Extracellular Stimulation of Mammalian Neurons Through Repetitive Activation of Na\textsuperscript{+} Channels by Weak Capacitive Currents on a Silicon Chip

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INTRODUCTION

The stimulation of neurons with extracellular electrodes is used in neural prosthetics, such as with brain stimulators (Wichmann and DeLong 2006), cochlea implants (Shepherd and Javel 1997), and retina chips (Stett et al. 2000; Weiland et al. 2005). It is also widely used in physiological studies with brain slices and cultured neurons on planar metal electrodes (Egert et al. 1998; Eytan et al. 2003; Gholmieh et al. 2006; Jimbo et al. 2003; Steidl et al. 2006; Wagenaar et al. 2005) as well as on silicon chips (Fromherz and Stett 1995; Hutzler and Fromherz 2004; Jenkner et al. 2001). In all cases, a capacitive or a Faradaic current across the electrode/electrolyte interface gives rise to a current and a voltage gradient in the extracellular space that polarizes the neuronal membrane such that action potentials are elicited. Theoretical investigations on cell models as well as the choice of suitable stimulus parameters have been considered in some detail (Kuncel and Grill 2004; McIntyre and Grill 2002; Merrill et al. 2005; Ranck 1975; Wagenaar et al. 2004). Although commonly used, standard protocols come at the risk of damaging the neurons by irreversible electroproetion (He et al. 2007; Rubinski 2007; Ryttser et al. 2000) as well as by electrochemical reaction products (Brummer et al. 1983; Harnack et al. 2004; Huang et al. 2001; Merrill et al. 2005).

Here we present electrophysiological experiments on safe extracellular stimulation under well-defined conditions where electroproetion and electrochemistry are avoided. Mammalian cells are studied in the geometry of adhesion on an electrolyte-semiconductor (EOS) capacitor as illustrated in Fig. 1. We used titanium dioxide on silicon to achieve a good insulation without significant Faradaic current for a wide range of voltages and with a relatively high capacitance (Ulbrich and Fromherz 2004; Wallrapp and Fromherz 2006). The system is an important model for the application of integrated chips fabricated by complementary metal oxide semiconductor (CMOS) technology where an insulating and biocompatible passivation layer is essential (Hutzler et al. 2006).

In previous experiments with snail neurons under similar conditions we found that action potentials could be elicited by rather weak falling and rising voltage ramps at the EOS capacitor (Schoen and Fromherz 2007). The data indicated, however, that a similar protocol would not be effective for rat neurons because of their smaller size. To gain insight into the effect of capacitive stimulation of mammalian cells and to develop a suitable stimulation protocol for rat neurons, we here use a model system with the sodium channel Na\textsubscript{v}1.4 from skeletal muscle (Trimmer et al. 1989) that was stably overexpressed in HEK293 cells (Schmidtner and Fromherz 2006).

The paper is organized as follows: after a METHODS section, we first characterize the capacitive polarization of HEK293 cells under whole cell current clamp. We consider the weak activation of sodium channels that is induced by falling voltage ramps. Then we describe the accumulative depolarization of the cells that is induced by a repetitive weak activation of sodium channels. These observations are compared with a numerical simulation. Finally, it is demonstrated that a protocol of repetitive weak stimulation is able to elicit action potentials in cultured rat neurons. In the DISCUSSION section, we consider the limits of safe extracellular stimulation on the basis of our experimental results. The APPENDIX describes theoretical issues of capacitive stimulation in the geometry of cell adhesion that are applied for the evaluation of the experiments and for the numerical simulation.

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were coated with poly-L-lysine (molecular weight 70 to 150 kD, Melsungen, Germany), and exposed to UV light for 40 min. The chips were sterilized with 70% ethanol for 1 h, rinsed with aqua ad (Braun, many) was used that stably expressed the \( \text{Na}^+ \) -subunit of the NaV1.4 channel. The chips were cleaned as for HEK293 cells. They supported by cautious wiping with a tissue wipe (Tork F1; SCA Tissue, Neenah, WI). They were rinsed with Millipore water, dried by a nitrogen jet, sterilized by UV light for 40 min, and coated for 2 h with fibronectin (F2006; Sigma-Aldrich, St. Louis, MO) at a concentration of 10 \( \mu \)g/ml in phosphate-buffered saline (PBS, 14040; Gibco Invitrogen) containing 1% bovine serum albumin (BSA, A8806; Sigma-Aldrich) and 0.1 mg/ml papain (P4762; Sigma-Aldrich) for 15 min in an incubator at 36.4°C. Excess medium was removed and cells were resuspended in 1 ml Dulbecco’s modified Eagle’s medium (DMEM, 61965; Gibco Invitrogen) with 10% fetal bovine serum (FBS, 10270; Gibco Invitrogen). After centrifugation at 300 \( g \) for 10 min, excess medium was removed and cells were preplated in 10 ml DMEM onto a 92-mm-diameter cell culture dish (150350; Nunc A/S, Roskilde, Denmark) for 60 min in an incubator at 36.4°C with 10% CO\(_2\). The supernatant was centrifugated, excess medium was removed, and cells were resuspended in 1 ml DMEM. Cell density was determined and adjusted to approximately 250,000 ml\(^{-1}\) with DMEM. After dilution at the ratio of 1:2 with Leibovitz-15 (31415; Gibco Invitrogen) containing 5% FBS, 3 ml of adjusted cell suspension was applied to the chip. After 1 h in the incubator, the medium was changed to 3 ml of the culture medium (NB, 14913; Gibco Invitrogen). Cells were superposed to a bias voltage from the computer interface card (National Instruments, Austin, TX).

**METHODS**

**Stimulation capacitors**

We used silicon chips (4 x 4 mm\(^2\)) with TiO\(_2\)/silicon capacitors (Wallrapp and Fromherz 2006). The circular capacitors had a diameter of 250 \( \mu \)m and were etched in a 1-\( \mu \)m-thick field oxide on highly boron doped silicon. They were insulated with 15.6 nm TiO\(_2\) made by atomic layer deposition. That material has a dielectric constant of 34. Faradaic current of the electrolyte/TiO\(_2\)/p-Si capacitors was \(<5 \mu\text{A/cm}^2\) in a voltage range of \( V_C = 0–6.5 \text{ V} \) with respect to a Ag/AgCl bath electrode. The capacitance was voltage dependent. It saturated above 3 V in the accumulation region of p'-silicon at \( c_s = 1.5 \mu \text{F/cm}^2\). It dropped below 3 V in the depletion region and increased again in the inversion region.

**Cell culture**

HEK293 CELLS. The chips with the culture chamber were cleaned with a detergent (5% Tiskopur R36, Bandelin, Berlin) at 80°C supported by cautious wiping with a tissue wipe (Tork F1; SCA Tissue, Neenah, WI). They were rinsed with Millipore water, dried by a nitrogen jet, sterilized by UV light for 40 min, and coated for 2 h with fibronectin (F2006; Sigma-Aldrich, St. Louis, MO) at a concentration of 10 \( \mu \)g/ml in phosphate-buffered saline (70013; Invitrogen) and kept in the incubator. Once a week, 0.4 ml NB medium was added to compensate for evaporation. Experiments were carried out after 19 to 25 days in vitro.

**Numerical simulation**

A numerical simulation of capacitive stimulation with voltagedriven sodium channels was implemented on the basis of a twomain model that distinguishes an attached and a free membrane area of adherent cells (see the APPENDIX). The equations for the current in the cell and in the cell–capacitor junction were combined with the equations for the dynamics of sodium channels. We evaluated the dynamics of the intracellular voltage \( V_C \), the time-dependent profile of the extracellular voltage \( V_E \) in a circular cell–capacitor junction, and the sodium conductances and the sodium currents in the free and attached membranes. For capacitive stimulation, voltage was applied between the lipid bilayer of the cells and silicon dioxide on chips was separated by a narrow cleft (\( 20 \text{ nm} \)) filled with electrolyte. The covered and free areas of the capacitor have to be distinguished as well as the attached and free areas of the membrane. For the measurements, the cell is contacted by a patch pipette under clamp. The voltages with respect to bulk electrolyte are \( V_C \), \( V_E \), and \( V_M \). The covered and free areas of the capacitor have to be distinguished as well as the attached and free areas of the membrane. For the measurements, the cell is contacted by a patch pipette under current clamp. The voltages with respect to bulk electrolyte are \( V_C \), \( V_E \), and \( V_M \).

**Electrical measurements**

With HEK393 cells as well as with rat neurons, we replaced the culture medium by an extracellular recording solution containing (in mM) 135 NaCl, 5.4 KCl, 1.8 CaCl\(_2\), 1.0 MgCl\(_2\), and 5 HEPES. It was adjusted to pH 7.3 with NaOH and had a specific resistivity of 65 \( \Omega \text{cm} \). The pipette solution consisted of (in mM) 140 KCl, 1.0 CaCl\(_2\), 1.0 EGTA, and 5 HEPES and was adjusted to pH 7.3 with KOH. Extra- and intracellular solutions were adjusted to an osmolality of 0.3 and 28 mol/kg with D\(_2\)O, respectively (all reagents from Sigma). Whole cell patch-clamp recordings were performed according to standard procedures (Hamill et al. 1981). Micropipettes were fabricated from borosilicate glass (GB150T-10; Science Products, Hofheim, Germany), fire polished, and filled with intracellular solution. An EPC10 patch-clamp amplifier (HEKA Electronics, Lambrecht, Germany) was connected to a chlorinated silver wire in the pipette and to a Ag/AgCl electrode (EP05; WPI, Sarasota, FL) in the bath at ground potential. The resistances of the pipettes were in the range between 2.5 and 3.0 M\( \Omega \). After seal formation and breakthrough, the serial resistance was compensated by >70% to minimize voltage errors in voltage-clamp mode. Leakage and capacitive currents were subtracted by a p/4 protocol. For current-clamp recordings, the resting voltage was adjusted by injection of a bias current. Recorded data were low-pass filtered at 8.4 kHz. All experiments were performed at room temperature, 22–24°C. For capacitive stimulation, voltage was applied between the capacitor and a Ag/AgCl electrode. Specific voltage pulses (waveform generator 33220A; Agilent, Palo Alto, CA) were superposed to a bias voltage from the computer interface card (National Instruments, Austin, TX).

**FIG. 1.** Capacitive stimulation of a cell in the geometry of cell adhesion (not to scale). A cell (diameter \( \sim 20 \mu \text{m} \)) is attached to an insulated substrate (capacitor diameter 250 \( \mu \text{m} \)) from which it is separated by a narrow cleft (\( \sim 35 \text{ nm} \)) filled with electrolyte. The covered and free areas of the capacitor have to be distinguished as well as the attached and free areas of the membrane. For the measurements, the cell is contacted by a patch pipette under current clamp. The voltages with respect to bulk electrolyte are \( V_C \), \( V_E \), and \( V_M \) in the electrolyte around the cell, \( V_M \) in the cell, \( V_J \) in the cell–capacitor junction, and \( V_M \) in the substrate. A falling voltage ramp applied to the substrate evokes a capacitive current that creates a voltage profile around the cell that polarizes the attached and free membranes. The center of the attached membrane is strongly depolarized (red), whereas the upper membrane is weakly hyperpolarized (blue).
the membrane (see the appendix). The sodium conductances in the
attached and free membrane were described as $g_{Na}^{M} = p_{Na}^{M} \times \bar{g}_M$ and
$g_{Na}^{FM} = p_{Na}^{FM} \times \bar{g}_M$, respectively, with a maximum conductance $\bar{g}_M$
and probabilities $p_{Na}^{M}$ and $p_{Na}^{FM}$ of the channels to be in their open state.
For the voltage-dependent dynamics of the open probabilities, a
multistate model of the rat brain sodium channel was used (Kuo and
Bean 1994). To account for the different gating voltage of the NaV1.4
channel, we displaced the voltage dependence of all rate constants by
+18 mV. We solved the coupled equations for $V_J$, $V_M$, $p_{Na}^{M}$, and $p_{Na}^{FM}$
in cylindrical coordinates by iterative integration steps using the
NDSolve function of Mathematica 5.0 (Wolfram Research, Oxford-
shire, UK).

RESULTS

NaV1.4 channels in HEK293 cell

We characterized the NaV1.4 channels in HEK293 cells by
common voltage-clamp and current-clamp experiments. A
HEK293 cell on a circular EOS capacitor is depicted in Fig. 2
A. The measurements presented in Figs. 2 to 4 were performed
with that particular cell. We contacted the cell with a patch
pipette and determined the capacitance and resistance of its
membrane to 12.6 pF and 2.6 GΩ, respectively. The respective
passive time constant was about 30 ms. With an area-specific
membrane capacitance of 1 µF/cm², these values indicate a
membrane area of 1,260 µm² and a specific leakage conduc-
tance of 0.03 mS/cm².

Starting from a holding voltage of −80 mV, we applied
depolarizing voltage pulses of 10 ms duration and recorded the
membrane currents (Fig. 2B). The signals were typical for
HEK293 cells with overexpressed NaV1.4 channels (Chen and
Heinemann 2001). The inward currents were completely abol-
ished by 0.1 µM tetrodotoxin (TTX). The small delayed
outward currents were due to endogenous potassium channels
(Zhu et al. 1998). They could be blocked in a separate exper-
iment by intracellular application of 20 mM cesium fluoride
(data not shown).

Next we applied a current pulse of 100 pA for 5 ms, starting
from −80 mV, and observed the resulting depolarization (Fig.
2C). The intracellular voltage was raised to about −40 mV
such that a self-excitation of the sodium channels was induced
in analogy to the upstroke of a neuronal action potential. The
intracellular voltage slowly decayed toward its starting value
due to a low density of endogenous potassium channels (Hsu
et al. 1993) and due to tiny leakage currents.

Capacitive activation of Na⁺ channels

We addressed the question whether capacitive stimulation is
able to activate sodium channels up to self-excitation similar to
an intracellular stimulus. In these experiments, we applied volt-
cage ramps to the capacitor and recorded the change of the
intracellular voltage with respect to the bath potential. As a
reference we determined the voltage above a capacitor without
cell.

VOLTAGE RECORDINGS. We adjusted the chip voltage to 6.2 V
and the intracellular voltage to −80 ± 1 mV. We applied
falling voltage ramps to the chip with a constant amplitude of
−5.5 V and durations of 0.3 to 0.7 ms and restored the voltage
with rising ramps of identical duration (Fig. 3A). At the onset
of the falling ramps, the intracellular voltage suddenly dropped
(Fig. 3B). Then it increased and decreased again during the ramps
with a maximum of about −30 mV for the shortest ramp. There
was an inverted response with positive changes of the intracellular
voltage during the rising ramps (Fig. 3B). Small residual depolar-
izations were observed after the stimulation pulses.
As a reference, we applied the same stimulation protocols to
a capacitor without cell and measured the voltage near the
capacitor with a micropipette electrode. The voltages in the
electrolyte resembled the intracellular voltages in their wave-
form, however, with a distinctly lower amplitude (Fig. 3C).
The residual voltages after the stimuli are plotted in Fig. 3D.
The intracellular depolarizations were enhanced up to 2.3 mV
with shorter ramps. There were no residual voltages for the
recordings without cell.
In the following three sections we consider the electrophys-
iological interpretation of the measurements.
EXTRACELLULAR VOLTAGE. The voltage transient near a cell-free capacitor (Fig. 3) is caused by the current in the electrolyte without cell. The shape of the signals resembled the intracellular transients, although with a smaller amplitude and without residual after the falling and rising voltage ramps were identical.

We identify the voltage that is recorded near the center of a cell-free capacitor with the extracellular voltage that is induced around and above a cultured cell by the same capacitive stimulus. This interpretation relies on analytical and numerical computations as described in the appendix. For a large capacitor, the voltage drop around the cell is dominated by the current through the free capacitor area with a minor contribution of the current beneath the cell that spreads at the periphery of adhesion. The voltage from the capacitor to the bulk electrolyte decays with a characteristic length that corresponds to the large radius of the capacitor. The voltage drop across the height of a cell is small and electrolyte around and above the cell is almost isopotential.

CAPACITIVE TRANSIENTS OF MEMBRANE VOLTAGE. We use the recordings of the intracellular voltage and the voltage without cell, which reflects the extracellular voltage, to estimate the capacitive polarization of the free and attached membrane, and also the extracellular voltage in the cell–capacitor junction.

The voltage change across the free membrane is given by the difference of the intracellular and extracellular signals. From the minima during the shortest falling ramp in Fig. 3, we obtain a hyperpolarization of $-10 \text{ mV}$.

To estimate the concomitant depolarization of the attached membrane, we consider the voltage drop across the serial capacitances of free and attached membranes (see Eqs. A7 and A8 in the appendix). When we assume areas of 250 and 1,000 $\mu\text{m}^2$ for the free and attached membrane, as estimated from the size of the adhesion area and the capacitance of the whole cell, we obtain an average depolarization of $+40 \text{ mV}$. Thus the average voltage between the extracellular space in the junction and the extracellular space above the cell is $-50 \text{ mV}$. The same average voltage $-50 \text{ mV}$ exists between the junction and the periphery of cell adhesion because the surround of the cell is almost isopotential.

More important, the voltage between the center of the junction and the periphery of cell adhesion is distinctly larger than the average voltage: there is a profile of the extracellular voltage along the junction that arises from the current flow along its sheet resistance. For a circular junction, that voltage profile is parabolic (see Eq. A5 in the appendix), and the voltage in the center is twice the average voltage.

In summary, a falling voltage ramp leads to a strong depolarization in the center of the attached membrane and a far
weaker hyperpolarization of the free membrane. Vice versa, a rising ramp induces a strong hyperpolarization in the center of the attached membrane and a far weaker depolarization of the free membrane. Voltage-gated ion channels are quite differently affected in the attached and free membranes.

**ACTIVATION OF NA$^+$ CHANNELS.** The strong depolarization during a falling voltage ramp is able to activate sodium channels in the center of the attached membrane. The resulting inward flow of sodium ions depolarizes the cell. The strong hyperpolarization during the rising ramp induces a fast deactivation of the channels. (The concomitant depolarization of the free membrane is weak and not able to activate sodium channels there.) As a consequence, a triangular voltage stimulus gives rise to a pulse of inward current. That current leads to the residual depolarization of the cell that is observed after the stimulus (Fig. 3D) that decays with the passive time constant of the membrane.

We did not study the residual depolarization of the cells for the whole strength–duration plane of capacitive stimulation. Instead, a constant voltage amplitude of $5.5 \text{ V}$ was chosen that was rather high but well below the threshold of Faradaic current. It corresponds to an applied charge of $c_3 \Delta V_S = 7.5 \mu \text{C/cm}^2$. We varied strength and duration of the capacitive current in a range that was well below the threshold of electroporation. The stimulation pulses with higher strength and shorter duration induced a larger residual depolarization (Fig. 3D): the larger depolarization in the center of the attached membrane induces an accelerated opening of sodium channels that overcompensates the effects of a shorter duration of opening and of a lower driving force of the sodium current.

Compared with intracellular stimulation (Fig. 2C), the effect of the capacitive stimuli (Fig. 3B) is rather modest. Whereas the pipette current depolarizes the whole membrane up to the threshold of self-activation, a capacitive stimulus depolarizes only a small patch of the attached membrane efficiently, and only for a short time. The resulting pulse of sodium inward current is not sufficient to induce a high depolarization of the whole membrane that is needed for self-activation of sodium channels.

**Repetitive activation of Na$^+$ channels**

Considering the weak response to individual capacitive stimuli, we investigated the effect of repetitive stimulation with the goal of achieving a large residual depolarization of the cell through a summation of many pulses of sodium inward current.

We applied up to 30 triangular voltage pulses with an amplitude of $-5.5 \text{ V}$ and a duration of 0.7 ms. The records of the intracellular voltage are shown in Fig. 4A. All pulses gave rise to biphasic capacitive transients. The first pulse left a residual depolarization by about 2 mV. An application of up to 15 pulses raised the residual intracellular voltage almost linearly up to a depolarization of $+35 \text{ mV}$. At a threshold of 17 pulses, the intracellular voltage autonomously increased after the pulse train. With longer trains, that rapid depolarization was initiated during the stimulation. We assign the autonomous depolarization to a self-excitation of the sodium channels in the attached and free membranes in analogy to intracellular stimulation (Fig. 2C).

We added TTX and repeated the stimulation by 30 pulses. In Fig. 4B the resulting record (gray trace) is compared with the record without TTX (black trace). The capacitive transients remained unchanged, whereas both the residual enhancement of the intracellular voltage and the autonomous depolarization after 17 pulses were suppressed. The experiment proves that both the stepwise depolarization and the autonomous depolarization were mediated by sodium channels. As an additional test, we investigated the role of the initial intracellular voltage in a range of $-80$ to $-119 \text{ mV}$. Figure 4C shows the records for a train of 30 stimulation pulses. Starting at $-88$ and $-93 \text{ mV}$, the threshold of self-excitation was reached after a larger number of pulses. When the initial voltage was lowered further, the induced depolarization was insufficient to activate ion channels.

The experiments on a repetitive activation of sodium channels were repeated with six different cells. With the same stimulus parameters, the hyperpolarization of the free membrane ranged from 7 to 12 mV, the net depolarization per pulse varied between 1.5 and 4 mV, and it took 10–25 pulses to reach the threshold. These deviations may be attributed to variations in the size of the cells, in the number of expressed sodium channels, and in the quality of the cell–chip contact.

**Numerical simulation**

To confirm the interpretation of the experimental data, we performed a numerical simulation. Cell size and membrane parameters were adopted from the data of a selected HEK293 cell (Figs. 2–4). The maximum sodium conductance $g_{Na}$ was adjusted to reproduce the results of intracellular stimulation (Fig. 2). The dynamics of the sodium channels was described by a complete kinetic scheme (Kuo and Bean 1994). All parameters are summarized in Table 1.

The response of the intracellular voltage to trains of triangular voltage pulses with an amplitude of $-5.5 \text{ V}$ and a duration of 0.7 ms is plotted in Fig. 5A, starting from a membrane voltage of $-80 \text{ mV}$. Each pulse induced a biphasic transient with an amplitude of $+9 \text{ mV}$. A residual depolarization appeared with an increasing number of pulses and a sudden depolarization occurred after about 15 pulses. When we lowered the starting voltage, the sudden depolarization was delayed and finally suppressed as shown in Fig. 5B. Obviously, the numerical simulation reproduces all key features of the experiments in Fig. 4. Thus it may provide a view on the hidden dynamics in the capacitor–cell junction.

Figure 6A shows time course (left) and radial profile (right) of the extracellular voltage and of the open probability of the sodium channels in the attached membrane for the first voltage pulse of a train. The voltage (top row) settled to a negative parabolic profile within a few microseconds of the falling ramp and to a positive parabola during a few microseconds of the rising ramp. The opening of the channels (bottom row) was induced within several hundred microseconds after the onset of the falling ramp. It was limited to the central region of the cell–chip junction. After the onset of the rising ramp, the deactivation of the channels was completed within a few microseconds due to the strong hyperpolarization. Fast channel inactivation played a minor role because the duration of the falling ramp was short and because the rising ramp induced fast deactivation.

The computed effect of a train of voltage pulses is shown in Fig. 6B. Each falling ramp induced an opening of the channels...
and each rising ramp promoted complete closing (first trace, red). The repetitive activation induced a significant inactivation of the channels in the attached membrane (second trace, red). For each voltage pulse, an inward current appeared with two components: a smoothly increasing current during the falling ramp and a short tail current at the onset of the rising ramp (third trace, red). These repetitive current pulses pumped the intracellular voltage upward (fourth trace). After about 14 pulses, the intracellular voltage was raised so far that the channels in the free membrane became activated (first trace, blue). The large inward current through the free membrane (third trace, blue) initiated a self-excitation of all channels. Subsequently, the channels became inactivated (second trace, red and blue) such that further voltage pulses induced mere capacitive transients during a slow passive repolarization of the cell (fourth trace).

### Rat neurons

The experiments with NaV1.4 channels in HEK293 cells suggest a protocol for the capacitive stimulation of mammalian neurons: a repetitive weak activation of intrinsic sodium channels in the center of the attached membrane may give rise to a depolarization of the whole cell that is sufficient to elicit an action potential.

### Table 1. Parameters of the numerical simulation

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<th>Parameter</th>
<th>Description</th>
<th>Value</th>
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<tr>
<td>$A_M$</td>
<td>Total membrane area</td>
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<td>$A_J$</td>
<td>Attached membrane area</td>
<td>250 $\mu$m$^2$</td>
</tr>
<tr>
<td>$d_J$</td>
<td>Width of cleft</td>
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<td>Oxide capacitance</td>
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<tr>
<td>$c_{M,\text{max}}$</td>
<td>Maximum sodium conductance</td>
<td>8.5 mS/cm$^2$</td>
</tr>
<tr>
<td>$g_M$</td>
<td>Potassium conductance</td>
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We studied neurons from rat hippocampus that were cultured on a TiO₂/silicon capacitor. Figure 7A shows a micrograph of a capacitor with neurons after 20 days in culture. The neurons were interconnected by a network of neurites and often showed spontaneous activity. Their resting voltage lay typically between −60 and −70 mV. The recordings of Fig. 7B refer to a neuron with a whole cell capacitance of 30 pF and a membrane resistance around 100 MΩ. Its spontaneous activity was sparse. We adjusted the resting voltage to −70 ± 1 mV prior to capacitive stimulation under current clamp.

NEURONAL EXCITATION BY REPETITIVE STIMULATION. In analogy to the experiments with HEK293 cells, we investigated the effect of an individual triangular voltage pulse. A symmetric pulse with an amplitude of −5.5 V and a duration of 0.4 ms induced a biphasic modulation of the intracellular voltage with a slight residual depolarization after the stimulus (Fig. 7B). Due to its short duration, the capacitive transient was distorted by the low-pass filtering of the amplifier. Pulses of longer duration caused a smaller residual depolarization (data not shown).

When we increased the number of pulses, the depolarizing effects accumulated and the intracellular voltage continuously rose. After 25 pulses, the threshold for an action potential was reached. With longer pulse trains, the action potential appeared after a similar number of pulses with a superposition of capacitive transients. We varied the starting voltage between

[FIG. 5. Numerical simulation of capacitive stimulation by a train of voltage pulses. Intracellular voltage of a cell with NaV1.4 sodium channels with respect to the voltage in the electrolyte near the cell. The stimulating triangular voltage pulses have an amplitude of −5.5 V and a duration of 0.7 ms. A: an increasing number of pulses evoked an increasing depolarization of the cell. Beyond 15 pulses, the threshold for self-excitation was reached. B: stimulation with 30 voltage pulses with different starting voltages from −80 to −120 mV.]

[FIG. 6. Internal variables of a cell–capacitor junction obtained from numerical simulations. A: voltage in the cell–capacitor junction (top) and fraction of open Na⁺ channels in the attached membrane (bottom) during the 1st pulse of a pulse train with an amplitude of −5.5 V and a duration of 0.7 ms. Left: time course at selected points along the radial axis. Right: radial profile at selected time points. B: response to a pulse train with 30 pulses. The repetitive opening and closing of Na⁺ channels in the attached membrane (1st trace, red) with concomitant pulses of inward current (3rd trace, red) drove an increasing depolarization (bottom trace) until self-activation of the sodium channels in the upper membrane set in after 14 pulses (1st and 3rd traces, blue). There is an increasing inactivation of the channels in the attached membrane during stimulation (2nd trace, red) and complete inactivation on self-activation (2nd trace, red and blue).]
−60 and −102 mV. More stimulation pulses were required to elicit an action potential at lower voltages as shown in Fig. 7C. Below −76 mV, the threshold was not reached.

We performed similar experiments for five neurons. In all cases, the residual depolarization after similar pulse trains was distinctly smaller than that with HEK293 cells. In three neurons, action potentials could be elicited as shown in Fig. 7. In two cases, the depolarization was insufficient to overcome the threshold of an action potential.

**RAT NEURONS VERSUS HEK293 CELLS.** The response of rat neurons to capacitive stimulation by repetitive voltage pulses matches all key features observed for the activation of NaV1.4 channels in HEK293 cells. This correspondence suggests that the stimulation of rat neurons is dominated by the same mechanism—i.e., by a repetitive activation of sodium channels and a summation of small inward current pulses. Of course, certain contributions of other ion channels cannot be excluded.

The similar size of neuronal cell bodies and of HEK293 cells may lead to a comparable activation of sodium channels in the attached membrane. Nevertheless, the efficiency of the stimulation protocol is lower for two reasons. 1) The extended arborizations are weakly coupled to the capacitor because of their low seal resistance and thus contribute to the free membrane. As a result, the local sodium current in the area of

**FIG. 7.** Capacitive stimulation of a rat neuron by a train of voltage pulses. A: DIC microscopic image of rat neurons cultured on a TiO₂/silicon capacitor coated with polylysine after 20 days in vitro. B: response of the intracellular voltage to an increasing number of voltage pulses (parameter indicated at the top). The resting voltage was adjusted to −70 ± 1 mV. C: stimulation with 30 voltage pulses with different resting voltages from −60 to −102 mV.
applied voltage ramps with an amplitude up to 6 V and a slope.

Faradaic current and electroporation are not induced by the electrode, the limits for safe stimulation are rather well defined.

of cell adhesion on an insulated titanium dioxide/silicon elec-

Protocol of repetitive stimulation

Stimulation voltages or stimulation currents are enhanced until extracellular stimulation, these limits are usually not known.

the limits of safe stimulation. In practical applications of local depolarization of the neurons cannot be achieved within a large capacitance, the same current can be induced with a lower voltage slope such that a smaller voltage amplitude may be applied.

Extracellular stimulation is limited by two constraints if damage of the cells is to be avoided: 1) the electrode voltage must be below the onset of Faradaic current and 2) the local depolarization of the membrane must be below the threshold of irreversible electroporation. To achieve safe stimulation, it is important to apply defined voltage pulses to the electrode that have a certain amplitude $\Delta V_s$ and a certain slope $\Delta V_s/\Delta t$. The slope determines the current $c_s \Delta V_s/\Delta t$ (electrode capacitance, $c_s$) and the amplitude defines the applied charge $c_s \Delta V_s$. For a large capacitance, the same current can be induced with a lower voltage slope such that a smaller voltage amplitude may be applied.

For a given system, it is well possible that a sufficiently high local depolarization of the neurons cannot be achieved within the limits of safe stimulation. In practical applications of extracellular stimulation, these limits are usually not known. Stimulation voltages or stimulation currents are enhanced until action potentials are elicited or until a desired clinical effect appears (Hamani et al. 2005). In theoretical studies, these issues are usually not taken into account.

Protocol of repetitive stimulation

In our model system with sodium channels in the geometry of cell adhesion on an insulated titanium dioxide/silicon electrode, the limits for safe stimulation are rather well defined. Faradaic current and electroporation are not induced by the applied voltage ramps with an amplitude up to 6 V and a slope up to $-20$ V/ms. The applied stimuli effectively activate sodium channels in the central region of cell adhesion. Yet, due to the small area of efficient opening, the resulting depolarization of the whole cell is not sufficient to elicit self-excitation.

On the basis of these observations, we suggest a protocol for safe stimulation that takes advantage of the well-known asymmetric voltage dependence of sodium conductances (Hodgkin and Huxley 1952). Even for weak capacitive currents, a large depolarization of the whole cell is achieved by repetition: pulses of sodium inward current are induced in a small area of the cell membrane during the cathodic phases, whereas no compensating outward current appears in the anodic phases. By superposition, the injected charges depolarize the cell such that the threshold of self-excitation is reached if the passive relaxation of the cell is not too fast and if the inactivation of sodium channels is not too high.

The frequency of repetitive stimulation is not directly related with the dynamics of the ion channels. It is determined by the nature of the electrode and by the cell-electrode geometry: the duration of a voltage pulse is determined by the chosen amplitude and slope that are determined by the limits of safe stimulation (i.e., by the onset of Faradaic current and electroporation).

The protocol for extracellular stimulation by repetitive weak activation of sodium channels resembles a theoretical model for intracellular stimulation (Bromm 1968) that was proposed to explain the physiological effect of periodic electrical stimulation (Bromm and Lullies 1966; Gildemeister 1944). In a certain sense, our protocol is an implementation of this so-called Gildemeister effect on a cellular level.

Relevance for metal electrodes

Our experiments were performed with insulated titanium dioxide/silicon electrodes that have a relatively low capacitance. Usually metal electrodes are used for the extracellular stimulation of cultured neurons as well as of brain tissue. Within the range of capacitive stimulation, all conclusions of our study remain valid. The constraints for safe stimulation that limit the capacitive current and the applied voltage also have to be considered here. The capacitance of bare metal electrodes is high due to a rather thin insulating Helmholtz layer between the metal electrons and the electrolyte. By the same token, however, the threshold for Faradaic current may be rather low. For a comparison, let us identify the capacitive current of a titanium dioxide/silicon electrode and of a metal electrode for the same geometry of cell adhesion. When the capacitance is enhanced from 1.5 to 30 $\mu F/cm^2$, we achieve the same current with the same duration when the amplitude of the voltage ramp is lowered from $-5$ to $-0.25$ V. If we take advantage of the protocol of repetitive weak activation of sodium channels, rather low voltages may be applied and Faradaic current is avoided even for metal electrodes.

Outlook

We have investigated the mechanism of extracellular capacitative stimulation in the geometry of cell adhesion under conditions where cell damage by Faradaic current and electroporation are excluded. Our study is focused on planar insulated electrodes, compact cell somata, and overexpressed sodium...
channels. In that model system all relevant electrical features are well defined such that a complete electrophysiological analysis of extracellular stimulation could be attained. Weak capacitive stimuli open sodium channels in a small area of the adherent membrane and give rise to a low depolarization of the cell. A large depolarization is achieved by a repetitive application of weak capacitive stimuli. That protocol gives rise to a summation of sodium inward currents such that the threshold for action potentials is reached under conditions that avoid cell damage.

Certainly, an extrapolation of our results from individual cells to high-density cultures and to brain tissue is an involved issue. With arborized neurons, the position of soma, axon, and dendrites relative to the electrode plays a certain role (McIntyre and Grill 1999; Rattay 1986; Warman et al. 2005; Ziv et al. 2005). When the neurons are separated from the electrode by neuroglia or necrotic cell layers, the induced membrane polarization presumably is quite different. Another aspect is the difficulty of achieving precise timing and high frequency of neuronal excitation with a burst of weak stimulation pulses. Irrespective of these problems, the characterization of both the primary polarization of neurons and the secondary activation of sodium channels as well as the implications with respect to the upper bounds for the voltage and the current of extracellular stimuli are of fundamental importance for more complicated systems. The electrophysiological analysis in a defined model system and the protocol for a summation of subthreshold stimuli may be a guide for improving long-term applications of neuroprosthetic devices.

APPENDIX

Extracellular stimulation of neurons relies on a gradient of the extracellular electrical potential that depolarizes parts of the plasma membrane. As a basis for experiments with cells attached to a planar capacitor, we summarize some theoretical relations 1) on the extracellular voltage above a circular capacitor without and with cell, 2) on a two-domain model of capacitive stimulation, and 3) on the capacitively induced voltage across the attached and the free cell membrane.

Circular capacitor without and with cell

We consider a circular capacitor (radius, \(a_S\); area-specific capacitance, \(c_S\)) on an insulated substrate in a semiinfinite electrolyte (resistivity, \(\rho_E\)). A stationary voltage ramp \(dV_E/dt\) gives rise to a displacement current per unit area \(i_z = c_S dV_E/dt\) across the capacitor/electrolyte interface. The voltage in the electrolyte obeys a Laplace equation: \(\nabla^2 V_E = 0\). In cylindrical coordinates, the boundary condition at the substrate is \(\partial V_E/\partial z|_{z=0} = -i_z \rho_E\) for \(0 \leq a < a_S\) and \(\partial V_E/\partial z|_{z=0} = 0\) elsewhere. For a vanishing voltage at infinity, we obtain Eq. 1 as a solution with the Bessel functions \(J_0\) and \(J_1\) (Carslaw and Jaeger 1959)

\[
V_E(a, z) = i_z \rho_E a_S \int_0^a d\lambda J_1(\lambda a_S) J_0(\lambda a) \frac{\exp(-\lambda z/a)}{\lambda} (AI)
\]

The voltage is visualized in Fig. A1A as a contour plot and as a section along the radius and normal direction. It has its maximum \(V_E(0, 0) = i_z \rho_E a_S\) at the center of the capacitor. There it changes along the normal with an electrical field \(-\partial V_E/\partial z|_{z=0} = i_z \rho_E\), and is constant along the radius with \(\partial V_E/\partial a|_{a=0} = \rho_E\). With \(a_S = 250 \mu m\), \(c_S = 1.5 \mu F/cm^2\), and \(\rho_E = 65 \Omega cm\), a falling voltage ramp \(dV_E/dt = -20\)

FIG. A1. Calculated voltages above a free capacitor and around an attached cell. Radii of the capacitor and of the cell were 125 and 10 \(\mu m\), respectively. The voltage in the electrolyte was normalized to its maximum \(V_E(0, 0)\) at the center of a free capacitor. A: contour plot with a profile along the normalized radius \(a/a_S\) of the capacitor at a height \(z = 0\) (bottom) and a profile along the normalized vertical axis \(z/a_S\) in the center \(a = 0\) (left), from the analytical result given in Eq. 1. B: finite-element simulation of the voltage with and without attached cell. The equipotential lines refer to the system with cell (solid) and to the capacitor without cell (dashed). The voltage in the cleft of 50 \(\mu m\) cleft between cell and capacitor is by far larger and not depicted in the plot. \(V/\text{ms}\) gives rise to a maximum voltage of \(-50\) mV at the capacitor and to a field strength of \(-0.2\) mV/\(\mu m\). When a cell is cultured on a large capacitor, we distinguish the regions of covered and free capacitor, and the regions of attached and free membrane (Fig. 1). The stimulation current through the covered capacitor is forced to flow along the cell–capacitor junction before it spreads into the bath. As a result there is a voltage drop \(V_J - V_E\) along the attached membrane from the junction to the surround. Along the free membrane, the voltage is dominated by the current through the free capacitor. We implemented a finite-element model with FEMLAB 3.1 (Comsol AB, Stockholm). The cell was described as an impermeable half-ellipsoid with a radius of 10 \(\mu m\).
and a height of 2 μm. It was positioned 50 nm above a circular electrode with radius 125 μm on an insulated substrate that formed the bottom of a cylindrical electrolyte (radius 1.25 mm; height 1.25 mm; resistivity, 65 Ωcm). Top and side walls of the electrolyte were held on ground potential. A constant current was applied to the electrode. The stationary solution was calculated with cell and without cell. The computed voltages were normalized to the maximum voltage without cell. The isopotential planes around the cell and for the cell-free capacitor are plotted in Fig. A1B. (The voltage profile in the cell–chip junction is not plotted.)

The finite-element calculation shows that the voltage drop around a cell is rather small. Further it demonstrates that the presence of the cell changes the voltage around and above the cell only little as compared with the cell-free capacitor. At the periphery of cell adhesion, the voltage is enhanced by +10% due to the current that spreads from the area of cell adhesion. Above the cell, the voltage is lowered by about −4% with a bending of the equipotential planes toward the cell pole (Maswiwat et al. 2007). On the basis of these results, we assume in a first approximation that the voltage around a cell is constant and that it can be expressed by the voltage on a cell-free capacitor.

Two-domain model

For a cell on a capacitor, we distinguish two domains of the plasma membrane for the attached and for the free region of the cell. We consider the effect of a capacitive stimulus on the extracellular voltage \( V_J \) in the cell–capacitor junction, on the intracellular voltage \( V_M \), and on the extracellular voltage \( V_E \) near the free membrane for certain area specific ion conductances in the attached and free membranes, \( \kappa'_{IM} \) and \( \kappa'_{FM} \), respectively. We take into account the extended area of the cell–capacitor junction in contrast to a previous study where a one-compartment model was used (Schoen and Fromherz 2007).

As considered above, we approximate the extracellular voltage near the free membrane by the voltage near the center of a cell-free capacitor with

\[
V_E = \rho_E a_o c_s \frac{dV_s}{dt}
\]

The extracellular voltage in the cell–capacitor junction is governed by the current balance per unit area. The distributed nature of the system is described by a two-dimensional analog of the cable equation for a core-coat conductor (Weis and Fromherz 1997) according to Eqs. A3 with the displacement current across the capacitor, the capacitive and ionic current across the membrane, and the current along the junction (area-specific capacitance of the membrane, \( c_{M} \); reversal voltages, \( V_o \); sheet resistance, \( r_J \)). The boundary condition at the periphery of the junction is \( V_J = V_E \).

\[
c_s \frac{d(V_s - V_J)}{dt} + c_M \frac{d(V_M - V_J)}{dt} + \sum_i \kappa'_{IM}(V_M - V_J) = -V_J \frac{1}{r_J} \nabla V_J
\]

The intracellular voltage \( V_M \) is determined by the balance of capacitive and ionic currents through the attached membrane with an area \( A_J \) and through the free membrane with an area \( A_M - A_J \) according to

\[
\begin{align*}
\int_{A_J} \left[ c_M \frac{d(V_M - V_J)}{dt} + \sum_i \kappa'_{IM}(V_M - V_J) \right] \\
+ (A_M - A_J) \left[ c_M \frac{d(V_M - V_E)}{dt} + \sum_i \kappa'_{FM}(V_M - V_E) \right] = 0
\end{align*}
\]

Here Eqs. A2–A4 are used to describe the passive response of the attached and free membranes to a capacitive stimulus. They also build the basis for a numerical simulation with voltage-gated sodium channels (see METHODS).

Passive response

We consider the extracellular and intracellular voltages for a junction with a circular radius \( a_o \) without membrane conductances. When a step of capacitive current is applied, the extracellular voltage in the electrolyte is established within a time that is determined by the electrode capacitance and by the access resistance. In our system, the time constant \( \tau_E = R_E C_s \) is <1 μs. The response time of the extracellular voltage in a cell–capacitor junction \( \tau_J = (c_S + c_M) a_J^2 r_J/5.78 \) can be derived from Eq. A3 (Braun and Fromherz 2004). In our system, it is in the order of 1 μs. Thus the passive response of the system is distinctly faster than the dynamics of ion channels and the stationary response provides a reliable estimate for the effect of a capacitive current on the polarization of the membrane.

From Eq. A3 without membrane conductances, we derive a parabolic profile of the voltage drop along a junction with a constant sheet resistance \( r_J \) according to Eq. A5. The average voltage drop is given by Eq. A6

\[
V_J - V_E = \frac{r_J c_s a_o^2}{4} \left( 1 - \frac{a_J^2}{a_o^2} \right) \frac{dV_s}{dt}
\]

\[
(V_J - V_E) = \frac{r_J c_s a_o^2}{8} \frac{dV_s}{dt}
\]

The average voltage drop along the junction is identical with the total voltage change across the attached and free membrane with \( (V_J - V_E) = -\Delta(V_M - V_J) + \Delta(V_M - V_E) \). The voltage changes across the serial capacitances of attached and free membranes are related by \( -A_J(V_M - V_J) = (A_M - A_J)(V_M - V_E) \). By combining these two equations, we can express the voltage changes across the attached and free membranes by the average voltage drop in the junction according to Eqs. A7 and A8, respectively

\[
\Delta(V_M - V_J) = -\frac{A_J}{A_M}(V_J - V_E)
\]

\[
\Delta(V_M - V_E) = \frac{A_J}{A_M}(V_J - V_E)
\]

A negative voltage drop \( (V_J - V_E) \) in the junction—as it is induced by a falling voltage ramp (Eq. A6)—gives rise to a depolarizing effect on the attached membrane (Eq. A7) and to a hyperpolarizing effect on the free membrane that is smaller for \( A_J \ll A_M \) (Eq. A8).

Two further aspects may be noted. 1) The voltage changes across attached and free membranes are independent of the voltage \( V_E \) in the surround of the cell according to Eqs. A7 and A8 together with Eq. A6. 2) A change of the intracellular voltage \( \Delta V_M \) with respect to the bath potential—that is recorded with a patch pipette—reflects the change \( \Delta V_E \) of the extracellular voltage and a fraction of the voltage drop \( (V_J - V_E) \) in the junction according to Eq. A8.

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