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Original article

Validation of the use of field effect transistors for extracellular signal recording in pharmacological bioassays

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Abstract

The use of field effect transistors (FETs) in biomedical research has been in rapid progression in recent years. The present study aims to demonstrate a quantitative use of these devices in pharmacological bioassays. FETs were made as a 4×4 matrix of gates with a width of 200 μm separating each gate. The surface of the FETs (silicon oxide), covered with a layer of laminin, fibronectin, or nitro cellulose was suitable for cell adhesion. The cultured dissociated cardiac myocytes were spontaneously active within 24 to 48 h after initial plating. Simultaneous intracellular patch clamp recordings were used to verify the electrophysiological signals of cells that were coupled to the gates. All positive chronotropes (isoproterenol, norepinephrine) and negative chronotropes (verapamil, carbamylcholine, SDZ PCO400) showed their characteristic effects on heart cells in terms of changes of beat frequency. As the myocytes were in a complete syncitium on each FET, the cells need not be directly coupled to the gate in order to detect any ionic changes. This enables global cellular responses to be analyzed. The system also offers an opportunity to study the interconnections and communications between different cells. Furthermore, the changes of signal shapes in the presence of different agents could also be detected. The present study demonstrates how versatile and sensitive this recording system is in distinguishing different ionic signal shapes. The authors believe that this system has the potential to replace some currently employed in vitro methods, offering an alternative, which can substantially reduce animal use in pharmacological experiments.

Keywords: Cardiac myocytes; Heart rate; Extracellular recording; Field effect transistors; Methods; Bioassay; Rat

1. Introduction

For many decades, evaluation of the effects of pharmacologically active agents has been based on a variety of functional in vitro bioassays. These could be in a form of a tissue-organ bath to monitor mechanical and pressure changes (e.g., Langendorff preparations) or electrophysiological techniques (e.g., patch clamp recordings). However, all the currently available methods can only be used for short-term recordings (8 to 10 h at most) and, in most cases, the tissues or cells are irretrievably damaged. Furthermore, all the drug studies that utilize electrophysiological techniques are extremely time-consuming. A reliable long-term recording system, which is noninvasive, efficient, reproducible, and durable (> 24 h to days), would be especially useful when the recovery of cellular functions upon interventions are important to the investigation. The recent use of biomicroelectronic interfaces may provide the answer. Not only do these devices provide a means of long-term recording for the monitoring and assessment of cellular functions, but they also significantly reduce the use of animals.

The use of field effect transistors (FETs) and multielectrode arrays with live cells recordings in recent years has been well documented (Fromherz et al., 1991; Gross et al., 1995; Krause et al., 1999; Offenhäusser et al., 1997a, 1997b; Sprössler et al., 1998, 1999). Preliminary studies have utilized basic pharmacological agents to characterize their effects on cells cultured on the microelectrodes (Denyer et al., 1998, 1999). Despite many attempts to demonstrate the use of these microelectronic devices in the past few years, few have shown any real indication as to their reliability in in vitro functional study applications. Furthermore, the signal strength is often only in the range of

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tens to hundreds of microvolts and, coupled with low signal-to-noise ratios, no identifiable signal shapes can be recognized. The system used in the current study is capable of performing an on-line sampling of 16 channels (with the potential of recording up to 64 simultaneously). Further reduction of the noise level was achieved due to the introduction of a new modified power supply (Krause et al., 1999). We are now able to detect signals from a syncitium of cardiac myocytes, which have been cultured on different chemically modified surfaces (fibronectin, laminin, or nitrocellulose) (Lochner et al., 1990).

This paper presents the steps that have been taken to increase the reliability of the system and shows high amplitude extracellular recordings of dissociated cardiac myocytes on FETs with excellent signal-to-noise ratios. For the first time, quantitative and reproducible recordings of the effects of drugs on myocytes cultured on the FETs have been obtained. We aim to illustrate the use of the FET system as a future pharmacological investigative tool.

2. Methods

2.1. Experimental setup

Recordings of cellular signals were obtained using n- or p-channel FET arrays or planar metal-microelectrode arrays (gold electrode surface), which were electrically connected to a commercially available FET system to form an extended gate electrode (EGE) array (Krause et al., 1999). The EGEs were used with an unmodified bare metallic gold surfaces with diameters down to 6 μm. The surface layers of both types of devices were made of SiO_2. The fabrication and encapsulation processes for the FET and EGE devices (Fig. 1) have been described elsewhere (Offenhäusser et al., 1997a; Krause et al., 1999; Sprössler et al., 1999). Both types of device were mounted and encapsulated together with a glass ring fixed onto the chip carrier to form a small culture dish. The free surface areas (maximum fluid volume) of the devices are 0.06 cm^2 (300 μl) and 0.38 cm^2 (1500 μl) for the FET and EGE, respectively. All measurements were carried out with an Ag/AgCl wire as a reference electrode, which defines the gate potential. The devices were mounted in special preamplifier headstages, which were connected to a main amplifier system (Krause et al., 1999), operated by a standard PC (500 MHz, Pentium III). Readings were taken using a multifunction I/O Board (PCI-6071 E, National Instruments, Austin, TX, USA), which was controlled by the MED64-conductor software (Ver. 2.1, Panasonic, Japan).

2.2. Cell preparation

Hearts of embryonic day 15 to 18 Sprague Dawley (Charles River, Sulzbach, Germany) rats were used. The procedure for culturing dissociated cardiac myocytes has been described previously (Denyer et al., 1998). In brief, the hearts were removed, minced, and trypsinized. The resulting supernatant was collected and added into the stop solution (to block trypsinization) of HAMS F10 solution containing 36% foetal calf serum (FCS), 0.5% insulin, tranferrin, selenite, ITS solution, 6 mM of L-glutamine, and 2% of penicillin/streptomycin mixture (20 mM stock) 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, 6 mM glutamine, and 2% of antibiotic mixtures. About 20 μl of

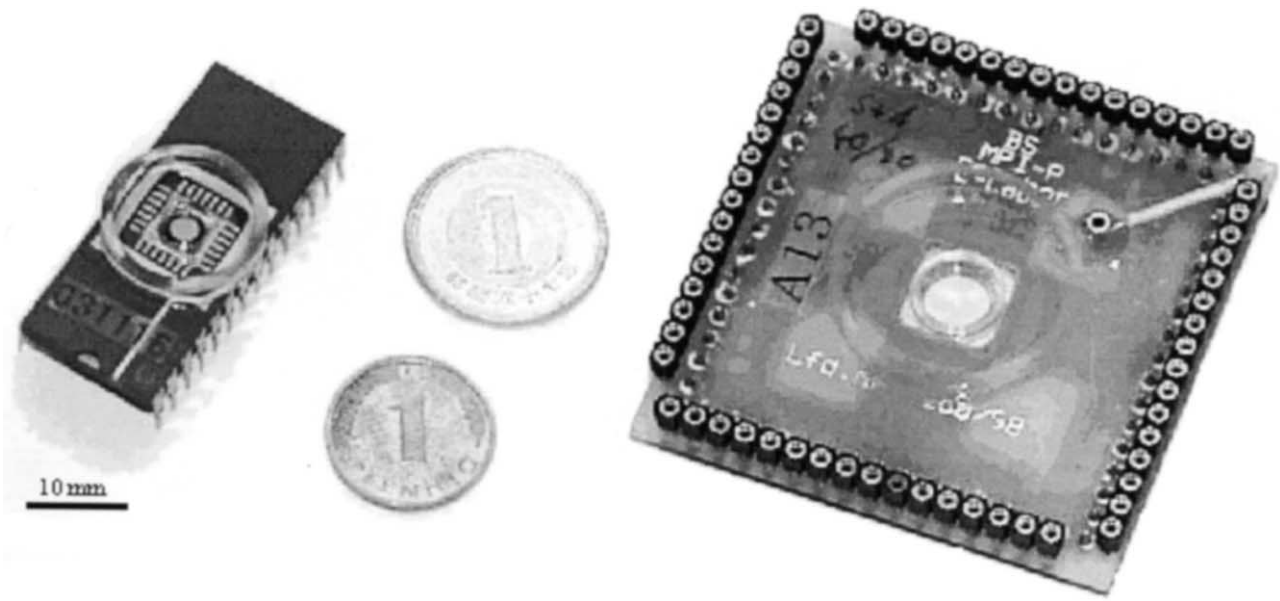

Fig. 1. Bioelectronic devices (MPIP-1999) Left: FET device (16 channels); right: EGE device (64 channels). The larger coin is a Japanese one yen coin.

1 to 2 million cells per ml suspension was plated onto FETs or EGEs with extracellular matrix proteins.

2.3. Patch clamp recording

The culture medium on FETs was replaced with standard patch clamp external solution (in mM: KCl, 5; NaCl, 150; $MgCl_2$, 1; HEPES, 10; $CaCl_2$ 2.5; glucose, 10; pH 7.38 at 24°C, adjusted with 1 N NaOH) and equilibrated in the incubator for 30 min before experimentation. For patch clamp recordings, the cardiac myocytes were bathed in external solution under a constant temperature of 35°C using a thermostatically controlled platform. Intracellular recordings were made by patch clamp technique using glass tips (Hilgenberg, Germany) filled with standard internal patch clamp solution (in mM: $C_6H_{11}O_7K$, 125; KCl, 20; $CaCl_2$, 0.5; $MgCl_2$, 2; HEPES, 10; EGTA, 5; pH 7.3 at 24°C, adjusted with 1 N KOH.). The tip impedance usually fell between 3 to 7 MΩ. The signals were monitored using an EPC9 patch clamp amplifier and the TIDA software, version 4.11 (both from HEKA Elektronik, Germany) operated by a standard PC (500 MHz, Pentium III).

2.4. Pharmacology

To validate the applicability of FETs in pharmacological bioassays, some well-established positive chronotropic agents: isoproterenol (ISO) and norepinephrine (NE) and negative chronotropes verapamil (VP); carbamylcholine (CARB); and SDZ PCO400 (SDZ) were used. Atropine (ATROP, 10 μM) was used to antagonize the response of CARB in drug combination experiments. The different concentrations of all the pharmacological agents used in this study were made up using the external solution. The stock solutions of all the agents were made up using appropriate vehicles (in bracket): ISO (1/10 N HCl), NE (1/100 N HCl), VP (water), CARB (water), and ATROP (water). The appropriate concentrations of the pharmacological agents used were prepared using the external solution. A simple drug-cycle protocol was employed. A basal beat frequency of cultured myocytes on a particular FET or EGE was recorded for 60 s. This was followed by completely replacing the external solution with one that contained either ISO (10 nM, 100 nM, 1 μM) or NE (10 nM, 100 nM, 1 μM). Recordings were made in the presence of drug for 60 s. The cell layer recovery was achieved by washing gently 5 times at 1 min intervals. The same procedures were carried out with the negative chronotropes. In drug combination experiments, different agents were administered on the cell layer in each corresponding group (A to D) in the following order as indicated: A: NE (0.1 μM) vs. CARB (1.0 μM) vs. ATROP (10 μM); B: VP (1.0 μM) vs. NE (10 μM);C: VP (1.0 μM) vs. ISO (10 μM); D: NE (0.1 μM) vs. CARB (10 μM).

2.5. 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), and DNAse II (D8764). ITS (51300-036) was obtained from Gibco. Isoproterenol bitartrate, arterenol bitartrate verapamil hydrochloride, carbamylcholine chloride, and atropine sulphate were all purchased from SIGMA. SDZ PCO400 was from Leo Pharmaceuticals.

2.6. Calculations and statistics

The results are expressed as beat frequency (bpm) mean ± S.E.M. of the control (in the absence of drugs) and test (in the presence of drugs). The differences were analysed using Student's unpaired t test; $P < .05$ was taken as statistically significant.

3. Results

3.1. Signal shapes of the extracellularly recorded signals

Cardiac myocytes were cultured on the FET surface and an optical imagine is shown in Fig. 2. The sensor spots (indicated by circles) of the FET sensor can be seen through the cell layer. The system detects the current in the cleft between the cell and the recording device. Therefore, the signal shapes obtained with the present extracellular recording system differ from the shapes of the intracellular action potentials obtained using patch clamp. Fig. 3 shows the intracellular patch clamp signal of a single cardiac myocyte on the sensor (upper trace) and the extracellular signal recorded with the FET chip simultaneously (lower trace). The action potentials were verified by visually identifying the cellular contractions of the cardiac myocytes. However, depending on the age of the culture and the cell density, the force of contraction and beat frequency vary. The increase in beat frequency in the presence of ISO (0.1 μM) is shown in Fig. 4. In the first 29 s the heartbeat was stable at 20 ± 3 bpm (n = 11). Between 29 and 50 s the drug was administered by exchanging the bath solution with extracellular solution containing the drug. During this time period (not shown) the baseline of the signal was unstable. After 50 s the heartbeat frequency was 215 ± 6 bpm. Channels 3, 10, 11, 15, and 16 had no electrical contact to the chip.

All the obtained extracellular responses on different FET chips showed various signal shapes and amplitudes of up to 24 mV. These shapes can be divided into four categories, D1–D4 (Sprössler et al., 1999). It has been demonstrated that the different extracellular signal shapes are related to different ion current densities between cell layer and FET device surface. It is therefore possible to distinguish the corresponding ion channel activity in the presence of

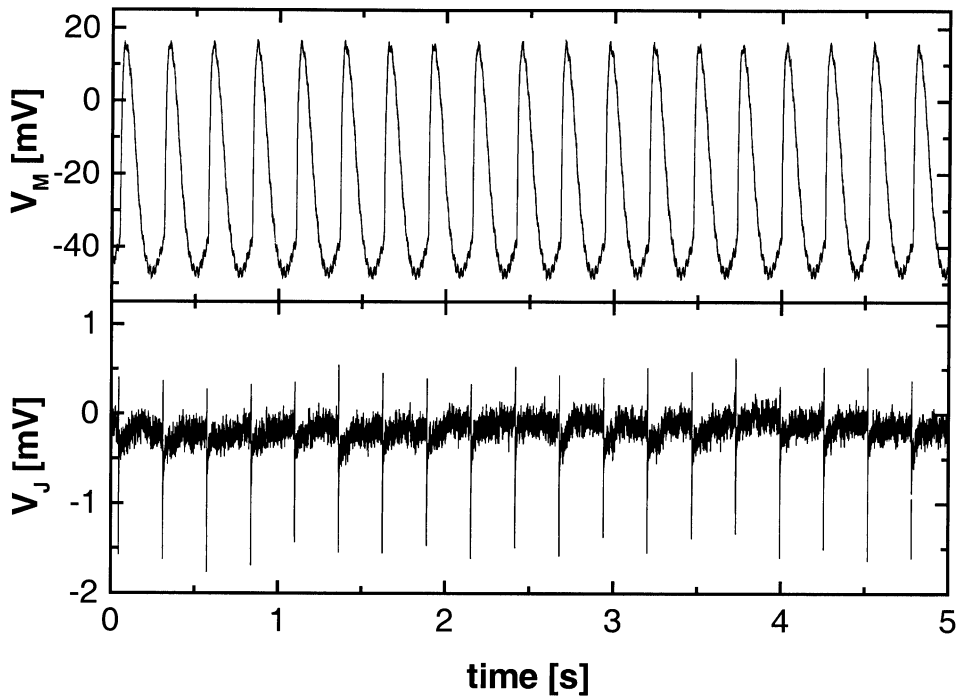

Fig. 2. Traces showing simultaneous heart cell recordings. Upper trace: Intracellular action potentials recorded with patch-clamp. Lower trace: Extracellular signals recorded with FET device.

different positive and negative chronotropic agents. Once a particular drug is administered, the activity of the ion channels on the membrane is either hindered or elevated. We were able to detect the signal shape changes due to the presence of these agents by comparing the signal shapes before and after drug administration (Fig. 5). When 1 μM

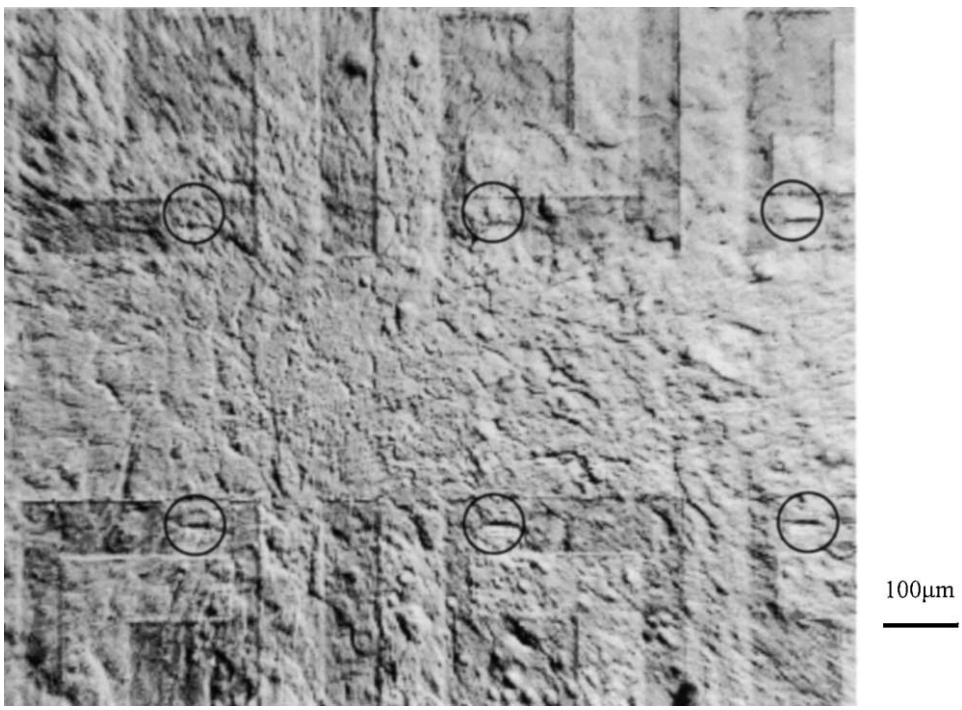

Fig. 3. Optical image of a cardiac myocyte cell layer on a FET sensor surface. The six sensor spots (indicated by circles) of the FET sensor can be seen through the cell layer.

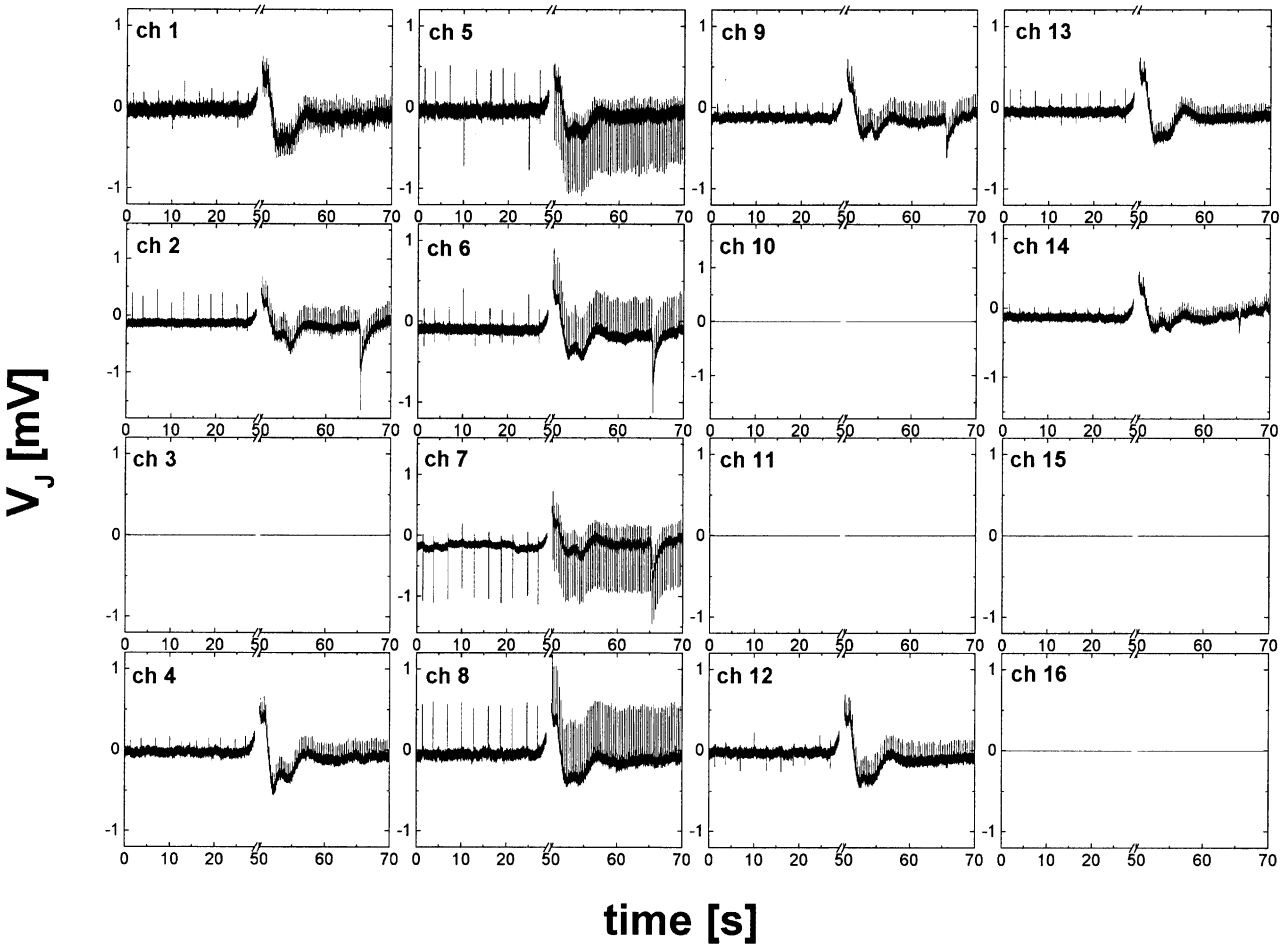

Fig. 4. The effect of 0.1 μM isoproterenol on cells cultured on transistor chip. In the first 29 s the beat frequency was stable at 20 ± 3 bpm. Between 29 and 50 s, the drug was administered by exchanging the bath solution with extracellular solution containing the drug. During this time the baseline of the signal is unstable due to temperature changes or undefined gate potentials (not shown in plot). After 50 s the heartbeat frequency was about 215 ± 6 bpm. Channels 3, 10, 11, 15, and 16 had no electrical contact.

NE (gray box) was added to the cell layer, the Ca^{2+} signal increased (right inset). This is due to an increased in Ca^{2+}-channel activity in the cell-sensor contact area (Sprössler et al., 1999).

3.2. The positive chronotropic effects of isoproterenol and norepinephrine on myocytes

The partial concentration-response curves for both ISO and NE are shown in Fig. 6. The results are expressed as percentage increase of the basal beat frequency. ISO was shown to be slightly more potent than NE in elevating the beat frequency, although it was not significant ($P > .05$).

3.3. The negative chronotropic effects of carbamylcholine, verapamil, and SDZ PCO400 on myocytes

Administration of these negative chronotropes caused the beat frequency of the cells to decrease. The partial concentration-response curves for the three drugs are shown in Fig. 7. CARB was the most potent with a relative potency of CARB $\geq$ VP > SDZ in this preparation.

3.4. The effects of sequential administration of isoproterenol, carbamylcholine, atropine, SDZ PCO400, and verapamil on myocytes

In four independent experiments (A–D), the ability of the cells to undergo rapid sensing to the changes of the environment and the stability in long-term experiments were investigated:

Experiment A: NE (0.1 μM), CARB (1 μM), ATROP (10 μM) After the administration of NE the heart rate elevated from 16 ± 2 (n = 9) to 235 ± 12 bpm (n = 9) ($P < .001$), which was rapidly reduced to 7 ± 1 bpm (n = 9) by adding CARB. The inhibitory effect of CARB was reversed to 150 ± 14 bpm (n = 9) after ATROP was administered.

Experiment B: VP (1.0 μM), NE (10 μM) Verapamil reduced the initial beat frequency from 250 ± 20 bpm

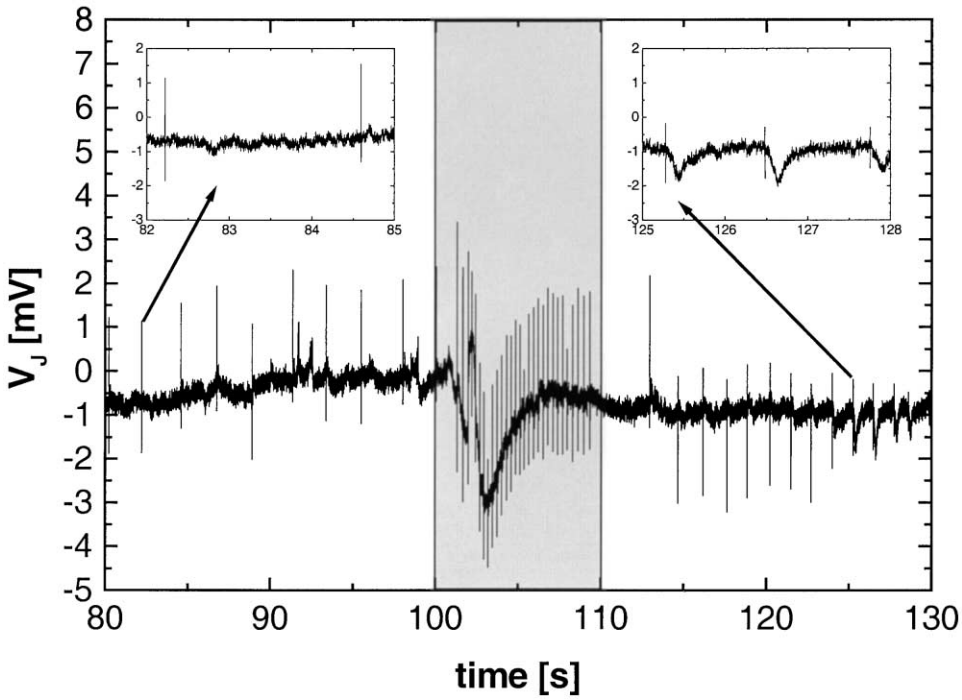

Fig. 5. Administration of 1 μM norepinephrine (gray box) changed the signal shape of the extracellular recording. The slow part of the signal (right inset) is due to elevated Ca^{2+}-channel activity of the cell in the sensor contact area.

(n = 11) to 8 ± 1 bpm (n = 11). The heart rate was partially restored (110 ± 10 bpm, n = 11) when NE was subsequently administered.

Experiment C: VP (1.0 μM), ISO (10 μM) The addition of a high concentration of VP caused a complete cessation of the basal beat frequency (105 ± 8 bpm, n = 6). After 3 min

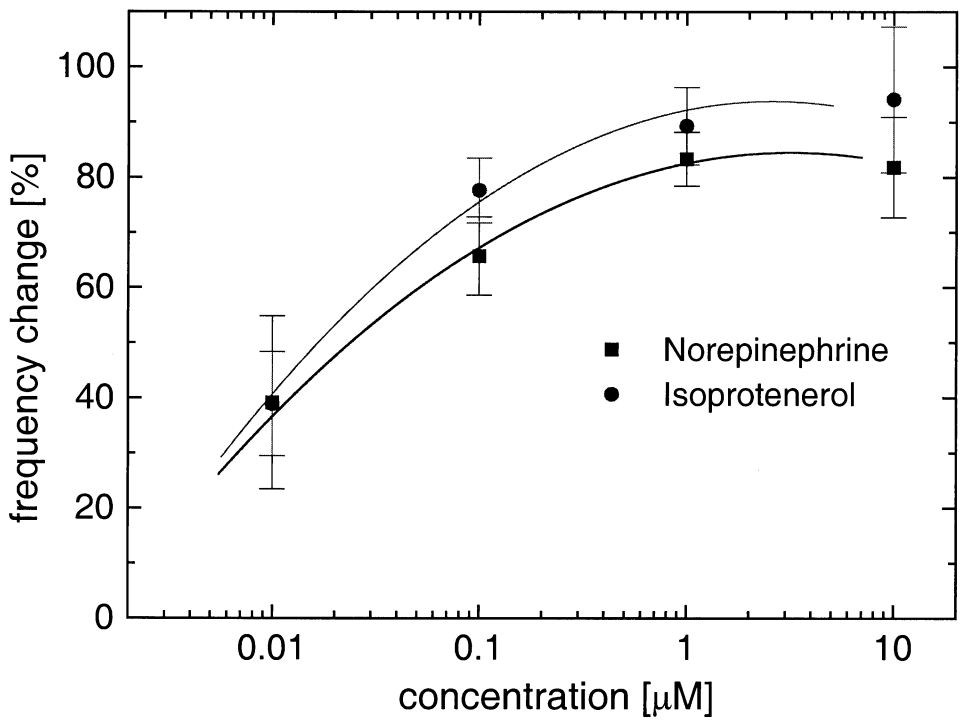

Fig. 6. The changes in the beat frequency of heart cells in the presence of norepinephrine and isoproterenol (0.01–10 μM).

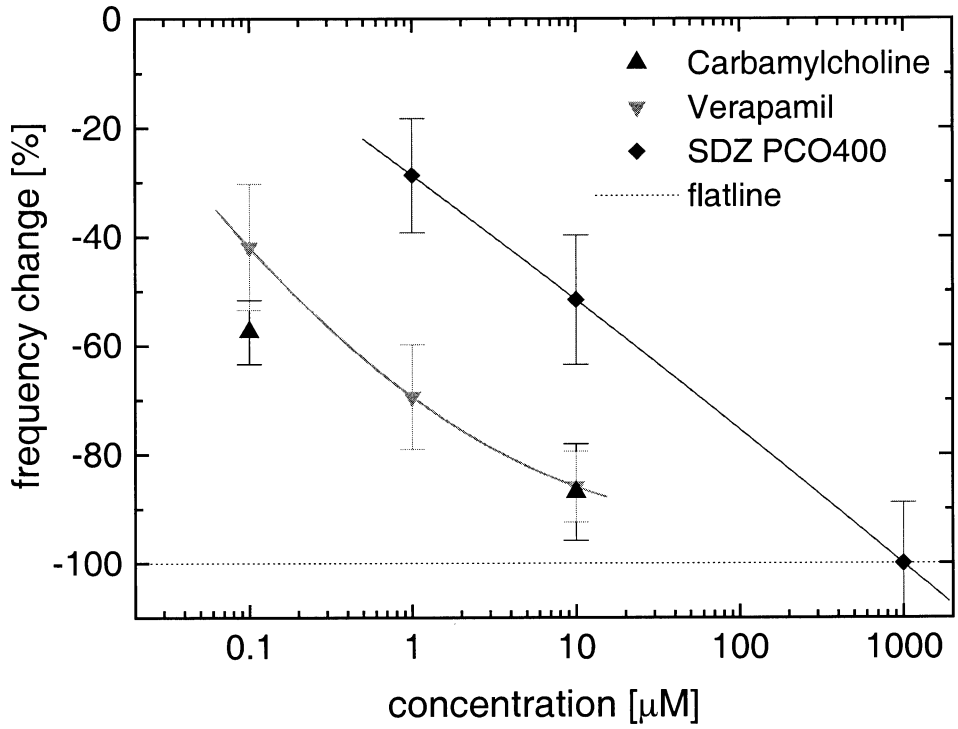

Fig. 7. Concentration-response curves of the three relaxants. Carbamylcholine and verapamil appeared to have similar negative chronotropic effects on cells cultured on the chips. SDZ PCO400 appeared to be the least potent. The dashed line indicates complete cessation of heartbeats.

flat line, some activity of the cell layer was restored, albeit very slowly, by adding ISO (17 ± 3 bpm, $n = 6$).

Experiment D: NE (0.1 μM), CARB (10 μM) The heart rate of 14 ± 3 bpm was elevated to 96 ± 8 bpm ($n = 5$) whenNE was administered. This effect of NE on the beating heart cells was inhibited by CARB to 7 ± 2 bpm ($n = 5$).

The responses to the drugs administered showed that the sensor could detect rapid cellular physiological changes. The cells were stable over several hours and in some cases the cells completely recovered after washing.

4. Discussion

The present study demonstrates that some basic information, which is of direct physiological/pharmacological importance, can be obtained from cells cultured on the FET. The dissociated syncitium of cardiac myocytes was chosen because it provided an electrogenic means to evaluate the sensitivity of the FET system without the use of any artificial stimulation. The use of relevant and well-established cardiac positive and negative chronotropic agents further substantiates the ability of this system as an in vitro pharmacological bioassay. Most importantly, the present study provides a quantitative functional analysis of biological data using microelectronic devices. These devices may be used as a substitute for some of the existing in vitro methods. A review on the use of cell-based biosensors has been provided by Pancrazio et al. (1999).

The cell sensor coupling can be explained by the point contact model (Fromherz et al., 1991; Krause et al., 1999; Pancrazio et al., 1999; Regehr et al., 1989; Sprössler et al., 1999) and has been investigated over several years. Depending on the coupling mechanism and on the relative position of the cells, electrophysiological signals from the device differ in shape and intensity. It is crucial that the cells are in tight contact with the sensor. The signal-to-noise ratio depends strongly on the cell-to-sensor distance and the surface coating. The use of extracellular matrix proteins as contact materials aids cell adhesion onto the silicon chip surface. It was possible to achieve signal-to-noise ratios of up to 25 as the mean noise with the FET system fell in the range of 100–200 μV, with 1–5 mV recording amplitude.

Electrophysiological techniques, such as patch clamp, have been used for many years. Patch clamp recording is proven to be of tremendous value when it comes to detecting physiological/ionic changes and, with the appropriate use of agonists and antagonists, the mechanisms of action of a number of agents can be elucidated. However, this method is very time-consuming and cannot be performed over a long period of time. Dissociated cells are highly motile and often move away from the recording electrode in less than an hour, making long-term recording of a single cell impossible. Unless multiple patch clamps are being employed, there is no way of evaluating the conductivity or connectivity between cells in the same culture. It is difficult to do even when multiple patch clamps are available. In addition, there is a lack of long-term detection methods as the cells are being

damaged after patch clamp recording. The FET system can provide the answer. It can be used as a standard in vitro means of evaluating the presence of different receptors by using appropriate antagonists (e.g., the effect of carbamylcholine was antagonized by atropine). It can characterize the relative potency of the adrenoceptor agonists on the heart (ISO > NE on β_1-adrenoceptors, Fig. 6). It can also detect signal shape changes, such as that observed when NE was administered onto the cells (Fig. 5). The bathing volume is small (300 μl) and only a very small amount of drug is required. The addition benefit of this system is that it can pinpoint the focal point (or foci) of the syncitium and can be used to determine the propagation velocity and the direction of propagation. The bathing solution can also be further analyzed biochemically (e.g., dialysis, high press liquid chromatography) to look for substances that are being released by the cells before and after treatments. Depending on the objective of the investigation, the cells on the device can also be collected for further molecular analysis. In addition, as the gates on each chip are basically a network of detection electrodes and the cells on each chip are interconnected in one way (e.g., by gap junctions) or the other, the recordings thus obtained can be used as a reflection of the physiological responses of the whole or part of an organ. In other words, dissociated cultures from different parts of an organism in vitro can be viewed as a representation of those parts of the organism in vivo. One single culture yields enough myocytes to prepare nearly 100 chips. The use of cell lines is also an option. The combination of cell lines and reusable chip can reduce the use of animal tissues significantly.

The present study demonstrates that the FET system could be used to perform some standard functional studies by detecting extracellular changes of biological signals. Many pharmacological bioassays rely heavily on the appropriate use of in vitro techniques to acquire data of drug profiles before these drugs can proceed to further stages of evaluations. Most of these methods are invariably short term and are not suitable for long-term monitoring of drug actions or cellular recovery, which may take many hours. The use of bioelectronic interfaces, such as the FETs, could provide a system that is sensitive enough to detect changes of the extracellular electrochemical gradients, durable enough to be reused many times, and capable of recording for many hours in the absence of an operator. In principle, it would be possible to keep the cells on the chip and execute a long-term experiment over several days as long as sterility is maintained.

The immediate goals are to further improve the longevity and sensitivity of the chips. Although more improvements and refinements are required, the system at its current status is more than adequate and capable of performing certain basic pharmacological investigations, evaluations, and disease investigation. There is little doubt that this approach can substitute or even replace some of the existing electrophysiological techniques in the near future.

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