



An impedimetric biosensor based on PC 12 cells for the monitoring of exogenous agents

Gymama E. Slaughter^{a,b,*}, Rosalyn Hobson^{a,c}

^a Center for Biosystems and Engineering, School of Engineering, Science, Technology, Virginia State University, P.O. Box 9212, 1 Hayden Drive, Petersburg, VA 23806, United States

^b Department of Engineering, Virginia Commonwealth University, 601 West Main Street, Richmond, VA 23284, United States

^c Department of Electrical and Computer Engineering, Virginia Commonwealth University, 601 West Main Street, Richmond, VA 23284, United States

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ABSTRACT

The effect of exogenous agents on the complex impedance of PC 12 cells that were cultured to confluency on 250- μm gold microdot electrodes fabricated within 8-well cell culture biochips was studied. Surface attachment of PC 12 cells to gold microelectrodes was accomplished using cysteamine SAMs covalently derivatized with laminin. The impedimetric response of PC 12 cells that undergo calcium exocytosis in the presence of calcimycin, nifedipine, mannitol and carbachol were identified. Treatment with carbachol induces muscarinic receptor-dependent rises in free cytosolic Ca^{2+} . Experiments with calcimycin and nifedipine were carried out to clarify the relationship between these two receptor-triggered events. In particular, it is believed to mediate intracellularly the release of Ca^{2+} from non-mitochondrial stores. We also examined cellular impedance responsiveness of PC 12 cells in response to phenotypic alteration especially with regard to modulation of ion fluxes using nerve growth factor (NGF), dexamethasone and forskolin. Our results demonstrate that a change in electrophysiological behavior, such as exocytosis of cytosolic Ca^{2+} is detectable using impedance spectroscopy, and therefore support the results of impedance fluctuation to be attributed to ion-fluxes.

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1. Introduction

Traditionally, toxicity assessments involve animal studies, which is both time-intensive and costly (Zurlo et al., 1994). More importantly, the use of animals in research when alternative technologies exist may be socially objectionable. This creates opportunities for the development of cell-based, high-throughput screening techniques that may be applied to toxicity assessments and drug development. Cell-based biosensors are a key component in the development of practical methods for the screening of drugs for possible toxic side effects and for the monitoring of the effects of biochemical warfare agents; thus minimizing the use of experimental animals. Our laboratory has long-standing interest in the development of a cytotoxicity biosensor based on differentiated ES J1, neuronal stem cells and PC 12, clonal pheochromocytoma cell line (Greene and Tischler, 1976) that may emulate components of the central nervous system (Bieberich and Guiseppi-Elie, 2004).

The fundamental aspects of cell-surface interactions and electrical cell signal responses related to the improvement of neuron-to-electrode surface attachment (NESA) is utilized with the goal of building a cytotoxicity cell-based biosensor systems suitable for detecting changes in the cell membrane impedance characteristics, as previously described by Slaughter et al. (2004). NESA is important in building cell-based biosensors suitable for detecting chemically stimulated changes in cell membrane impedance, is vitally important in the performance of deep stimulating electrodes used in the treatment of Parkinson's disease and is a concern in the performance of prosthetic devices such as retinal and cochlear implants. The prerequisite for monitoring the electrical impedance of cells is to have a tight neuron-to-electrode interface, which has been demonstrated in our previous work, where electrode arrays utilizing ω -amine alkanethiols, cysteamine (CA) and 11-amino-1-undecanethiol (11-AUT) surface modification and protein derivatization techniques were developed to serve as the substrate for the culture of cell types that are dependent on adhesion for confluency and proliferation. Cell adhesion proteins, collagen, fibronectin and laminin, were covalently coupled to the aminoalkanethiol decorated gold electrodes via acid-amine hetero-bifunctional cross-linking. Cells interacting with adhesion proteins anchored via chemisorbed SAM layers form tight neuron-

* Corresponding author at: Center for Biosystems and Engineering, School of Engineering, Science, Technology, Virginia State University, P.O. Box 9212, 1 Hayden Drive, Petersburg, VA 23806, United States. Tel.: +1 804 524 8989; fax: +1 804 524 6732.

E-mail address: gslaughter@vsu.edu (G.E. Slaughter).

to-electrode and tight cell-to-cell contacts by virtue of the covalent attachment of the protein layer to the electrode. Thus, promoting adhesion of laminin to gold microelectrodes enhances neuron-to-electrode contact of PC 12 cells. The converse, observed on physisorbed protein layers, is that cells likely similarly attach to these proteins but do not establish tight neuron-to-electrode and tight cell-to-cell contacts. However, cells that become attached to the covalently immobilized proteins also become immobilized and remain anchored over the time scale of these experiments. On the other hand, cells that become attached to the physisorbed proteins continue their motility, exercising motility forces that remove these proteins from the electrode and do not lead to tight neuron-to-electrode and tight cell-to-cell contacts. Confluent layers of PC 12 cells cultured on both non-modified and SAM-modified gold microelectrodes were examined by AC impedance (50 mV p-t-p at 4 kHz). The CA SAM-modified surfaces were identified as being best suited for optimal neuron-to-electrode surface attachment using laminin. The observed stability of the impedimetric response of PC 12 cells cultured on laminin derivatized CA SAMs offers a promising approach for the development and application in cell-based sensing.

Against this backdrop, we describe here the use of PC 12 to investigate the effect of exogenous agents on their impedance profile by examining the short-duration fluctuations that result from physiological events. The PC 12 cell line has been widely used as a neuronal model system and offers many advantages over other in situ systems (Chalfie and Perlman, 1977; Greene and Rein, 1978). These cells synthesize and store neurotransmitters and exhibited polarization-induced secretion of catecholamines and acetylcholine (Greene and Rein, 1977a,b; Schubert and Klier, 1977). They can be differentiated into neuronal-like cells by exposure to nerve growth factor (NGF) and transmitter release is confined to the presynaptic terminals (Zerby and Ewing, 1996), and can be also differentiated by exposure of synthetic glucocorticoids into chromaffin cells, in which somatic transmitters release is observed (Elhamdani et al., 2000; Taylor and Peers, 1999a,b; Tischler et al., 1983). In addition, exogenous agents modulate the activity of calcium channels and may cause substantial influence on the course of neuronal growth and differentiation. Thus, for calcium ions to work as a messenger, conditions in which there are a dynamic balance between the processes leading to a rise in intracellular calcium, $[Ca^{2+}]_i$ and those restricting this rise must exist. The loss of regulation of cytosolic $[Ca^{2+}]_i$ level results in dramatic changes in cell activity, and cell death (Faber, 1981; Kater and Mills, 1991).

On the other hand, forskolin has been observed to perturbed lipid organization in rat liver plasma membranes (Whetton et al., 1983) due to its lipophilic nature and alters the function of the nicotinic acetylcholine receptors, possibly by increasing the rate of closure of open channels upon exposure. It exerts its effect primarily by directly activating the catalytic unit of adenylate cyclase (Seamon and Daly, 1981) and hence, elevates intracellular levels of the second messenger cAMP, which is essential in neurophysiological mechanisms such as ion channel gating. Moreover, these PC 12 cells have voltage-dependent Ca^{2+} channels that open after either electrical (high extracellular K^+) or chemical (cholinergic agonist) depolarization. In the present studies, we demonstrate the effect of Ca^{2+} influxes elicited by high-voltage-activated Ca^{2+} channel blocker, nifedipine, by stimulation of muscarinic cholinergic receptors with carbachol, and by the ionophore A23187, all of which induces physiological events such as constitutive exocytosis. The exocytotic release of neurotransmitters and hormones from neurons and endocrine cells (Angleson and Betz, 1997; Kasai, 1999; Travis and Wightman, 1998) are responsible for the recycling and renewal of plasma membrane components and for the secretion of molecules into the extracellular environment (Alberts et al.,

1989). In neurons the exocytosis of neurotransmitter molecules can be monitored non-invasively from populations of cells in real time using impedance spectroscopy.

D-Mannitol is a cell-impermeant and nonmetabolized sugar that is utilized as a hypertonic solution and is known to elicit intracellular calcium transients (Harik and LaManna, 1988; Martin et al., 1988). The beneficial effects of mannitol have been attributed to the consequent increase in intravascular volume and coincident decrease in extracellular fluid volume. Mannitol and other hyperosmotic agents may possibly activate inflammatory mediators (Shapiro and Dinarello, 1997) and produce toxic effects on certain cell types (Richmon et al., 1995). Little is known about mannitol's effects on the function of PC 12 cells. Recently, hyperosmotic stress has been shown to activate a number of mitogen-activated protein kinases (MAPKs) (Berl et al., 1997), specifically stress kinases such as p38 and c-Jun N-terminal kinase (JNK) (Galcheva-Gargova et al., 1994). The present report investigates and contrasts the effects of mannitol and exogenous agents on PC 12 cell impedance signatures on gold microelectrode arrays.

The impedimetric responses of the effect of exogenous agents on PC 12 cells were examined on CA SAMs covalently derivatized with laminin surfaces. Impedimetric responses of PC 12 cells that undergo calcium exocytosis and ion modulation in the presence of calcimycin, nifedipine, mannitol, carbachol, NGF, dexamethasone, and forskolin were obtained. Our results demonstrate that a change in electrophysiological behavior, such as exocytosis or modulation of ions, is detectable using temporal impedance monitoring.

2. Experimental

2.1. Materials and methods

2.1.1. Materials

Eight-well array cultureware (ECIS 8W1E) consisting of one active electrode (250 μ m diameter) and one large area counter electrode (100 cm²) per well were purchased from Applied Biophysics (Troy, NY). The rat (*Rattus norvegicus*) pheochromocytoma cell line was obtained from the American Type Culture Collection [ATCC designation/number PC 12/CRL-1721] (Manassas, VA). All chemicals reagents and laminin were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted. CA was used without further purification. Water used was sterile Milli-Q quality (Millipore, Bedford, MA).

2.2. Preparation of SAM-modified ECIS

The ECIS 8W1E cell culture arrays were functionalized with self-assembling CA using conditions optimized for the formation of organized monolayers (Sang et al., 2003; Slaughter et al., 2004). Briefly, the ECIS substrates were prepared by subsequent washing with sterilized Milli-Q water followed by UV sterilization. The SAMs were prepared by transferring aliquots of deaerated (10 min with N_2) aqueous solutions of 2-mM CA into the designated wells and allowed to adsorb for 3 h at room temperature. Following incubation, the solutions were removed by aspiration and the electrodes were immediately and thoroughly rinsed with Milli-Q water. All wells were modified with CA SAM and followed by covalent immobilization of laminin to the SAMs monolayers.

2.3. Protein functionalization of ECIS

Surface functionalization by derivatization with extracellular protein, laminin of the CA ECIS electrode surfaces were utilized. Each of these wells were then incubated under hetero-bifunctional coupling conditions with 500 μ l of 100 μ g/ml laminin via the

hetero-bifunctional reagent 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) and incubated for 2 h. This results in an NHS-activated site on laminin and improves the coupling efficiency of laminin to the CA ECIS electrodes. This produced protein functionalization by derivatization conditions in all wells. This served to monitor the impedance signatures generated by PC 12 cells due to their response to the agent. Dedicated 8-well plates (each comprising a combination of reference electrode containing medium only, two control electrodes (positive and negative) containing cells + medium only, and the remaining wells with cells receive the appropriate aliquot of agent) were used for the evaluation of each agent in cell culture conditions.

2.4. PC 12 cell cultures

PC 12 cells, rat pheochromocytoma (CRL-1721), were cultured in T-50 culture flask in RPMI 1640 medium (Cellgro) supplemented with 25 mM HEPES, 2 mM L-glutamine from Mediatech, Inc (Herdon, VA) for 10–14 days. The medium also contained 10% fetal bovine serum, 8.5% horse serum, penicillin (100 µg/ml), streptomycin sulfate (100 µg/ml), fungizone (1.25 µg/ml) and 1% sodium pyruvate that were all obtained from Fisher Scientific (Malvern, PA). Cells were grown at 37 °C in a humidified atmosphere of 95% air and 5% CO₂ and subcultured every 2–3 days at 80% confluency. Cells were plated in the modified ECIS cultureware at a typical density of 1.6×10^6 cells/ml and were first grown to confluence over a period of 18 h, followed by impedance interrogations.

2.5. AC impedance measurements

Impedance measurements were performed using a Model 1260 Impedance/Gain-Phase Analyzer (Schumberger Technologies, Houston, TX) that was interfaced through an HP 34970A Data Acquisition/Switch Unit (Agilent Technologies, Palo Alto, CA) that allowed multiplexed addressing of the eight separate electrodes of the array. Arrays were placed in a Model 3546 incubator (Forma Scientific, Marjetta, OH). Electrodes were subject to a 4000 Hz interrogating sine wave of 50 mV p-t-p voltage. The electrode resistance (real part of the complex impedance) of each electrode was monitored over time.

2.6. Measurement procedures

Prior to the experiment, the impedance of all wells containing adherent PC 12 cells were measured for 18 h before any addition of exogenous agent commenced. The medium in each well was then replaced gently by tilting the dish, removing the medium, and then adding fresh 500 µl serum-free medium (Banker and Goslin, 1990) at 37 °C to the side of the well. At time zero, the reference (blank) and the negative control (control) wells received a small volume (10 µl) of the appropriate solvent and (10 µl) of serum-free medium respectively, whereas the others wells received the same exogenous agent (10 µl). The positive control well received 30 µl of 30% sodium hypochlorite (final concentrations are noted in the description of agent treatment). The ECIS wells were immediately rocked gently back and forth and placed into the impedance interrogation unit at 37 °C and impedance interrogation was then performed within 2 min of the addition of the vehicle for ca. 9 h at 37 °C. The collected media were transferred to centrifuge tubes and were centrifuged at $3000 \times g$ for 5 min to remove any cells that might have washed off the wells during the manipulations. There was no visible cell loss from the wells, nor was there any visible pellet in any of the sample tubes after centrifugation.

2.6.1. Calcimycin treatment

After achieving confluency, PC 12 cell cultures medium was replaced with serum-free medium. Calcium ionophore A23187, stock solution of 250 µM was made in 100% DMSO and filter (0.22 µm)—sterilized prior to treatment. A final concentration of 5 µM was applied directly into well containing cells in the dark (at low light intensity) as a single bolus. Impedance was monitored for 15 min before and 9 h after A23187 application. The same treatment application was utilized for *carbachol* (applied from a stock of 2.5 mM in medium) final concentration 50 µM, *nifedipine* (applied from a stock of 250 µM in DMSO) final concentration 5 µM, *nerve growth factor* (applied from a stock of 10 µg/ml in medium), final concentration 200 ng/ml, *dexamethasone* (applied from a stock of 2 mM in DMSO) final concentration 40 µM and *forskolin* (applied from a stock of 1 mM in DMSO) final concentration 20 µM. Experiments were performed as above.

2.6.2. D-Mannitol treatment

D-Mannitol experiments were conducted at three different concentration levels (100 mM, 200 mM and 300 mM). The medium in the wells were replaced gently by tilting the dish, removing the medium, and then adding freshly prepared 500 µl D-mannitol to the side of the well. Impedance was monitored for 24 h after D-mannitol application. Experiments were performed as above. All agents used were tested in the presence of the bare ECIS electrode without cells to ensure the electrode impedance was not directly affected by the agents tested.

3. Results and discussion

Ca²⁺ is one of the most important signaling agents in mammalian cells and has the ability to control diverse cellular functions. Intracellular free Ca²⁺ acts as a messenger to regulate growth, differentiation, and cell death. Coordination of all these signaling functions requires precise regulation of intracellular free Ca²⁺ levels. A sustained high intracellular free Ca²⁺ level may initiate a cascade of signals leading to activation of phospholipases, endonucleases, and Ca²⁺-dependent proteases, which threaten cell survival (Trump and Berezsky, 1995). The ability of calcium ionophore A23187 (calcimycin) to transport divalent cations with greatest specificity for Ca²⁺ across the cell membrane is known to increase the intracellular free Ca²⁺ levels (Erdahl et al., 1994; Reed and Lardy, 1972). Calcimycin is widely used to stimulate Ca²⁺-dependent secretion from cells on the assumption that permeabilization of the cell membranes to Ca²⁺ ions leads to a rise in concentration of cytosolic Ca²⁺ ([Ca²⁺]_i), which in turn serves as a signal for secretion. We challenged the PC 12 cells with calcimycin for ca. 9 h, which produced a robust exocytosis of Ca²⁺ ions (Fig. 1). These cells treated in Ca²⁺-free medium were depleted of their cytoplasmic Ca²⁺ stores by treatment with calcimycin. The impedance profile of cells treated with calcimycin versus the control cells indicates that the cells that underwent treatment responded with an increase in impedance and attenuation in impedance fluctuations, whereas the control showed no attenuation of impedance fluctuations. Calcium exocytosis appears to occur instantaneously and can be detected 5 min after ionophore application and reaches a plateau ca. 6.17 h after stimulation with a maximum normalized impedance of 1.04 ± 0.07 . The lessen fluctuations observed in this impedance profile can be explained by the local occurrence of exocytosis and the direct mobilization of Ca²⁺ ions with calcimycin. It is known that physiological stimuli can produce a localized rise in postsynaptic Ca²⁺ concentration. Under such conditions, one may expect localized exocytosis, thereby ensuring spatial specificity to this process. It has been widely assumed since then that calcium ionophores intercalate in membrane lipids and act as diffusible Ca²⁺

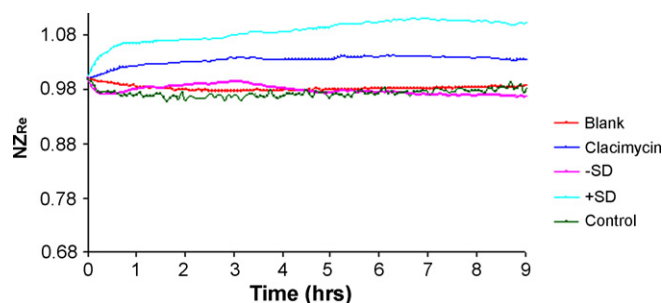


Fig. 1. Real-time changes in the normalized electrical resistance in response to dosing with 5 μ M A23187 in confluent cultured PC 12 cells on covalently immobilized laminin on CA-SAM/Au. The reference well containing medium only (blank), and the negative control well containing cell and medium (control) received a small volume (10 μ l) of the appropriate solvent and (10 μ l) of serum-free medium respectively. Values represent mean $\pm$ S.D. ($n=5$).

carriers that permit redistribution of Ca^{2+} ions as dictated by the concentration gradients of free Ca^{2+} . The permeability of the plasma membrane to high concentration of external Ca^{2+} ions and, possibly, to the release of Ca^{2+} from mitochondrial and other membrane-bound stores of Ca^{2+} is attributed to observed impedance profile.

The cell exposed to NaOCl responded with a steep decrease in impedance followed by a gradual increase and then a gradual decrease, as the cells membranes are lysed/ruptured. This impedance profile for NaOCl is typical, however, the degree of the change in impedance differs from one sample to the other, although the profile remains relatively the same. Calcium exocytosis was blocked by nifedipine (Fig. 2), an agent known to block exocytosis and forskolin (Fig. 3), an agent known to disrupt lipid membrane structures and aiding in the closure of the channel once it has opened.

In Fig. 4, the carbachol-induced $[\text{Ca}^{2+}]_i$ rise was slow rather than abruptly, and long-lived with considerable short fluctuation in impedance responses. $[\text{Ca}^{2+}]_i$ can rise even in cells incubated in Ca^{2+} -free medium, due to redistribution from intracellular stores. The rise in normalized impedance is significant with a maximum at 1.01 ± 0.07 , and $[\text{Ca}^{2+}]_i$ remained elevated for several hours. The erratic fluctuations and different size of the carbachol-induced responses suggest that different Ca^{2+} sources were involved, for example (1) redistribution from the intracellular stores in the Ca^{2+} -free medium and (2) redistribution plus increased influx across the plasma membrane. Also in this case, a considerable part of the response was maintained when the cells were exposed to carbachol. Thus, the increase in ion uptake at 50 μ M of agonist is feasible and the probability of channel opening is increased with the ago-

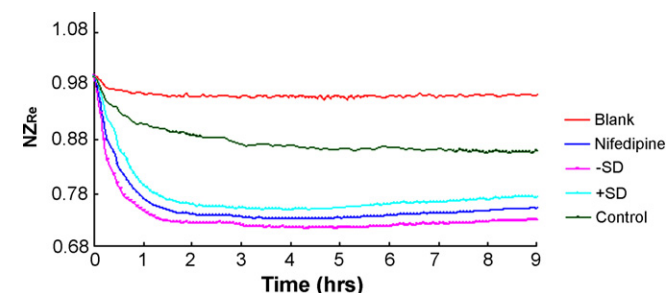


Fig. 2. Real-time changes in the normalized electrical resistance in response to dosing with 5 μ M nifedipine in confluent cultured PC 12 cells on covalently immobilized laminin on CA-SAM/Au. The reference well containing medium only (blank), and the negative control well containing cell and medium (control) received a small volume (10 μ l) of the appropriate solvent and (10 μ l) of serum-free medium respectively. Values represent mean $\pm$ S.D. ($n=5$).

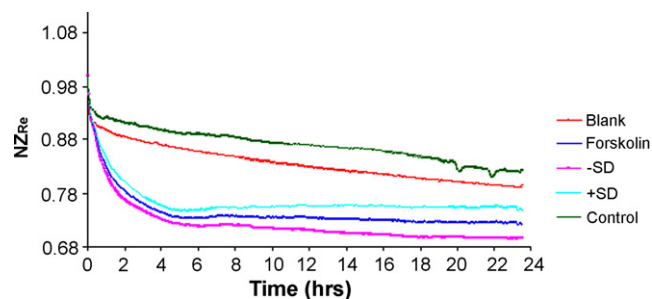


Fig. 3. Real-time changes in the normalized electrical resistance in response to dosing with 20 μ M forskolin in confluent cultured PC 12 cells on covalently immobilized laminin on CA-SAM/Au. The reference well containing medium only (blank), and the negative control well containing cell and medium (control) received a small volume (10 μ l) of the appropriate solvent and (10 μ l) of serum-free medium respectively. Values represent mean $\pm$ S.D. ($n=5$).

nist concentration, thus the facilitation of closure would be likely to decrease ion flux. Treatment with carbachol induces muscarinic receptor-dependent rises in free cytosolic Ca^{2+} as well as hydrolysis of membrane phosphoinositides. When cells were treated with carbachol, recovery of the exocytotic response was not observed within 9 h, during which cells were stimulated with carbachol.

PC 12 cell line processes voltage-dependent calcium channels that bind organic calcium antagonist, such as nifedipine. Fig. 2 shows the impedance profile of 5 μ M nifedipine and its inhibition of depolarization-induced calcium uptake in PC 12 cells. Upon introduction of the vehicle, the normalized temporal impedance decayed sharply due to the specific blockage of the high-voltage activated (L-type) calcium channels achieving a minimum at 0.745 ± 0.02 after 1.68 h. After 4 h, at 5- μ M nifedipine a considerable increase in impedance was detected. This increase in normalized impedance is attributed to the specific blockage of the L-type calcium channels (noting not all calcium channels are blocked), which in turn decreases their open-state probabilities (Catterall and Striessnig, 1992; Wie et al., 1989). Since the effects of nifedipine are known to be a decrease in mitotic activity and reduction in $[\text{Ca}^{2+}]_i$ (Wie et al., 1989), the impedance profile obtained allows us to deduce that the impedance signature is a result of decrease $[\text{Ca}^{2+}]_i$ in the presence of nifedipine and the binding of nifedipine to the membranes in order to block the calcium channels, thus eliciting its inhibition of potassium-induced Ca^{2+} uptake into the cells.

Previous studies (Mattson et al., 1988; Morad et al., 1988) have demonstrated in PC 12 clonal cell line that calcium antagonists bind to membranes in various tissues and inhibit calcium-induced actions. These studies almost certainly demonstrate that the cal-

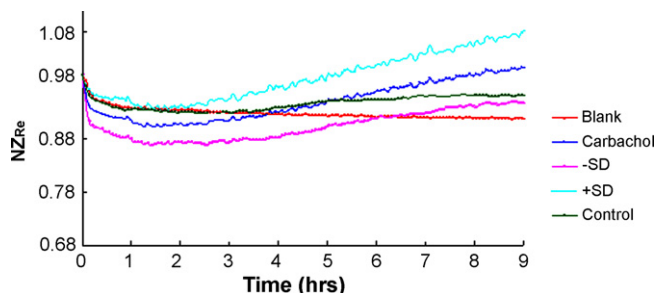


Fig. 4. Real-time changes in the normalized electrical resistance in response to dosing with 50 μ M carbachol in confluent cultured PC 12 cells on covalently immobilized laminin on CA-SAM/Au. The reference well containing medium only (blank), and the negative control well containing cell and medium (control) received a small volume (10 μ l) of the appropriate solvent and (10 μ l) of serum-free medium respectively. Values represent mean $\pm$ S.D. ($n=5$).

cium antagonist, nifedipine, in fact, binds to the calcium channel protein, thus inhibiting the potassium-induced accumulation of Ca^{2+} . The 5- μM nifedipine vehicle caused not only a marked decrease in impedance, but also worsened the functional conditions of cells by substantially reducing the proliferation activities of the cells. The present results show that PC 12 in the presence of 5- μM L-type calcium channel blocker, nifedipine suppresses proliferation activity. Studies of intracellular free calcium concentration changes (Goldberg, 1988; Greene and Tischler, 1982) allow us to conclude that these processes are related to a decrease of $[\text{Ca}^{2+}]_i$ level during cell cultivation.

To determine the response of PC 12 cells to osmotic stress, confluent monolayers of PC 12 cells were exposed to 100 mM, 200 mM, and 300 mM mannitol (Fig. 5). Within 2.5 h after addition of mannitol, the normalized impedance began to increase due to cells rounding up, thus resulting in a change in their membrane integrity (compare 100 mM and 300 mM with 200 mM). This change in membrane integrity phenomenon was evident at 100 mM mannitol but was more dramatic at 200 mM mannitol. PC 12 cells rounded up by the addition of 200 mM mannitol (maximum at 0.900 ± 0.04 , after 6.16 h) appear to recover their original volume after 10 h to baseline impedance of the previously confluent monolayer. However, with the addition of 100 mM mannitol, the relative change in impedance profile is slightly larger (maximum at 0.926 ± 0.02 , after 17.3 h) than that of 200 mM (maximum at 0.900 ± 0.04 , after 6.16 h) and appears to recover their original volume after 20 h. On the other hand, the addition of 300 mM mannitol impedance profile (maximum at 0.844 ± 0.01 , after 10.1 h) is comparable to that of 100 mM mannitol, however the recovery to original volume was not observed during the course of the experiment. This may not be surprising, as both 100 mM and 300 mM (Fig. 5) induced the same change in cell membrane integrity as 200 mM mannitol, with a less definite rise in normalized impedance. However, the more concentrated mannitol appeared to cause insignificant change in cell membrane integrity than did less concentrated mannitol.

In their undifferentiated state, PC 12 cells have been shown to synthesize catecholamines (Greene and Tischler, 1976, 1982; Tischler et al., 1983). Upon differentiation with NGF or glucocorticoids, PC 12 cells express properties characteristic of sympathetic neurons or mature chromaffin cells respectively (Greene and Tischler, 1976; Tischler et al., 1983). We examined how PC 12 cells cellular impedance responsiveness is affected by phenotypic alteration especially with regard to modulation of ion fluxes. After NGF treatment, PC 12 cells exhibited a dramatically altered mor-

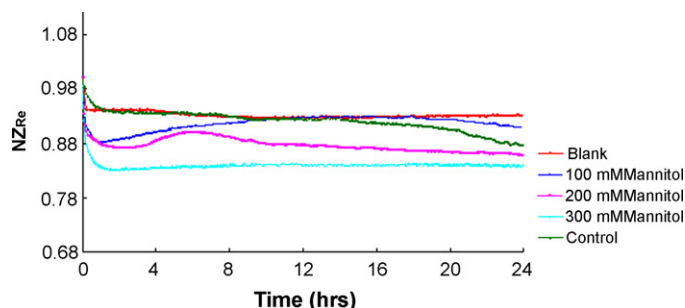


Fig. 5. Real-time changes in the normalized electrical resistance in response to dosing with D-mannitol in confluent cultured PC 12 cells on covalently immobilized laminin on CA-SAM/Au. Values represent mean $\pm$ S.D. electrical resistance change for a sample size of 6. The concentration effect of 100 mM D-mannitol, 200 mM D-mannitol and 300 mM D-mannitol on PC 12 cell growth and osmotic stress. The reference well containing medium only (blank), and the negative control well containing cell and medium (control) received a small volume (10 μl) of the appropriate solvent and (10 μl) of serum-free medium respectively. Values represent mean $\pm$ S.D. ($n = 5$).

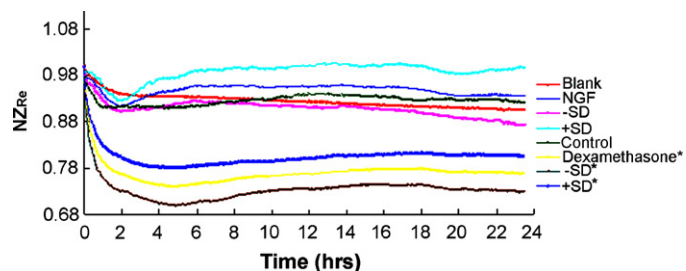


Fig. 6. Real-time changes in the normalized electrical resistance in response to dosing with 200 ng/ml NGF and 40 μM of dexamethasone, separately in confluent cultured PC 12 cells on covalently immobilized laminin on CA-SAM/Au. The reference well containing medium only (blank), and the negative control well containing cell and medium (control) received a small volume (10 μl) of the appropriate solvent and (10 μl) of serum-free medium respectively. Values represent mean $\pm$ S.D. ($n = 5$) ($\pm$ S.D., NGF and $\pm$ S.D., Dexamethasone).

phology, displaying neurites within 12 h and prominent branching within 48 h. Unlike the impedance profiles in undifferentiated cells, NGF-treated cells show a sharp decrease in normalized impedance (minimum at 0.913 ± 0.0 , $t = 2.08$ h), followed by a gradual increase and stabilization (Fig. 6). This is attributed to the physiological events within the cells upon exposure to NGF. Cells were also exposed to 40 μM dexamethasone to enhance catecholamine secretion (Tischler et al., 1983). Fig. 6 shows that exposure of PC 12 cells to 40 μM of dexamethasone evokes marked secretion, which is wholly dependent on Ca^{2+} entry through voltage-gate Ca^{2+} channels (Taylor and Peers, 1999a,b), since it could be completely inhibited by either removal of extracellular Ca^{2+} . Upon dexamethasone treatment, cells increased in size without concomitant neurite development. Dexamethasone-treated cells impedance profile (minimum at 0.740 ± 0.04 , $t = 4.82$) is similar to that of NGF-treated cells (minimum at 0.913 ± 0.01 at $t = 2.08$ h), however it differs in the reduction in initial impedance after introduction of the vehicle. This reduction in impedance is also attributed to physiological changes within the cells in preparation for differentiation. Reappearance of catecholamine release did not occur within the time span of experiments.

In undifferentiated PC 12 cells, forskolin significantly elevated cAMP. The impedance profile of the effect of forskolin evoked cAMP in undifferentiated cells (Fig. 3) was similar to that of NGF-treated and dexamethasone-treated cells, where forskolin attenuated exocytotic activity significantly. Forskolin is thought to be a highly specific activator of adenylyl cyclase. When this possibility was investigated with neuronal nicotinic receptors in PC 12 cells using impedance spectroscopy, significant change in normalized impedance (minimum at 0.718 ± 0.02 , $t = 6.3$ h) was obtained and the acute elevation of cAMP can alter the ion conducting properties of the neuronal nicotinic acetylcholine receptors. However, during the course of this study, it was observed that 20 μM forskolin caused a slight increase after 10 h. Thus, it is possible that the effect of forskolin on PC 12 cells was due to a direct effect on the membrane structure or the nicotinic receptor. Given the lipophilic nature of forskolin, it probably acts to perturb the plasma membrane lipid structure and alter the function of the nicotinic acetylcholine receptors, possibly by increasing the rate of closure of open channels. The activation of receptor occurred instantaneously whereas cellular cAMP content did not increase for a measurable period of time. This impedance profile demonstrates that activation and/or ion flux through neuronal nicotinic acetylcholine receptors of PC 12 cells is directly inhibited by forskolin. These observations emphasize the need to consider all of the possible biochemical actions of new chemicals, even those that appear to be as specific as forskolin.

Considering the very lipophilic nature of the forskolin molecule, the most likely explanation for our results is that forskolin is entering the plasma membranes, disrupting the lipid structure, and acting like an anesthetic molecule. General anesthetics are known to inhibit nicotinic acetylcholine receptor activation, possibly by facilitating the closure of the channel once it has opened (Lechleiter and Gruener, 1984). Such a mechanism would explain the results shown in Fig. 3. The membrane-perturbing properties of forskolin have been shown previously with physical chemical techniques applied to liver plasma membranes (Whetton et al., 1983). Changes in membrane physical properties, however, did not become very noticeable until a concentration of 1 μ M forskolin. The fact that changes in nicotinic receptor function are observed even at 1 μ M forskolin implies that either the receptors are a much more sensitive marker of membrane perturbation or that a specific membrane–lipid–receptor interaction is disturbed by forskolin at low concentrations.

4. Conclusions

Like normal precursor cells arising from the sympathoadrenal region of the neural crest (Landis and Patterson, 1981), PC 12 cells, which resemble immature chromaffin cells, can be induced by exogenous factors to give rise to differentiated phenotypes resembling either sympathetic postganglionic neurons or mature adrenal chromaffin cells. We have shown that changes in phenotype induced by growth factor, glucocorticoid or forskolin treatment lead to differential impedance responsiveness. In contrast to undifferentiated PC 12 cells, which were unresponsive to the addition of carrier solvent. Induction of a sympathetic neuronal phenotype with NGF was accompanied by a sharp decrease in impedance. In this study, we have also shown that PC 12 cells differentiated to a mature chromaffin phenotype with dexamethasone were responsive upon impedance interrogation. After treatment with dexamethasone, a lower shift down in impedance on the impedance curve. This decrease in impedance may reflect a change in receptor expression after induction of a mature chromaffin phenotype, but it is difficult to evaluate the meaning of such a shift without further studies.

In contrast to our results with NGF or dexamethasone-differentiated PC 12 cells, forskolin binds to the cell membrane and disrupts the opening of Ca^{2+} channels. Several possible mechanistic interpretations can be given, such that forskolin could work as a channel blocker, but this seems unlikely because most channel blockers have positive charged and is not as lipophilic as forskolin. Another possibility is that forskolin binds to adenylyl cyclase and some direct interaction between the enzyme and the nicotinic receptors takes place to inhibit receptor activation. We believe this is unlikely but cannot be totally ruled out by the presented data.

In contrast to undifferentiated cells, the treated cells significantly differ in their impedance profiles. Control experiments showed no effect on exocytosis. Cells that received the appropriate vehicle responded with exocytosis when stimulated. Limited numbers of events, which were comparable to those recorded from cells stimulated with a vehicle, were also recorded from cells which were stimulated with 3% sodium hypochlorite. After deple-

tion of releasable vesicles by repeated stimulation with all vehicles was able to evoke release in all cells. Nifedipine- and forskolin-treated cells depicted a crucial temporal impedance response. This indicates that the blockage calcium channels resulted in minimal exocytosis.

These results, however suggest that this cell line and the use of impedance spectroscopy represent a useful model system to investigate receptor-mediated responses and biological assays/cholinergic stimulation/muscarinic regulation.

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