



Cardiomyocyte-transistor-hybrids for sensor application

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Abstract

An extracellular recording system has been designed for the detection of electrical cell signals using p-channel or n-channel field-effect transistor (FET) arrays with non-metallized gates. Signals from rat heart muscle cell were recorded by these devices and the results described on the basis of an equivalent circuit. This technique is sensitive enough to detect minute changes of the extracellular membrane voltage and has potential applications in drug screening. We show that known cardiac stimulants (isoproterenol, norepinephrine) and relaxants (verapamil, carbamylcholine) have characteristic effects on the heart cells in terms of the changes of beat frequencies in the absence or presence of corresponding agents. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Field effect transistors; Extracellular recording; Dissociated cardiac myocytes; Pharmacological biosensor

1. Introduction

The functional coupling of biologically active elements, such as proteins, whole cells or even tissue slices, to elements of micro-electronic circuits opens up exciting new perspectives for the development of new biosensors. The high sensitivity and selectivity of biological recognition systems with signal-amplification cascades that have been optimized by evolution, coupled with a man-made signal-detection and processing system, will open up new possibilities. By using living cells, we have access to a set of receptors that detect with very high sensitivity external signals of, e.g. chemical nature and process them by a physico-chemical machinery that includes signal amplification cascades, which finally results in a set of output signals that allows the cell to communicate with its environment and with other cells. These signals can be of chemical (organic molecules or inorganic ions) or physical nature (mechanical contraction, change in electrical potential, etc.). Whole-cell biosensors (McConnell et al., 1992; Pancrazio et al., 1999) provide the opportunity to elicit such information, for applications in areas such as

pharmacology, cell biology, toxicology, and for monitoring environmental factors. In addition, the coupling of a 2D cellular network with an extracellular recording system might allow the structure–function relationship of such a network to be studied in detail.

For extracellular signal recording from electrogenic cells *in vitro*, the use of microfabricated microelectrode arrays (MEAs) or field-effect transistors (FETs) is of increasing interest. In case of the MEAs these devices employ glass or silicon substrates, onto which electrode arrays made of gold, platinum or indium tin oxide are fabricated. Miniaturized FET arrays can be used in two slightly different configurations: as a standard field effect transistor with ordinary *poly*-Si, silicide or metal gate and as an open gate field effect transistor configuration. With such systems, simultaneous recordings from multiple sites of electrogenic cultures have been reported (Thomas et al., 1972; Pine, 1980; Jobling et al., 1981; Gross et al., 1985; Novak and Wheeler, 1988; Fromherz et al., 1991; Offenhäusser et al., 1997; Bove et al., 1998; Egert et al., 1998; Thiébaud et al., 1999; Sprössler et al., 1999). Sufficient electrical coupling between the cell and the electrode for extracellular signal recording is achieved only when a cell or a part of a cell is located directly on top of the electrode.

In this paper, we present experiments where dissociated cardiac myocytes were cultured in high density on

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FET devices. Recordings of beating myocytes syncytium were taken using the FET system and if necessary patch clamp techniques were used simultaneously to verify the intracellular signals of cells coupled to the gates. Cardiac stimulants (isoproterenol, norepinephrine) and relaxants (verapamil, carbamylcholine) were used to verify effects on the heart cells in terms of the changes of extracellular signal shapes in the presence of different agents. The present study demonstrates the sensitivity of the recording system in discriminating different ionic signal shapes.

2. Materials and methods

2.1. Solutions and reagents

Cell culture reagents were obtained from Sigma: F10 HAMS (N1387), HBSS (H6648), FCS (F7524), pen-strep mixture (P0906), L-glutamine (G7513), trypsin-EDTA (T4049), DNase II (D8764). ITS (51300-036) was obtained from Gibco.

Standard recording solution was made from 5 mM KCl, 150 mM NaCl, 1 mM MgCl₂, 10 mM HEPES, 2.5 mM CaCl₂, 10 mM Glucose, pH 7.38 at 24 °C, adjusted with 1 N NaOH.

The different concentrations of all the pharmacological agents used in this study were made up using the standard recording solution. The stock solutions of all the agents were made up using appropriate vehicles (in bracket): Isoproterenol bitartrate (1/10 N HCl), arterenol bitartrate (1/100 N HCl), verapamil hydrochloride (water), carbamylcholine chloride (water), and atropine sulphate (water). They were all purchased from SIGMA.

2.2. Cell preparation

Embryonic cardiac myocytes were prepared as described before (Denyer et al., 1999). Briefly, hearts of

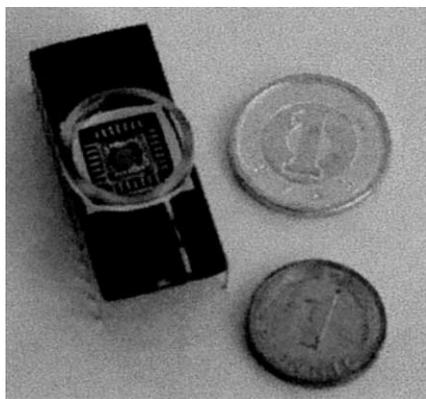


Fig. 1. FET recording devices: the die in the middle of the ceramic chip carrier forms together with the two glass rings a cell culture dish.

embryonic day 15–18 Sprague–Dawley (Charles River, Sulzbach, Germany) rats were removed, minced and placed into ice cold Ca/Mg-free Hanks balanced salt solution (HBSS). The chopped hearts were then pooled and washed gently for three to four times to remove traces of blood and other non-heart tissues. After washing, the HBSS was replaced with crude 0.05% trypsin and after 8 min incubation at 37 °C, the supernatant was discarded. The dissociation cycle then began with 2 ml of 0.05% crude trypsin for 8 min preceded by the addition of 100 µl DNase type II solution (10 000 units per ml) for 1–2 min. The resulting supernatant was collected and added into the stop solution (to block trypsinization) of HAM F10 solution containing 36% fetal calf serum (FCS), 0.5% insulin, transferrin, selenite, (ITS) solution, 6 mM of L-glutamine and 2 ml of penicillin/streptomycin mixture (5000 U per 5 mg/ml) for every 100 ml of F10. This cell suspension was then centrifuged at 1500 rpm for 5 min. The pellet was resuspended using HAMS F10 containing 10% FCS, 0.5% ITS and 6 mM glutamine and 2 ml of antibiotic mixtures. The above dissociation was repeated four to five times or until all the heart tissue had been dissociated. All the procedures were carried out under sterile conditions.

The subsequently yielded cell suspensions were pooled and incubated for 1.5–2 h at 37 °C. This incubation allowed cell debris and fibroblasts to adhere to the flask surface preferentially over cardiac myocytes leaving a higher myocytes to fibroblasts ratio. The final cell suspension was counted and adjusted accordingly. About 20 µl of 1–2 million cells per ml suspension was used, plated onto pre-cleaned and extracellular matrix (10 µg/ml fibronectin in HBSS) primed FETs.

2.3. Extracellular recording

Recordings of the extracellular membrane voltage were obtained using n- or p-channel FET arrays with non-metallized gates. The size of the gates of the 16 FET's are from 16 × 3 µm² down to 5 × 1 µm² and are arranged in a 4 × 4 matrix on 200 and 100 µm centers. The FET arrays were fabricated using conventional integrated circuit technology. The arrays were mounted on a 28 DIL chip carrier and partially encapsulated to form an electronic culture dish (Fig. 1). The details of the fabrication process and the electronic amplifier systems are described elsewhere (Offenhäusser et al., 1997; Sprössler et al., 1998, 1999).

All measurements were carried out with an Ag/AgCl wire as a reference electrode. The culture medium on FETs was replaced with standard recording solution and equilibrated in the incubator before experimentation. The devices were mounted in preamplifier headstages, which were connected to the main amplifier system, operated by a standard PC (500 MHz, Pentium

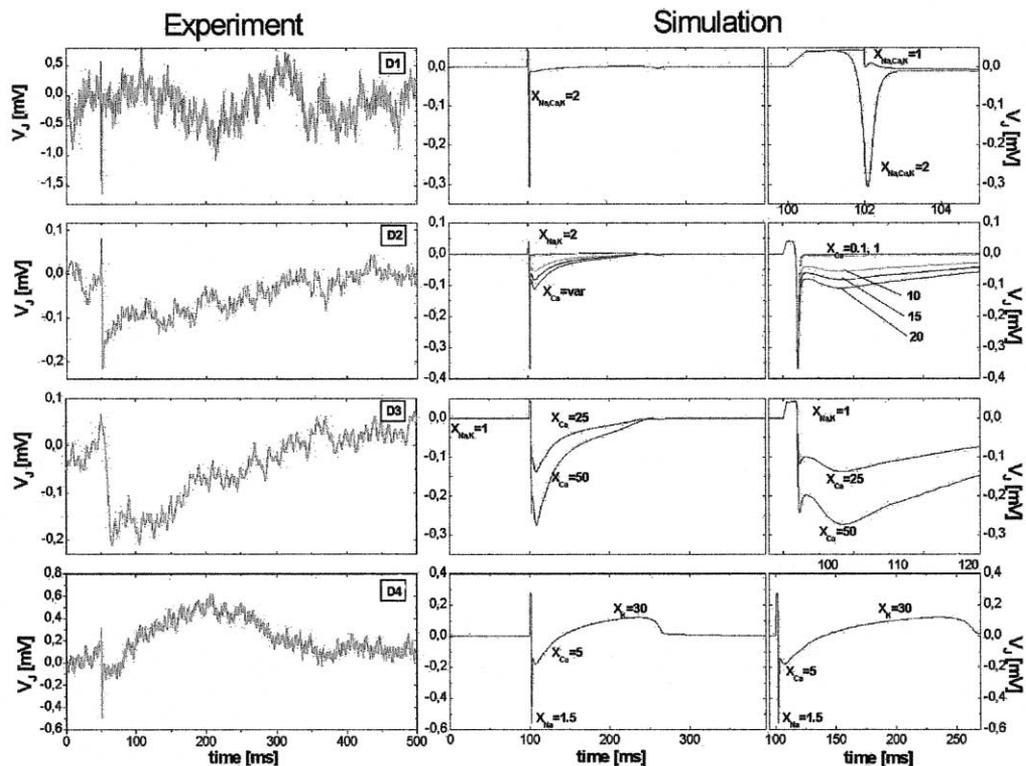


Fig. 2. Extracellular recordings (left) of action potentials from rat cardiac myocytes with FET and their simulation (right) with a simple point-contact model. The D1, D2, D3 and D4 subtypes were caused by different scaling of the ion current densities in the active area (for details see text). The frequency to observe the subclasses D2–D4 under standard recording solutions is below 5%.

III). Recordings were taken using a multifunction I/O Board (PCI-6071 E, National Instruments, Austin USA), which was controlled by the MED64-conductor software (Ver. 2.1, Panasonic, Japan).

2.4. Pharmacology

To stimulate changes in the various membrane currents of the cardiac myocytes, some well-established cardio-stimulants (isoproterenol, ISO and arterenol bitartrate (norepinephrine), NA) and relaxants (verapamil, VP; carbamylcholine, CARB) were used, which were prepared using the external solution. For the drug application a simple protocol was employed: first a basal recording of cultured myocytes on a particular FET was recorded for several seconds, followed by completely replacing the standard recording solution with one that containing either ISO, NA, VP, or CARB. After recordings in the presence of drugs for 60 s have been obtained, the cell layer was gently washed five times at 1 min intervals.

3. Results and discussion

3.1. Extracellular signal shape

The signal shapes, which were, differ from the shapes

of the intracellular recorded action potentials (e.g. patch-clamp). The extracellular signals obtain with our extracellular recording system show different shapes and amplitudes up to 25 mV as shown before (Sprössler et al., 1999). The observed FET-signals can be divided into three major categories A, B, and D (Sprössler et al., 1999). The categories A and B are based only on the contribution of the passive elements of the cell membrane in contact with FET, whereas signals with contribution of the ion current flow across the cell membrane in contact with the FET are categorized in D1–D4 (Fig. 2, left part). The positive upstroke at the beginning of the four signals is attributed to the stimulus current injected by neighboring cells. The fast negative spike is due to a coupled Na^+ ion current and the slower negative and positive continuation can be explained by different intensities of Ca^{2+} and K^+ ion currents in the junction area between cells and sensor. To describe the different shapes we used the time course of the ion-currents given by the HEART software V4.8 introduced by the Noble group (DiFrancesco and Noble, 1985; Noble, 1997) as discussed before (Sprössler et al., 1999).

In order to simulate the different signal shapes, we described the coupling of the cells to the FET-sensor by using the point-contact model (Regehr et al., 1989; Fromherz et al., 1991; Pancrazio et al., 1999; Sprössler

et al., 1999). By considering a simple equivalent circuit, it is possible to describe the principles involved in the recording using FET. Fig. 3 shows a schematic circuit of a cell in contact with the transistor including the electrical equivalent circuit. The separation of the cell membrane and the gate results in an extended cleft of electrolyte, which can be described by a specific conductance g_J ; the specific capacitance of the gate is c_{JG} . The membrane region beneath the transistor as well as the free part of the membrane is described by a specific membrane capacitance c_M . Incorporated in this membrane are various channels with time- and voltage-dependent and independent specific ionic conductances contributing to the current through the membrane. The extracellular voltage V_J is determined by Kirchoff's law with the current i_{JM} through the attached membrane, the capacitive current through the gate oxide and the current along the seal (Fig. 3).

$$c_{JG} \frac{dV_J}{dt} + g_J V_J = c_M \frac{d(V_M - V_J)}{dt} + \sum_i i_{JM}^i \quad (1)$$

The ion channels in the contact region may have different opening properties or different densities indicated by $i_{JM}^i = X_i i_M^i$ with X_i used as scaling factors. By assuming that the capacitive current $c_{JG}(dV_J/dt)$ through the gate can be neglected and that the voltage across the attached membrane is similar to the intracellular voltage Eq. (1) can be simplified as follows:

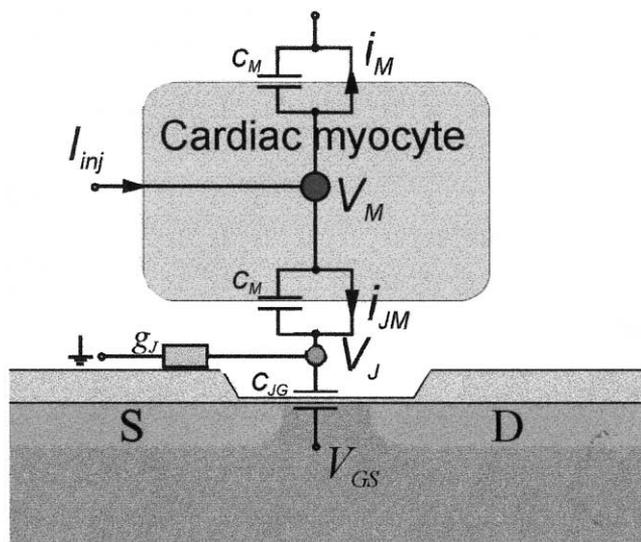


Fig. 3. Point-contact model: the attached and free membrane of the cell is described by the membrane capacitance c_M . The gate oxide is described by a capacitance c_{JG} and the junction by seal conductance g_J . The extracellular voltage V_J is recorded by the transistor and the intracellular voltage V_M can be monitored by micropipette. Further details can be found in the text.

$$V_J = g_J \left(c_M \frac{dV_M}{dt} + \sum_i X_i i_M^i \right) \quad (2)$$

We simulated the measured signal shapes with the given ion currents $i_M^{\text{Na}, \text{K}, \text{Ca}}$ for a single cardiac myocyte cell by different scaling factor $X_{\text{Na}, \text{K}, \text{Ca}}$, for the ion currents in the junction area using the time course of the ion-currents given by the HEART software V4.8 introduced by the Noble group (DiFrancesco and Noble, 1985; Noble, 1997) as discussed before (Sprössler et al., 1999). The specific membrane capacitance, respectively, the specific seal conductance were assumed to be $c_M = 1 \mu\text{F}/\text{cm}^2$, respectively, $g_J = 1 \text{ mS}/\text{cm}^2$. From these simulations, we are now able to assign the influence of particular current activity onto the extracellular signal shape. Subtype D1 describes a signal where all membrane currents in the contact area are scaled with the same factor X_i . Subtype D2 and D3 describe situations where the Ca-current is comparatively enhanced and dominates the extracellular signal. Subtype D4 shows a major contribution of K-current in the contact area. It should be noted that the most frequent recording subclass is D1 and that the subclasses D2–D4 are usually observed with a frequency below 5%.

In Fig. 4, the effects of different stimulants and relaxants on the extracellular signal shape are shown. Once a certain drug is administered, the activity of the ion-channels in the membrane is either reduced or enhanced. We were able to detect the signal shape change due to the drug response by comparing the signal shapes before and after drug administration. It shows that in case of ISO the signal shape changes from a D1- to a D2- or D3-subtype (the slow Ca-currents have not been simulated in Fig. 2). Isoprenolol is a β -adrenoceptor agonist and well-known cardioactive agent that stimulates G-proteins and enhances the L-type calcium channel activity, which causes an increase in Ca^{2+} signal. It can be clearly seen that after administration of ISO the Ca^{2+} signal increased (Fig. 4A and B). In addition an increase in beating frequency was observed. NA binds to a G-protein coupled receptor, which increases Ca^{2+} -current activity. Fig. 4C and D shows the extracellular recordings before and after administration of NA and clearly indicates the enhanced Ca^{2+} -current activity. Verapamil blocks the L-type calcium channel activity. In Fig. 4E, a typical D2 subtype is shown, which is characterized by an enhanced Ca^{2+} current. Application of VP led to a reduced Ca^{2+} -current activity (Fig. 4F). Carbamylcholine increases K^+ conductance in cardiac myocytes which can be seen in Fig. 4G and H, where the signal changes from a D1-subtype to a D4-subtype indicating an increased K^+ current. With the addition of stimulants/relaxants, the proportion of channels expressing D2, D3, and D4 signal types rose markedly from control levels of less than 5% to 20–50% (Table 1). These changes in the proportion of signal types were statistically significant,

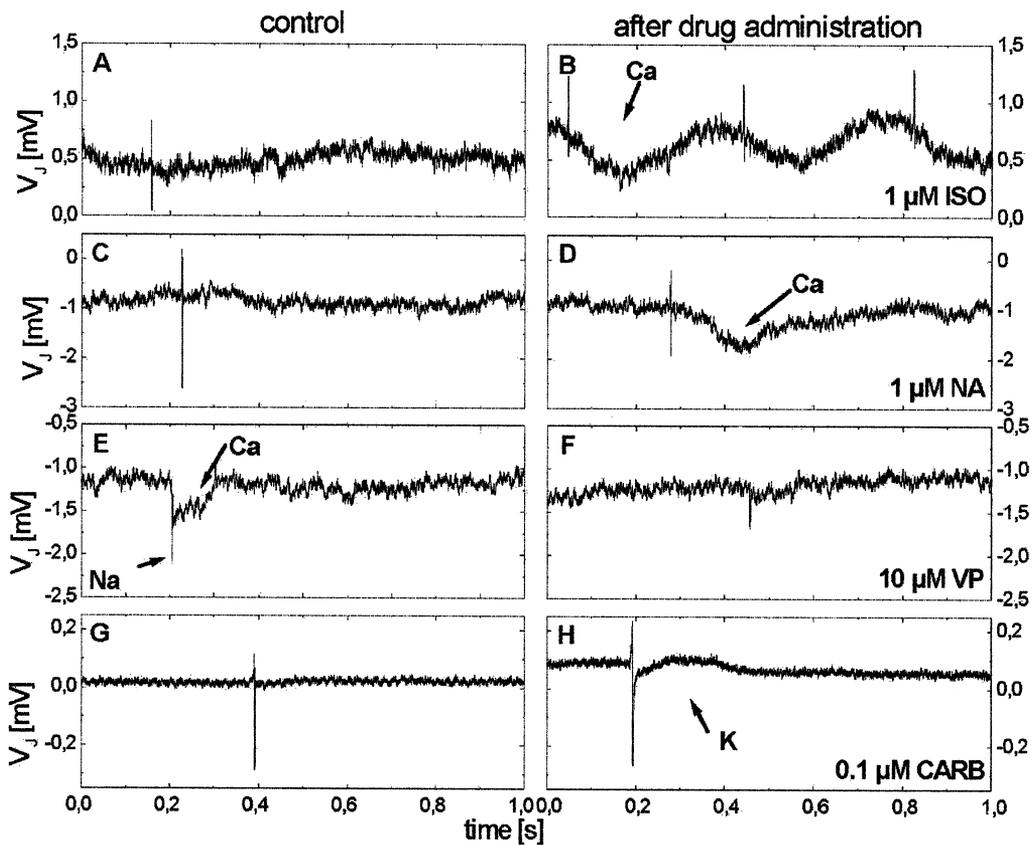


Fig. 4. Change in the extracellular signal shape after addition of isoproterenol, norepinephrine, verapamil and carbamylcholine.

Table 1

Proportion of changes in the extracellular signal shape due to drug administration

Stimulants/relaxants	# Chips	# Channels	Signal shape transition	Proportion of changes in signal shape ^a	Z	Confidence interval (95%)
Isoproterenol	10	58	D1–D2/D3	0.27*	4.0	0.16–0.38
Norepinephrine	11	90	D1–D2/D3	0.22*	3.5	0.13–0.31
Verapamil	9	90	D2–D1	0.30*	4.6	0.21–0.40
Carbamylcholine	5	39	D1–D4	0.51**	6.4	0.35–0.67

The confidence interval was calculated using the Wald equation: $p = \# \text{ changes} / \# \text{ experiments} = S/N$; $[p - (1.96\sqrt{p(1-p)/N})] \leq CI \leq [p + (1.96\sqrt{p(1-p)/N})]$. Under control conditions, the proportion of channels exhibiting D1, D2, D3, and D4 waveforms were 0.90, 0.05, 0.01, and 0.04, respectively.

^a * $P < 0.05$, ** $P < 0.01$.

suggesting that observed modulation of extracellular waveforms may be useful in distinguishing the basis of pharmacological modulation.

4. Conclusion

In summary, this paper describes the realization of cell-transistor hybrids for biosensor applications based on the direct coupling of cardiac myocytes with transistor based microelectronic devices. Depending on the cou-

pling mechanism and on the relative position of the cells seeded on the sensor spot, the signals on the device differ in shape and intensity, a fact which can be explained by the point contact model. Relevant and well-established cardio-stimulants and relaxants were applied in order to test the ability of this system as an in vitro pharmacological bioassay. It shows that isoproterenol and arterenol bitartrate (norepinephrine) caused an increase in Ca^{2+} signal as they are known to open Ca^{2+} channels of the cell membrane, whereas verapamil diminished the Ca^{2+} signal and carbamylcholine increased the K^{+} signal.

Acknowledgements

The authors gratefully thank Professor Dr Wolfgang Knoll (Max Planck Institute for Polymer Research in Mainz) for his generous support and Professor Dr Alfred Maelicke (Institute for Physiological Chemistry and Pathobiochemistry at the Johannes Gutenberg-University in Mainz) for the possibility to use animal facilities. The financial support of the Ministerium für Bildung, Wissenschaft, Forschung und Technologie (BMBF) project No. 0310895 is gratefully acknowledged.

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