's *t*-test. For each experiment the impedance measurement was carried out at least 10 times ($n \geq 10$). The presented data were obtained by using 15 different TiN MEAs.

Immunocytochemistry

To demonstrate that the impedance data were caused directly by the activation of PMA-mediated intracellular signaling pathways and not by a nonspecific intercalation of PMA within the cell membrane, impairment of cell-cell contacts, or by other gross morphological alterations, MCF-7 cultures were characterized by immunocytochemistry. A tight junction-specific occludin antibody was used to analyze changes in the cell membrane and in cell-cell contacts. Fluorescent-labeled phalloidin (Invitrogen, Karlsruhe, Germany), which stained exclusively F-actin filaments, was used according to manufacturer's instructions to detect changes in the cytoskeleton. For immunolabeling, MCF-7 cells were fixed with 4% formaldehyde for 30 min at room temperature. Immunofluorescence labeling was carried out as previously described (14). The rabbit polyclonal primary antibody anti-occludin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used at a concentration of 1 µg/mL. For detection of the primary antibody, a goat anti-rabbit Cy^{TM}3-conjugated secondary antibody was used at a concentration of 5 µg/mL.

Fluorescence Microscopy

Immunolabeled cells were visualized using a Nikon Eclipse TE2000 inverted microscope equipped with an EC-1 laser-scanning unit (Nikon, Düsseldorf, Germany). Photomicrographs were processed with LUCIA G (Nikon) and Adobe® Photoshop® 5.0.

RESULTS AND DISCUSSION

A commercial impedance gain-phase analyzer (Model 1260A;

Figure 2. Magnitude of the impedance of TiN electrodes and impedance of MCF-7 cells after PMA stimulation. (A) Nanocolumnar TiN electrodes ($\varnothing$ 30 μ m) showed a lower electrode impedance when compared with gold ($\varnothing$ 40 μ m) electrodes. (B) Frequency-dependent impedance spectrum of all electrodes measured without cells in culture medium. Average impedance (Z) of 59 electrodes of a single chip is shown ($\circ = 7 \times$ in use; $\square = 16 \times$ in use). (C) Impedance measurement of a chip that has been used 12 times for cultivation and impedance measurement. Impedance measurement ($n = 10$) of a single electrode over a period of 50 min with an interval of 5 min in culture medium without cells ($\circ$) and with MCF-7 cells ($\diamond$). (D) Frequency-dependent increase of the impedance in MCF-7 monolayer cultures after stimulation with 0.03 μ M PMA. (E) Relative impedance (Z_{rel}) related to the time point prior to 0.03 μ M PMA application. (F) Frequency-dependant impedance of MCF-7 cells after application of 0.3 μ M PMA. Statistical analysis is determined by a two-tailed Student's t -test. PMA, phorbol 12-myristate 13-acetate.

Solartron Analytical), combined with the dielectric interface (Model 1294; Solartron Analytical) was used for electrical impedance measurement. An overview of the measuring setup is given in Figure 1. In general, impedance was measured over a frequency ranging from 10 Hz to 1 MHz at constant culture parameters (5% CO_2 , 95% air, 37°C). The recorded impedance spectra (Figure 2, A–D) were measured between one of the 59 detecting electrodes and the counter electrode. The counter electrode is approximately 5000 times larger than the detecting electrode. Based on the significant smaller size, the detecting electrode dominates the measured impedance of the cell-electrode circuit.

First of all, we compared the impedance of the electrode-electrolyte interface of gold electrodes (frequently used for impedance recording) with nanocolumnar TiN electrodes applied in this study (see Figure 1C, inset). In Figure 2A, the impedances of gold electrodes (diameter 40 μ m; square

base approximately 1.6 μm^2) and TiN electrodes (diameter 30 μ m; round approximately 0.7 μm^2) are compared. Although the diameter of TiN electrodes is smaller than that of gold electrodes, the impedance of TiN electrodes was significantly lower. It seems very likely that the measured impedance of TiN electrodes is associated with the increased surface area that results from the nanocolumnar fabrication of TiN electrodes (Figure 1C). The average electrode impedance of all 59 electrodes of a single chip decreased as the frequency increased, whereby the overall impedance of the cell-free measurement mainly resulted from the electrode and the spread resistance of the medium. Apart from the significantly lower electrode impedance of nanocolumnar TiN electrodes, the recorded spectra showed the typical frequency-dependent decrease at high frequencies as reported in previous studies using gold, platinum, or other noble metals for impedance measurements (2,4,8,15–17). As illustrated in

Figure 2B, the variance of the overall number of electrodes was small if MEAs were used fewer than seven times, whereas the variance between all electrodes on a single chip slightly increased with the number of applications. However, if chips were used several times (10 times or fewer), the variance of a single electrode during measurement was remarkably small. If the impedance of a single electrode was measured 10 times over a period of 50 min (Figure 2C, $\circ$ = uncovered), the standard deviation of the impedance values was extremely small. Here, we observed a standard deviation of only 0.1% with respect to the average impedance measurements ($n = 10$) at 10 Hz and 100 kHz. Moreover, once the very same electrode was covered by cells and the impedance was measured every 5 min over a period of 50 min ($n = 10$), the standard deviation was 0.4% at 10 Hz and 0.04% at 100 kHz (Figure 2C, $\diamond$ = cell covered electrodes). This in turn demonstrated the outstanding properties of the nanocolumnar

Table 1. Peak Maxima of Z_{rel} of PMA-Treated MCF-7 Cultures

Time (min)	PMA (0.03 μ M)		PMA (0.3 μ M)	
	Z_{rel} (%)	Peak _{max} (kHz)	Z_{rel} (%)	Peak _{max} (kHz)
10	6	4.0	6	0.25
30	16	2.5	18	0.25
60	38	1.0	47	0.15
90	—	—	58	0.15
240	—	—	33	0.25

PMA, phorbol 12-myristate 13 acetate.

structured TiN MEAs for impedance measurements. Due to the extremely enlarged electrode-cell or electrode-electrolyte interface of nanocolumnar TiN electrodes, disturbing effects during impedance measurements are obviously minimized. This probably explains why TiN-based MEAs showed only very small variances when electrodes of the same or of different TiN chips were analyzed. For instance, the data presented in this study were obtained from more than 15 different TiN MEAs. Comparison of the impedance of cell-covered and uncovered electrodes showed that the contribution of cells to the overall impedance ranged between 100 Hz and 100 kHz, with a maximal amplitude close to 1 kHz (Figure 2C). Similar cellular impedance alterations have been reported in several previous studies (2,4,15–17).

Next, we investigated whether MEA-based impedance measurement was suited to detect PMA-induced extra- and intracellular alterations. For PMA experiments, growth and migration of MCF-7 cells were monitored every half hour over a period of 2–3 h until the cells showed a stable cellular state (i.e., showed a stable impedance spectra). This experimental strategy was necessary to exclude nonspecific effects that originate; for example, at the electrode-electrolyte and/or electrode-cell interface (electrode and tissue polarization). Therefore, all detectable alterations can be attributed to changes of intra- and/or extracellular compartments of 1–2 cells that covered a individual electrode. More importantly, for control experiments, the appropriate volume of PMA solvent (DMSO in a final dilution of 10^{-8} in

medium) was added to cultures, and the impedance was measured at least for 2 h. If no changes were observed (and thus no electrode impedance polarization could be detected), PMA was applied in a final concentration of 0.03 μ M, and impedance was measured after 10, 30, and 60 min (Figure 2, D and E). Here, alterations in cellular impedance were observed in a range of 100 Hz to 100 kHz, which was consistent with the contribution of cells to the measured impedance (cf. Figure 2C). Figure 2D showed an increase of impedance after 10 min, followed by further elevation 30 and 60 min after PMA addition (Table 1). To highlight changes in impedance, we used the normalized impedance Z_{rel} , which describes the relative impedance change after stimulation with PMA at different points in time (Figure 2, E and F, and Table 1).

After application of PMA, three major effects were observed: (i) Z_{rel} gradually increased within 60–90 min; (ii) the peak maximum was slightly shifted to lower frequencies during the time after PMA application; and (iii) at high PMA concentrations (0.3 μ M), cellular alterations were detected in a significantly lower frequency range in comparison to cells stimulated with 0.03 μ M PMA. The relative impedance changes after stimulation with 0.03 μ M PMA is shown in Figure 2E and Table 1. Here, Z_{rel} increased 10 min after PMA application by 6% with a maximal amplitude at 4 kHz, while after 30 min the impedance increased to 14% with a peak maximum at 2.5 kHz. Subsequently, the maximal alteration of Z_{rel} (38%) was detected after 60 min at 1 kHz. A similar effect was observed on MCF-7 cells that were stimulated with 0.3 μ M PMA (Figure 2F and Table 1). According to PMA-mediated PKC activation, we monitored cellular impedance and detected alterations of Z_{rel} of 6% at 10 min, 18% at 30 min, 47% at 60 min, 58% at 90 min, and 33% at 240 min. Comparison of data with 0.03 and 0.3 μ M PMA showed, as we expected, a similar time-dependent shift of the maximal amplitude to lower frequencies. Thirty minutes after PMA application, the peak maximum was detectable at 250 Hz, whereas during 60–90 min, a shift to 150 Hz occurred. Along with the general decline of Z_{rel} ,

Figure 3. Immunocytochemical analysis of MCF-7 cell morphology after PMA stimulation. MCF-7 cells were stained either with an anti-occludin antibody to visualize tight junctions (panels A–D) or with tetramethyl rhodamine isothiocyanate (TRITC)-phalloidin to stain actin filaments (panels E–G). The z-section (6 μ m) of MCF-7 cultures represents the distribution of tight junctions in the z-axis. Scale bar, 50 μ m. PMA, phorbol 12-myristate 13-acetate.

the peak maximum went back to the initial value of 250 Hz after 240 min. In respect to the overall impedance, a complete recovery of Z_{rel} down to the original level before PMA stimulation was not achieved.

Comparison of MCF-7 cells treated with 0.3 and 0.03 μM PMA revealed that the increase of Z_{rel} during the first 30 min after PMA stimulation was nearly identical (Table 1). Here, Z_{rel} increased to 6% after 10 min in cells treated with either 0.3 or 0.03 μM PMA. At 30 min, Z_{rel} was 16% for 0.03 μM PMA and 18% in the case of 0.3 μM PMA. In contrast to 0.03 μM PMA, a slightly increased Z_{rel} was measured after 60 min in cultures treated with 0.3 μM PMA. However, the most important observation is that the peak maximum Z_{rel} shifted to lower frequencies in terms of temporal- and concentration-dependent behavior of the cellular impedance after PMA stimulation. For instance, the peak maximum of Z_{rel} shifted from 4 to 1 kHz in the presence of 0.03 μM within 60 min, whereas a shift from 0.25 to 0.15 kHz was observed in the presence of 0.3 μM PMA (temporal-dependent impedance shift at a given concentration). Moreover, when Z_{rel} was compared directly to both concentrations, it was obvious that the major cellular contribution to impedance changes ranged between approximately 0.1–10 kHz at 0.03 μM PMA, while Z_{rel} at 0.3 μM was represented only at frequencies between approximately 0.01–1 kHz (Figure 2, E and F) (concentration-dependent impedance shift). To the best of our knowledge, such a phenomenon has not been described so far. At frequencies smaller than 1 kHz, the impedance of a cellular system is predominately characterized by a current flow around the cell and thereby represents the properties of the extracellular space. At high frequencies, the current also flows through the inner cell. In consideration of the concentration-dependent impedance shift of the two different PMA concentrations (Figure 2, E and F), we assume that PMA at low concentrations induces modifications in both the extracellular and intracellular space (peak maximum 1–4 kHz), whereas at high PMA concentrations the effect seems to be restricted exclusively to the

extracellular space (peak maximum, 0.15–0.25 kHz).

To correlate the impedance data with possible PMA-induced morphological changes, PMA-stimulated and nonstimulated MCF-7 cultures were stained with either an occluding-specific antibody to visualize tight junctions (Figure 3, A–D) or with tetramethyl rhodamine isothiocyanate (TRITC)-phalloidin to detect actin filaments of the cytoskeleton (Figure 3, E–G). As shown in Figure 3, A–D, occludin was found in the cell membrane where it is enriched in areas of the zonula occludens. When nontreated (Figure 3A) and treated (Figure 3, B–D) MCF-7 cultures were compared 60 min after PMA stimulation, no differences in the occludin staining patterns were observed. Moreover, z-sections of MCF-7 cells of 6 μm (at the bottom of Figure 3, A–C) showed intensely stained stripes representing occludin of tight junctions. Since the staining patterns of both stimulated and nonstimulated cultures were nearly identical for up to 4 h after PMA stimulation (data not shown), we assumed that PMA did not affect an impairment of tight junction on the morphological level at least during the very early period of PMA induction. However, when MCF-7 monolayer cultures were morphologically analyzed 24 h after PMA stimulation, gross alterations in the occludin expression were observed (Figure 3D). Here, the narrow band of occludin normally localized between cells (Figure 3, A–C) appeared more enlarged. Thus, these morphological changes occurred at late stages and probably were caused by an increased expression of occludin or eventually by an impairment of tight junctions that weakened the cell-cell contacts. Both possibilities have been reported in previous studies using phorbol ester for analyzing the role of occludin in epithelial barrier function (18–21).

To investigate intracellular alterations, actin stainings of control (Figure 3E) and PMA-stimulated cultures (Figure 3, F and G) were carried out. Here, no morphological alterations or impairments of the cytoskeleton were detected within the complete measurement period of 4 h and even after 24 h (data not shown). Although

a short-term effect on the cellular morphology has been described for porcine choroids plexus epithelial cells at high PMA concentrations (18), we could not observe such effects on MCF-7 cells.

All this brings up the question: how can the observed alterations of cellular impedance be explained, although apparently no gross morphological changes in cell shape occurred within the first few hours after PMA induction? One possible explanation is that the impedance spectroscopy represents a more sensitive tool to detect early and extreme small morphological changes, as opposed to optical techniques such as confocal laser-scanning microscopy. This hypothesis is supported by a number of previous studies (18–20). Here, the application of phorbol esters to different types of epithelial cells led to a fast alteration of the transepithelial electrical resistance (TER) without these morphological changes being detected on a microscopic level.

Although our impedance measurement was highly reproducible and sensitive, one has to consider that a small number of cell-covered microelectrodes did not respond to PMA. One explanation for this phenomenon probably depends on the actual cellular stage, which means that cells in the S-phase of the cell cycle were unable to respond to PMA.

Taken together, our study demonstrates for the first time the successful use of nanocolumnar structured TiN electrodes for cell-based impedance measurements. Moreover, we could show that commercially available multielectrode arrays in combination with a commonly used impedance analyzer allowed a feasible, reproducible, and highly sensitive detection of PMA-induced extra- and intracellular changes by multielectrode array-based impedance spectroscopy (MAIS).

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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Address correspondence to Andrée Rothmel, Center for Biotechnology and Biomedicine, Division of Molecular Biological-Biochemical Processing Technology, Faculty of Biosciences, Pharmacy and Psychology, University of Leipzig, Deutscher Platz 5, 04103 Leipzig, Germany. e-mail: andree.rothmel@bbz.uni-leipzig.de

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