Study on the mechanism of Bioelectric Recognition Assay: evidence for immobilized cell membrane interactions with viral fragments

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Abstract

The Bioelectric Recognition Assay (BERA) is a whole-cell based biosensing system that detects the electric response of cultured cells, suspended in a gel matrix, to various ligands, which bind to the cell and/or affect its physiology. Previous studies have demonstrated the potential application of this method for rapid, inexpensive detection of viruses in a crude sample. However, the understanding, so far, of the fundamental processes that take place during cell–virus interactions within the probe has been rather limited. In the present study, we combined electrophysiological and fluorescence microscopical assays, so that we can prove that animal and plant cells immobilized in BERA sensors respond to different viruses primarily by changing their membrane potential. The response of immobilized cells against different viruses did not depend on the virus ability to penetrate the cell, but was modified after binding each virus to a virus-specific antibody or removal of its coat protein after treatment with a protease. Consequently, we were able to assay the presence of a virus in its complete form or fragments thereof. Combination of immunological recognition with the electrophysiological response of immobilized cells allows for a considerable increase of the specificity of the BERA biosensory assay. In addition, rather than simply detect the presence of a protein or genomic sequence, the method can help gain information on the bioactivity of a virus.

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1. Introduction

The Bioelectric Recognition Assay (BERA) is a unique technology that detects the electric response of cultured cells, suspended in a gel matrix, to various ligands, which bind to the cell and/or affect its physiology. Preliminary studies (Kintzios et al., 2001a,b, 2002) have demonstrated the potential application of the method for ultra rapid (1–2 min), ultra cheap tests for infectious viruses in humans (Hepatitis B and C viruses, herpes viruses) and plants (tobacco and cucumber viruses). Assays have been carried in an entirely crude sample and a high sensitivity of the method (0.1 ng) has been indicated, making it an attractive option for routine sample screening that could help reduce the exceeding use of advanced and costly molecular techniques, such as the reverse transcription polymerase chain reaction (RT-PCR).
The electrophysiological response of immobilized cells in a BERA-sensor against a particular virus has been attributed to various mechanisms, such as alterations in cell membrane porosity and receptor function of immobilized cells. However, the understanding of the fundamental processes that take place during cell–virus interactions within the probe has been rather limited. This interaction has a number of putative components, which can be classified in two groups: (i) interaction with the cell membrane and (ii) influence on the whole cellular physiology. In the present study, we combined electrophysiological and fluorescence microscopical assays in order to investigate the response of animal and plant cell BERA-sensors against different viruses, under conditions that either allowed or prohibited virus interactions with the immobilized cell membranes. The improvement of our knowledge of the nature of virus-biosensor interaction will allow for optimization of the assay, particularly in respect to specificity and scope of applications.

2. Experimental

2.1. Biosensor system

In all experiments, mini BERA sensors consisted of a tube-like probe made of polypropylene (3.5 cm × 0.3 cm) and containing components of immobilized cells in a gel matrix (for a detailed description of the sensor configuration, see Kintzios et al., 2001b). A matrix volume of 50 µl was obtained. The electrodes were made from pure silver, electrochemically coated with an AgCl layer and had a diameter of 0.2 mm. Both electrodes were connected via coaxial cable to the recording device, which comprised an Advantec Adam-4017 PC I/O card. The Analog-to-Digital Converter (ADC) of this computer card was recording the signal (voltage). The ADC could accomplish an accurate range of 0.01 mV (accuracy = 0.009918 or 0.01 mV). The software responsible for the recording of the signal and processing of data was a modified version of the Advance Genie v. 3.0.

In theory, at the moment of sample application to the top of the probe the compounds under detection will interact with the part of the sensory cellular material in the area of the measuring electrode, causing a change of its electric properties. However, at the same time, the part of the sensory cellular in the area of the reference electrode will retain the initial value of these properties (rest potential). In this way, an electric potential will be created between the two electrodes.

2.2. Cell immobilization

Biosensors were constructed under sterile conditions by immobilizing either animal or plant cells, as described in following: Animal cells were derived from Vero (African green monkey kidney) cell line. After cell detachment from the culture vessel by adding trypsin/EDTA for 10 min at 37°C and cell concentration by centrifugation (6 min, 1200 rpm, 25°C), cells (at a density of 6 × 10^6/ml) were mixed with a 0.35% (w/v) low melting point agarose solution in saline at 37°C. Plant protoplasts were isolated from leaves of the tobacco (Nicotiana tabacum L.) cv. ‘Samsun’ as described previously (Reinert and Yeeom, 1982; Kintzios et al., 2001a). Isolated protoplasts were concentrated by centrifugation (6 min, 1200 rpm, 25°C), protoplasts (at a density of 6 × 10^6/ml) were mixed with a 0.35% (w/v) low melting point Bacto-agar solution in saline at 37°C. In all cases, cell–agarose mixtures were transferred into an appropriately configured sensor tube bearing Ag/AgCl electrodes, as described in 2.1.

2.3. Assay

2.3.1. Detection of herpes viruses

Solutions containing either Herpes Simplex virus (HSV-1) or Varicella Zoster virus (VZV) were obtained from the Hellenic Pasteur Institute. Two µl of negative control (PBS-Dulbecco’s medium) or samples containing either virus at a concentration of 4 or 250 ng/ml were applied on Vero cell sensors. Both viruses are virulent on Vero cells, but BERA is more specific for HSV-1 (Kintzios et al., 2002).

2.3.2. Detection of plant viruses

Virus isolates were purified from infected Nicotiana tabacum cv. Samsun leaves (a Greek isolate of tobacco rattle virus (TRV), TRV-GR) or infected cucumber (Cucumis sativus) leaves (the watermelon isolate PL104 of cucumber green mottle mosaic virus (CGMMV)), as described previously (Lister and Bracker, 1969; Tung and Knight, 1972; Bem, 1987; Brown et al., 1996; Bem and Vassilakos, 1996). TRV is virulent on tobacco, while CGMMV is not.

For the detection of CGMMV and TRV, 2 µl of control solution (phosphate buffer pH 7.4) or sample (buffer containing 4 ng/ml or 250 ng/ml purified virus) were applied on tobacco protoplast sensors.

2.3.3. Antibody complexation assay

2.3.3.1. Anti-VZV sera.

Two human sera were tested for the detection of IgG antibodies against VZV, using the Enzyme ImmunoAssay kit by Novatec (Dietzenbach, Germany). Both sera tested positive, with values equal to 3.3 and 1.6 Arbitrary ELISA Units.

2.3.3.2. Anti-HSV-1 sera.

Also, two human sera were tested for the detection of IgG antibodies against the HSV-1, using the HerpeSelect ELISA 1 kit, by FOCUS (Cypress, USA). The sera tested positive, with values equal to 8.4 and 8.0 Arbitrary ELISA Units.
In both cases (Sections 2.3.3.1 and 2.3.3.2), the samples are considered positive when they are greater than or equal to 1.1 and negative when they are lower than or equal to 0.9.

2.3.3.3 Anti-TRV and anti-CGMMV sera. Rabbit antisera (titre 10^2/24) against TRV and CGMMV were prepared by weekly intramuscular injections of 0.5 mg purified virus for two months. The obtained antisera were cross-absorbed against crude sap from tobacco or cucumber plants respectively. The IgG fractions were isolated from the prepared antisera by affinity chromatography on a protein A-Sepharose CL.4B (Pharmacia) column and concentrated to 1 mg IgG/ml.

One hundred microliters of samples containing each of the viruses described in Sections 2.3.1–2.3.3 above at their highest concentration (250 ng/ml) were mixed with an equal volume of antiserum against human (animal cell sensors) or plant (protoplast sensors) viruses. The viral concentration was determined as optimal for virus-antibody binding. Two microliters of the mixture were then applied on either animal cell or plant protoplast-BERA sensors and the response of each sensor was recorded.

2.3.4 Pronase treatment

One hundred microliters of samples containing each of the viruses described in Sections 2.3.1–2.3.3 above at their highest concentration (250 ng/ml) were mixed with an equal volume of pronase solution (Pronase E, from Streptomyces griseus, 1 mg/ml). The viral concentration was determined as optimal for pronase-mediated coat protein digestion. Two microliters of the mixture were then applied on either animal cell or plant protoplast-BERA sensors and the response of each sensor was recorded.

2.3.5 Recording and data processing

In every biosensor-based assay, the response of each sensor was estimated by recording the change of the sensor potential three seconds after sample application and until the response was stabilized. Depending on the virus assayed, a stable response of each biosensor was achieved 40–80 s after sample addition. The time required for signal stabilization depends on matrix porosity, being less than 120 s for Bactoagar concentrations lower than 0.5% (w/v) and coincides with maximum accumulation of viral particles on the surface of immobilized cells, as revealed by transmission electron microscopic observations (data not shown). Since different biosensors slightly varied in their original, steady-state potential, and in order to facilitate comparison of responses against different samples, the response of each sensor at the beginning of recording time was fixed to zero value. The final value of the sensor potential at the end of each assay was considered as the numerical value of each response.

2.4 Fluorescence microscopy assays

Immediately after completion of each biosensor-based assay, gel matrices with immobilized cells were carefully removed from each sensor and thin sections were made from the upper, 1-cm-deep part of each matrix. Sections were mounted on a Zeiss AxioLab fluorescent microscope equipped with a BP-546 excitation filter and an FT-580 chromatic beam splitter. A digital camera (SONY STV5 digital still camera) was attached to the microscope. Membrane potential changes were monitored by the cellular uptake of the lipophilic cationic fluorochrome, 3,3-dipropylthiadicarbocyanide iodide, the distribution of which is affected by the potential difference across the cell membrane (Eddy, 1989). After application of 5 μl of the dye, the fluorescence of the specimens was recorded for 5 min at 10-s intervals.

2.5 Chemicals

All solvents and chemicals used were of analytical quality (from Sigma Company, St. Louis). Water was double distilled. Vero cell cultures were originally provided from the American Type Culture Collection (ATCC).

2.6 Experimental design

Experiments were set up in a randomized complete block design and each experiment was repeated three times. In each application, a set of filter biosensors was tested against each individual sample.

3. Results

In each application, cell-immobilized sensors responded differently against different viruses two-fold: (a) in the average numerical value (in mV) of the sensor potential and (b) in the pattern of response. Application of virus-free control produced a response that was clearly different from any investigated virus. Results against each investigated virus are specified in following:

3.1 Antibody-mediated specification of the response of Vero cell-BERA sensors against herpes viruses

Vero cell-based biosensors responded differently against HSV-1 or VZV (Table 1), in a concentration-dependent pattern. Binding of HSV-1 with its specific antiserum resulted in an altered response pattern, with a decreased absolute value (Fig. 1). This was quite similar to the pattern of response against the pure HSV-1-specific antiserum and not considerably different from the response obtained after binding of HSV-1 with the VZV-specific antiserum, which also shows specificity against HSV-1 (Roizman, 1983). After binding with its specific antibodies, HSV-1 produced a response that
Table 1
Comparison of average response (in mV ± S.D.) of cell biosensors against samples containing different viruses, before and after treatment with virus-specific or virus-non-specific antisera, as well as before and after treatment with pronase (mean values, 15 biosensors, n = 45).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Virus</th>
<th>HSV-1</th>
<th>VZV</th>
<th>TRV</th>
<th>CGMMV</th>
</tr>
</thead>
<tbody>
<tr>
<td>No virus (control)</td>
<td>−3.3 ± 1.1</td>
<td>−3.3 ± 1.1</td>
<td>−5.9 ± 2.4</td>
<td>−5.9 ± 2.4</td>
<td></td>
</tr>
<tr>
<td>Virus only (4 ng/ml)*</td>
<td>−6.7</td>
<td>−0.1</td>
<td>4.2 ± 0.1</td>
<td>−1.2</td>
<td></td>
</tr>
<tr>
<td>Virus only (250 ng/ml)*</td>
<td>−12 ± 4.6</td>
<td>2.6 ± 0.7</td>
<td>2.7 ± 1.2</td>
<td>−7.4</td>
<td></td>
</tr>
<tr>
<td>Virus (4 ng/ml) + virus-specific antiserum</td>
<td>−3.6 ± 1</td>
<td>0.3 ± 0.1</td>
<td>1.2 ± 0.9</td>
<td>5.1 ± 1.5</td>
<td></td>
</tr>
<tr>
<td>Virus (4 ng/ml) + virus-non-specific antiserum</td>
<td>−4.60</td>
<td>0.6201</td>
<td>−</td>
<td>−1.5 ± 2.7</td>
<td></td>
</tr>
<tr>
<td>Virus-specific antiserum only</td>
<td>−4 ± 0.4</td>
<td>0</td>
<td>2.4 ± 0.7</td>
<td>−5 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>Virus (250 ng/ml) + pronase</td>
<td>4.6</td>
<td>0.4</td>
<td>3.8 ± 4.2</td>
<td>2.65 ± 2.45</td>
<td></td>
</tr>
<tr>
<td>Pronase only</td>
<td>−9.5</td>
<td>−9.5</td>
<td>3.6 ± 0.7</td>
<td>3.6 ± 0.7</td>
<td></td>
</tr>
</tbody>
</table>

* Different viral concentrations were used in order to optimize virus-antibody binding and pronase-mediated digestion of viral protein coat.

** HSV-1 also binds with its non-specific (VZV-specific) antiserum.

was roughly equal to negative control. On the contrary, binding of VZV with either antibody, modified the pattern of biosensor response but increased only slightly the final value of it. Consequently, treatment with antiserum did not affect considerably the response against VZV.

Imobilized VERO cells (Fig. 2A) incubated in 3,3-dipropylthiadicarbocyanide iodide emitted a bright red fluorescence upon contact with HSV-1 (Fig. 2B), which indicated extended cell membrane depolarization. On the contrary, binding of HSV-1 with its specific antiserum caused total quenching of fluorescence (Fig. 2C). A faint fluorescence was observed when HSV-1 was treated with the less specific VZV-antiserum (Fig. 2D), indicating partial membrane depolarization. Similarly, contact of immobilized cells with VZV resulted in membrane depolarization, which was only slightly decreased in antiserum-treated samples (data not shown). Therefore, cells immobilized in sensors demonstrated changes in their membrane potential that were in full accordance with the observed BERA-response (which increases with cell membrane potential and is inversely related to fluorescence).

3.2. Antibody-mediated specification of the response of tobacco protoplast-BERA sensors against plant viruses

Tobacco protoplast-biosensors responded differently against TRV or CGMMV (Table 1, Fig. 3A and B), in a concentration-dependent pattern. Binding of TRV with its specific antiserum resulted in an altered response pattern, with a decreased final value (Fig. 3A). Similarly, binding of CGMMV with its highly specific antiserum resulted in an entirely different pattern of response that
Fig. 2. The membranes of immobilized Vero cells were depolarized after attachment of the HSV-1 virus, an effect which was not observed when the virus bound with its specific antiserum. Treatment of the virus with a non-specific antiserum did not result in inhibition of the viral properties. (A) Vero cells immobilized in 0.35% Bacto-agar gel (B–D) Fluorescence micrographs of immobilized Vero cells incubated in 3,3-dipropylthiadicarbocyanide iodide after addition of either (B) HSV-1, (C) HSV-1 + HSV-1-specific antiserum, (D) HSV-1 + non-specific antiserum. Fluorescence intensity increases with increasing cell membrane depolarization. Individual figures correspond to different biosensors. Bar in (A) represents 100 μm.

was clearly distinguishable from the response of the pure antiserum (Fig. 3B).

Immobilized tobacco protoplasts (Fig. 4A) incubated in 3,3-dipropylthiadicarbocyanide iodide emitted a bright red fluorescence upon contact with TRV or CGMMV (Fig. 4B and C), which indicated extended cell membrane depolarization. On the contrary, binding of either virus with its specific antiserum caused a rapid decrease of fluorescence (Fig. 4D and E). This effect was not observed when CGMMV was treated with TRV-antiserum (Fig. 4F), which could not bind with the virus. Consequently, plant cells immobilized in sensors also demonstrated changes in their membrane potential that were in full accordance with the observed BERA-response.

3.3. Expression of the BERA response after removal of the viral protein coat

Removal of the viral protein coat after treatment with pronase resulted in considerably altered sensor responses against all viruses tested in the present study (Table 1 and Figs. 5 and 6 for HSV-1 and TRV, respectively). In the case of plant viruses (particularly TRV), these responses were not easily distinguishable from the response against a pure pronase solution. Although immobilized VERO cells demonstrated increased membrane depolarization upon contact with either HSV-1 (Fig. 7A) or VZV, this effect was not reproduced when viruses were treated with pronase (Fig. 7B). Similarly, the membrane depolarization of tobacco protoplasts upon contact with pronase-treated TRV (Fig. 7D) or CGMMV was reduced, compared to the response to untreated viruses (Fig. 7C).

4. Discussion

The results of the present study clearly demonstrate that detection of different viruses with BERA, was primarily based on changes of immobilized cell membrane potential. Some of the membrane functions operating in a cell suspension also exist within the cell-matrix system, although the immobilization procedure may affect membrane properties. In addition, cells are single immobilized in a gel matrix and more or less evenly distributed, whereas cells in suspensions are not homogenously exposed to viruses. Rosenthal and Shapiro (1983) also observed that changes in the membrane potential differed in time course and direction with respect to the capacity of B lymphocytes to internalize Epstein-Barr virus. Iwata et al. (1999) measured membrane currents using the whole cell patch clamp technique to study effects of rabies virus infection on ion channels in mouse neuroblastoma cells. By using the same technique, Wang et al. (1994) demonstrated that the influenza A virus M2 protein had an ion channel activity in mammalian cells.

In our experiments, the response of immobilized cells against different viruses did not depend on the virus ability to penetrate the cell. This was explicitly demonstrated in the case of tobacco protoplast sensors responding against the non-virulent CGMMV. In addition, it has been shown previously that BERA sensors are able for a virus-specific response, even if immobilized cells are not specifically susceptible to the virus under investigation (e.g. response of Vero cells against hepatitis B and C viruses—Kintzios et al., 2001b). Finally, Vero cell sensors used in the present study responded differently against HSV-1 and VZV, although both viruses can penetrate cells of this line. Obviously, binding of HSV-1 with antibodies inhibited its attachment to the cell membrane; consequently, the observed response was similar to the negative control. This was not observed with VZV, indicating that antibody-binding epitopes of this virus are not exclusive for obtaining a BERA-response. Indeed, VZV has a different mechanism of infectivity than HSV-1, at least in respect to the formation of syncytia, possibly because VZV specifies the smallest number of glycoproteins, having the smallest genome among herpesviruses (Cole and Grose, 2003). The progress of VZV infectivity after binding to neutralising antibodies is also different from HSV-1 (Wheeler and Canby, 1959). Furthermore, the response against all pronase-treated viruses never resembled negative control, thus indicating that immobilized cells could also respond against the products of the digestion process. It is reasonable to assume that cell penetration by the virus
is not required for a virus-plasmalemma interaction, however, lack of specific membrane receptors for a virus should limit the usefulness of the cell as a detector of this virus. Lack of receptors against molecules of interest is usually considered a major drawback for cell biosensors (Whelan and Zare, 2003), which can be partially overcome by injection into cells of mRNA, to induce the expression of receptors for molecules of interest (Shear et al., 1995). In spite of this, immobilized cells in BERA sensors were able not only to respond differently against different viruses, but also to
Fig. 4. The membranes of immobilized tobacco protoplasts were depolarized after attachment of TRV, an effect which was not observed when the virus bound with its specific antiserum. Although CGMMV is not virulent on tobacco, its attachment on protoplasts caused a partial depolarization of the cell membrane, which was also not observed when the virus bound with its specific antiserum. Treatment of the virus with a non-specific (TRV-specific) antiserum did not result in inhibition of the viral properties. (A–F) Fluorescence micrographs of immobilized tobacco protoplasts after addition of either (A) no virus, (B) TRV (C) CGMMV (D) TRV + TRV-specific antiserum, (E) CGMMV + CGMMV-specific antiserum or (F) CGMMV + TRV-specific antiserum. Fluorescence intensity increases with increasing cell membrane depolarization. Individual figures correspond to different biosensors. Bar in (A) represents 50 μm, bar in (F) represents 25 μm.

Fig. 5. Examples of the original responses of Vero cell-BERA sensors against HSV-1 (at a 250 ng/ml concentration), before and after treatment with pronase. Bold line—response against HSV-1, dashed line—response against HSV-1 + pronase. t = 0 corresponds to three s after sample application.
recognize modifications of the viral surface, either by antibody binding or pronase treatment. We are not yet able to offer satisfactory explanation for this phenomenon, which merits further investigation, although one possible mechanism may involve differences in the ion concentration in the vicinity of the electrodes caused by interactions of the virus with the cell or cell membrane. For example, virus-binding to cells could cause a local change in pH as part of an (even partial) fusion of the viral envelope with the cell membrane. In addition, potential changes could have resulted from changes of the lipid composition of the cell membrane in response to changes in the proximate cellular environment ('homeophasic adaptation responses') (Vigh et al., 1998). Preliminary experiments of our working group indicated that immobilized cells respond to viruses with a differential RNA accumulation, thus supporting the hypothesis of a biochemically active BERA-mechanism.

Assuming that cells were able to recognize individual elements or the viral structure, then we could use the assay in order to distinguish between the presence of a virus in its complete form or fragments thereof. As we have observed in recent experiments (unpublished results), the heat-inactivation of a virus resulted in an altered BERA-response; when the denaturation process was reversible, the original BERA-response against the virus was restored after certain period of time. Therefore, rather than simply detecting the presence of a protein or genomic sequence, the method could help gain information on the bioactivity of a virus, which is of particular importance for evaluating the actual status of infection and development of a disease. To our best knowledge, this is currently feasible only by culturing virus-infected cells and assaying viral replication and/or related cytopathic effects, which is both laborious and time-consuming (Burleson et al., 1992).

Cells with a broad susceptibility to different viruses, such as Vero, are easily available and suitable for mass...
particularly useful in drawing a first, rapid conclusion in the presence of a given virus in an unknown sample, therefore providing an effective sample “filter” prior to the employment of other analytical techniques.

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References


