

# Patch-clamping of primary cardiac cells with micro-openings in polyimide films

A. Stett V. Bucher C. Burkhardt U. Weber W. Nisch

NMI Natural & Medical Science Institute, Reutlingen, Germany

**Abstract**—Patch-clamping is a powerful method for investigating the function and regulation of ionic channels. Currently, great efforts are being made to automate this method. As a step towards this goal, the feasibility of patch-clamping primary cells with a microscopic opening in a planar substrate was tested. Using standard microfabrication and ion beam technology, small-diameter openings (2 and 4  $\mu\text{m}$ ) were formed in polyimide films (thickness 6.5  $\mu\text{m}$ ). Single cells (sheep Purkinje heart cells, Chinese hamster ovary cells) in a suspension were positioned on top of the opening and sucked towards the opening to improve adhesion of the cell to the planar substrate, hence increasing the seal resistance. Voltage/current measurements yielded a median seal resistance of 1.3 M $\Omega$  with 4  $\mu\text{m}$  openings ( $n=24$ ) and 26.0 M $\Omega$  with 2  $\mu\text{m}$  openings ( $n=75$ ), respectively. With 2  $\mu\text{m}$  openings, successful loose-patch recordings of TTX-sensitive inward currents and action potentials in sheep Purkinje heart cells ( $n=18$ ) were made. In rare cases, gigaseals ( $n=4$ ) were also measured, and a whole-cell configuration ( $n=1$ ) could be established. It was concluded that the simple planar patch approach is suitable for automated loose-patch recordings from cells in suspension but will hardly be suitable for high-throughput whole-cell patch-clamping with high-resistance seals.

**Keywords**—High-throughput screening, Ion channel, Electrophysiology, Patch-clamping, Loose patch, Automation

Med. Biol. Eng. Comput., 2003, 41, 233–240

## 1 Introduction

DRUG DISCOVERY nowadays is closely related to the screening of large numbers of compounds against multiple targets, which is achieved using high-throughput screening (HTS) technology. Owing to the increasing interest in membrane protein targets (membrane transporters, ion channels, receptors) in the pharmaceutical industry, the use of cell-based assays for functional screening gains in importance (GONZÁLEZ *et al.*, 1999). These assays provide information on the function and regulation of membrane-spanning proteins and their interactions with the molecular and cellular environment (JOHNSTON and JOHNSTON, 2002). Meeting the HTS need, expression cell lines are used for hosting ion channels and receptors, and, hence, adequate electrophysiological methods allowing for the monitoring of ion channel activity at a high throughput rate are required. This has resulted in the ongoing development of electrophysiological methods and instrumentation suitable for higher-throughput screening (OWEN and SILVERTHORNE, 2002; SIGWORTH and KLEMIC, 2002; XU *et al.*, 2001).

The state-of-the-art method for detailed characterisation of ion channel function and its regulation is the patch-clamp technique (HAMIL *et al.*, 1981). It enables us to control the voltage across

the membrane of the host cell and, hence, measure the ion channel gating. At the single-cell level, it allows the combination of analysis of the functional properties of ion channels and gene-expression analysis (DIXON *et al.*, 2000; MONYER and LAMBOLEZ, 1995).

The basic step when performing this method is to bring the tip of a glass pipette in close contact with a patch of the cell membrane. This gives rise to the formation of a tight seal with an ohmic resistance in the order of giga-ohms measured between the pipette solution and the electrolyte surrounding the cell and pipette. Finally, the membrane patch is disrupted, allowing electrical contact to the cytoplasm and enabling the recording of the ionic current across the entire membrane. The gigaseal provides low background noise on the recording and mechanical stability of the contact (NEHER, 1992). The true nature of this contact is still not understood in detail. It is assumed that it results from a tight contact between the membrane drawn into the pipette and the clean surface of the inner pipette wall. It can be calculated from geometric considerations that the distance between the cellular plasma membrane and the glass surface is in the range of ångströms (COREY and STEVENS, 1983; OPSAHL and WEBB, 1994; SAKMANN and NEHER, 1983).

The patch-clamp technique is time-consuming and requires skilled operators and well-equipped set-ups. Measurements are carried out sequentially at cells that are attached to the culture substrate, and, hence, it is slow. Because of these drawbacks, at present, the patch-clamp technique is advanced to meet the requirements for ion-channel HTS. The chosen way is the replacement of the patch pipette by micro-fabricated

Correspondence should be addressed to Dr Alfred Stett;  
email: stett@nmi.de

Paper received 25 June 2002 and in final form 17 December 2002

MBEC online number: 20033756

© IFMBE: 2003

micro-openings in planar substrates for mechanical and electrical cell contacts. The ultimate goal is the automation of the entire patch-clamp process, as described in the reviews by XU *et al.* (2001) and OWEN and SILVERTHORNE (2002). The automated patch-clamp devices are designed for recording suspended cells. They provide a means of positioning cells on the opening by suction (FERTIG *et al.*, 2002; MATHES *et al.*, 2001) and electrical fields (GUIA *et al.*, 2002; SCHMIDT *et al.*, 2000), respectively.

We tested the feasibility of patch-clamping primary cardiac cells and CHO cells with a microscopic opening (diameter 1–4  $\mu\text{m}$ ) in a planar polyimide film covered by a thin layer of silicon nitride ( $\text{Si}_3\text{N}_4$ ). The main questions we addressed were whether

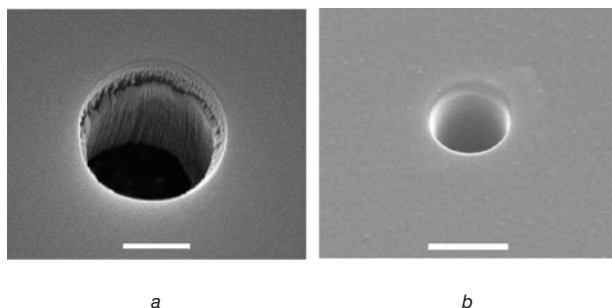
- (i) individual cells can be positioned on the opening by suction reliably
- (ii) high-resistance seals can be achieved by planar adhesion of native cells to the substrate surrounding the opening and whether the seal resistance can be improved by additional suction, as it is applied in the standard patch-clamp technique
- (iii) the membrane can be ruptured by suction impulses being applied to the opening to gain access to the cytoplasm for intracellular perfusion and electrical whole-cell recording.

## 2 Materials and methods

### 2.1 Fabrication of micro-openings

We fabricated micro-openings with a diameter of 4  $\mu\text{m}$  using photolithography and plasma etching. A polyimide layer (thickness 6.5  $\mu\text{m}$ ) was deposited on a float glass substrate (49  $\times$  49  $\text{mm}^2$ ) by spinning without using adhesion promoter. A 100 nm thick  $\text{Si}_3\text{N}_4$  layer was deposited on the polyimide by use of rf-sputtering Si in Ar/ $\text{N}_2$  plasma. On top, positive-tone photo-resist Shipley S1818 was spun over the  $\text{Si}_3\text{N}_4$  layer. This resist was structured by means of standard UV-photolithography, resulting in openings of 4  $\mu\text{m}$  diameter in the photo-resist layer. The latter serves as a mask when the  $\text{Si}_3\text{N}_4$  layer is etched in  $\text{CF}_4$ -plasma, resulting in a structured  $\text{O}_2$ -resistant  $\text{Si}_3\text{N}_4$  mask for etching the polyimide in  $\text{O}_2$  plasma. When the polyimide is etched, the photo-resist is also removed. As a result, openings with a diameter of 4  $\mu\text{m}$  were formed in the polyimide with a clean  $\text{Si}_3\text{N}_4$  layer on top (Fig. 1a).

To drill smaller openings (2  $\mu\text{m}$ ; see Fig. 1b) in the polyimide film, we used a focused ion beam (FIB) technique. In the FIB system (FIB 200, FEI), a beam of gallium ions,



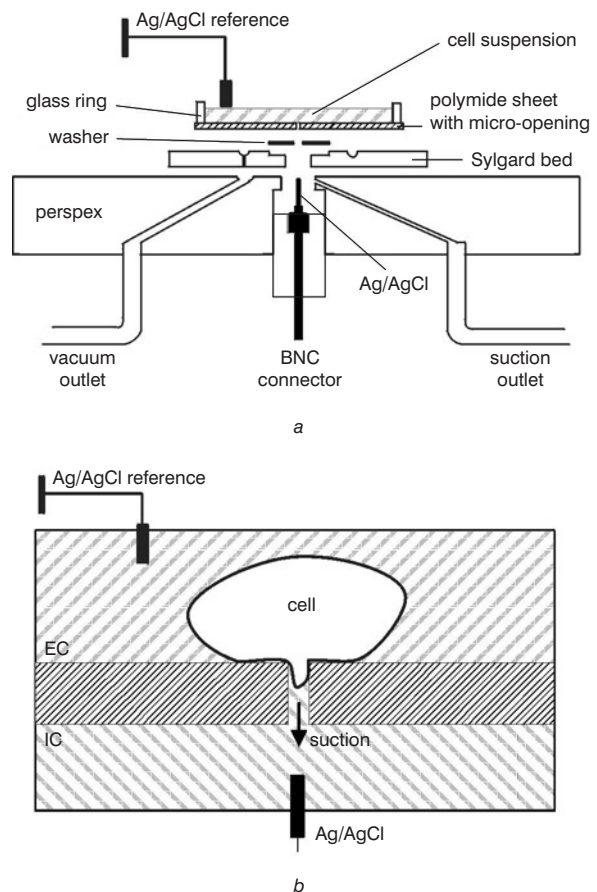
**Fig. 1** Micromachined micro-openings in polyimide films (thickness 6.5  $\mu\text{m}$ ) covered by thin layer of  $\text{Si}_3\text{N}_4$ . (a) SEM image of 4  $\mu\text{m}$  opening etched in  $\text{O}_2$  plasma. Note rough wall inside opening. (b) SEM image of opening with diameter of 2  $\mu\text{m}$  fabricated with focused ion beam. Scale bars = 2  $\mu\text{m}$

with energy of 30 keV and beam currents between 20 pA and 1 nA, was focused on a spot of less than 10 nm. To avoid charging by the ion beam, a thin layer of gold was deposited on the polyimide film. The ion beam was focused on the film and moved in a circular manner. By software control, the maximum outer diameter was confined to 2  $\mu\text{m}$ . After a few minutes, the polyimide in this circle was removed by physical sputtering. The process was controlled by imaging the openings at low beam current to minimise further milling. After successful milling of the openings, the gold layer was removed by argon etching. Thereafter, a 100 nm thick  $\text{Si}_3\text{N}_4$  layer was deposited on the polyimide, as described above.

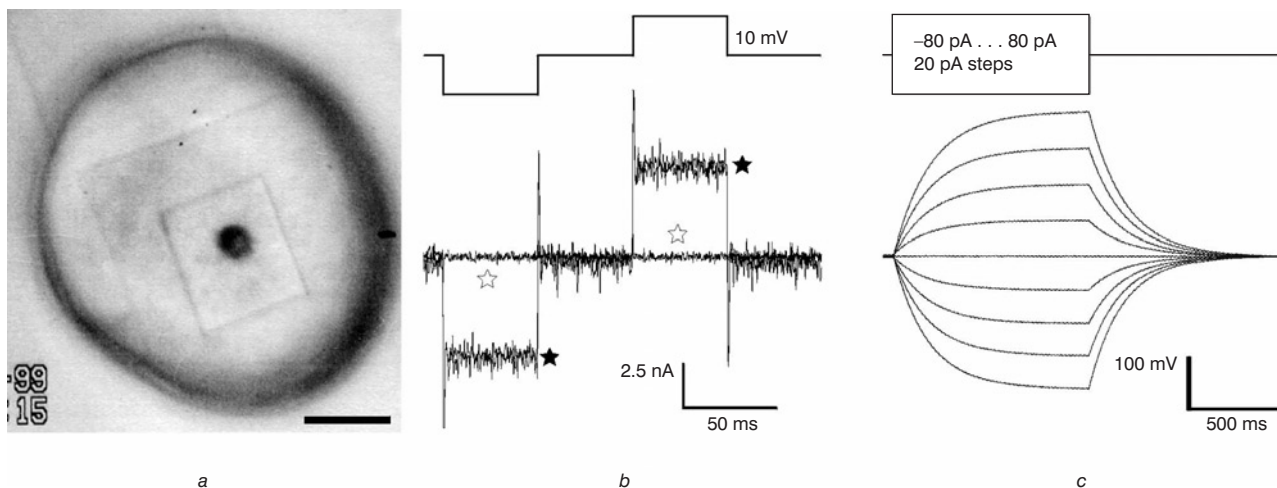
Finally, a glass ring was fixed using Sylgard to form a culture dish, and the film was stripped from the glass substrate with the help of phosphate buffered saline (PBS) solution.

### 2.2 Mechanical and electrical set-up

The polyimide film was fixed by vacuum application onto a Sylgard bed, with the micro-opening centred on a bore in the supporting Perspex mounting (Fig. 2a). This lower compartment was filled with pipette solution and was connected, by an



**Fig. 2** Cross-section through suction holder for polyimide film and contact site. (a) Holder consists of Perspex body with central bore for contacting lower side of micro-opening. Film is pressed onto Sylgard bed on top of Perspex by constant negative pressure applied to vacuum outlet. Fluids on both sides of film are in electrical contact through Ag/AgCl electrodes. Cells are sucked towards opening by negative pressure applied with syringe to suction outlet. Metallic washer prevents formation of curvature of film. (b) Compartment below film is filled with solution for intracellular (IC) recording; cells are kept in extracellular (EC) solution. Arrow indicates negative pressure



**Fig. 3** Characterisation of seal formation. (a) Cardiac Purkinje cell placed on opening with 2 µm diameter (scale bar 5 µm). (b) Leakage current measurement after voltage pulse application in voltage-clamp mode. (★) Superimposed current traces recorded without cell positioned on opening and with cell positioned but without suction applied (calculated seal resistance: 1.8 MΩ). (☆) Current trace recorded after application of suction. (c) Voltage traces recorded after current injection in bridge mode with same cell with constant suction (calculated seal resistance: 3.5 GΩ)

Ag/AgCl wire soldered to a BNC connector, with a single-electrode patch-clamp amplifier\*. Recordings were sampled with a software-controlled† interface board, with 16-bit analogue-to-digital converters‡.

Experiments were carried out under visual observation\*\* of an infrared patch-clamp set-up (LUIGS & NEUMANN).

### 2.3 Preparation and positioning of cells

**Cardiac Purkinje cells:** Sheep cardiac Purkinje fibres were cut out of either ventricle of the heart at the local slaughterer and brought into the laboratory in cold HEPES-buffered Tryode solution (mM: 137 NaCl, 5.4 KCl, 1 MgCl<sub>2</sub>, 1.8 mM CaCl<sub>2</sub>, 10 HEPES, 5 glucose). Single Purkinje cells were isolated from the fibres according to GLITSCH *et al.* (1989). Briefly, after an enzymatic treatment with protease and collagenase in nominally Ca<sup>2+</sup>-free Tyrode solution and subsequent bathing in mixtures of culture medium (M 199) and Tyrode solution, to increase Ca concentration stepwise, the Purkinje cells were squeezed out of the surrounding connective tissue. The cells were held in culture for 1–3 days in culture medium (M 199) at 37°C, 5% CO<sub>2</sub> and 90% humidity.

Freshly isolated Purkinje cells display a brick-like shape. They become spherical in culture after 1–2 days, with a diameter of 40–70 µm. As they do not show any deep invaginations of the cell membrane, Purkinje cells are particularly suited for patch-clamp experiments.

**Chinese hamster ovary DUKX cells:** For comparative sealing experiments, we used Chinese hamster ovary (CHO) cells cultured in MEM alpha medium with 10% FCS. After trypsinisation of the adherent epithelial-like cells, they become spherical, with a diameter of 15–50 µm. As CHO DUKX cells lack voltage-dependent ionic currents, they are particularly suited as an easily available passive membrane model.

**Positioning of cells on openings:** Cells were detached from the surface of the culture dishes by standard trypsin-EDTA

treatment. An aliquot of the cell suspension was positioned in the glass ring, and cells were allowed to settle. The moving of the cells to the opening was carried out either by the flow caused by application of negative pressure to the opening or by means of a suction pipette mounted to a micro-manipulator. In the latter case, the largest cell bodies with a smooth membrane were picked up and moved onto the opening. During this procedure, constant positive pressure was applied to the opening to prevent obstruction of the opening by debris. After placement of the cell (Fig. 3a), suction was applied to the opening (Fig. 2b) to increase adhesion of the cell to the surface and along the wall inside the opening. To improve adhesion, the Si<sub>3</sub>N<sub>4</sub> surface of the polyimide films was hydrophilised by plasma cleaning.

### 2.4 Electrophysiological recordings

During the positioning of the cells, current/voltage measurements were carried out between the two Ag/AgCl electrodes in the fluid compartments above and below the polyimide film (Fig. 2b). This allowed observation of the seal formation during suction either by measurement of the leakage current in the discontinuous voltage clamp mode of the amplifier (Fig. 3b) or by measurement of voltage traces after current injection in the bridge mode (Fig. 3c). From the records, the seal resistance was calculated according to Ohm's law.

All experiments were carried out at room temperature, using the following solutions both for CHO and Purkinje cells (in mM): bath solution: 143 NaCl, 5.3 KCl, 1.8 CaCl<sub>2</sub>, 0.8 MgCl<sub>2</sub>, 20 HEPES, 21 glucose; pipette solution 140 KCl, 2 MgCl<sub>2</sub>, 20 HEPES, 10 EGTA. Prior to use, the solutions were degassed to avoid bubble formation.

## 3 Results

### 3.1 Positioning of the cells

We tested whether controlled positioning of single cells on a micro-opening is possible by attracting suspended cells by a flow caused by negative pressure applied to the lower side of the opening. Applying negative pressure to openings with a diameter of 4 µm yielded a marked flow of extracellular solution

\*SEC-05L, npi electronic

†TIDA 3.0; HEKA elektronik

‡ITC 16, Instrutech

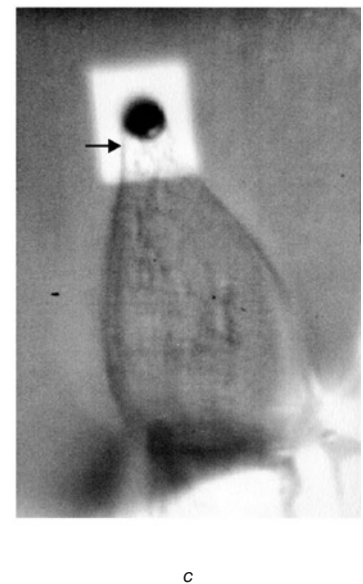
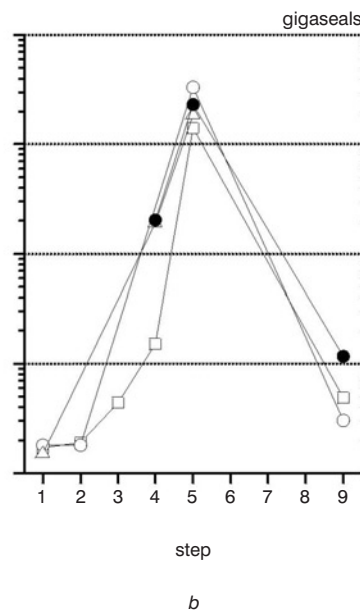
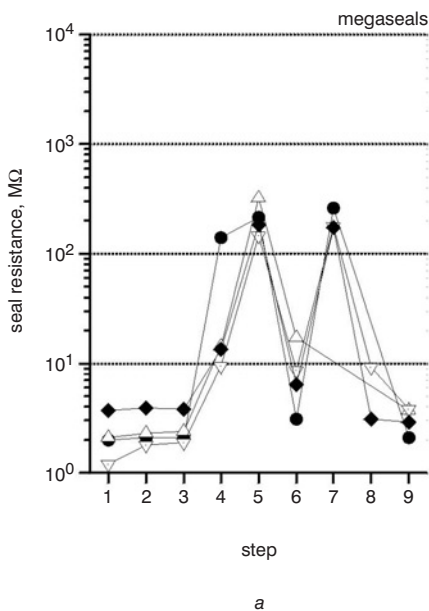
\*\*Zeiss Axiovert with upright illumination; Olympics water-immersion objective 40x/0.8, IR camera C2440, Hamamatsu

that was sufficient to attract cells from the suspension towards the opening. However, with steady negative pressure, cells accelerated and finally stroked the surface, resulting in an unpredictable position. Often, they were sucked into and through the opening, and, mostly, no clear increase in the seal resistance could be measured. With openings of 1 and 2  $\mu\text{m}$  diameter, cells could not be moved towards the opening. With dense suspensions, single cells attached to the opening by chance, but heavy suction (up to 500 mbar) was necessary to seal the openings with the attached cell.

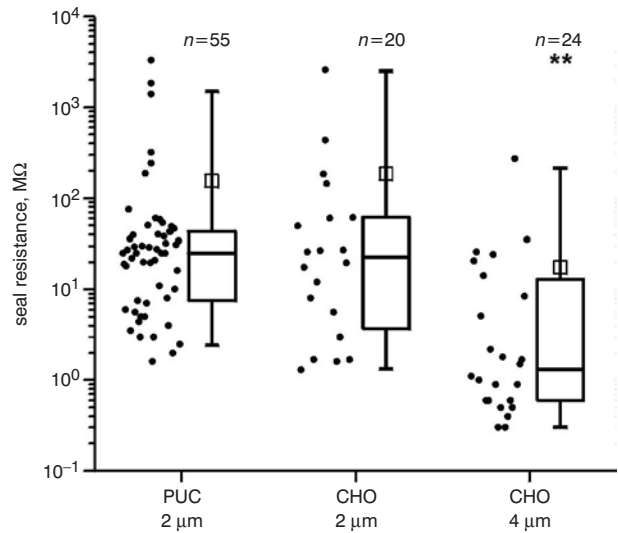
### 3.2 Seal formation

To investigate the seal formation and the dependence of the seal resistance on the applied suction, we placed cells by means of a suction pipette on openings with a diameter of 2  $\mu\text{m}$ . In the course of the experiments, we measured the seal resistance after different subsequent steps, as shown in Fig. 4. Placing the cells on the opening without suction did not increase the resistance in the majority of cases (Figs. 4a and b, steps 1–3). Only applying suction up to 500 mbar yielded a markedly increased seal resistance up to mega-ohms (Fig. 4a, step 5) and, in rare cases, up to giga-ohms (Fig. 4b). After the negative pressure was switched off, the megaseals decreased to a level initially reached by slight suction (compare steps 4, 6 and 8 in Fig. 4a). After the cells were removed from the opening, the resistance was comparable with that at the beginning of the experiment without a cell (compare steps 1 and 9). Sometimes, a membrane tube remained in the opening, as shown in Fig. 4c.

In contrast to the reversible formation of megaseals, the resistance of the gigaseals could only be reduced by removing the cell with a suction pipette (Fig. 4c). Removal by application of positive pressure failed. In all these cases, a membrane tube remained in the opening, and the final resistance after the cells had been removed remained above the resistance of the initial value without cell (compare steps 1 and 9).

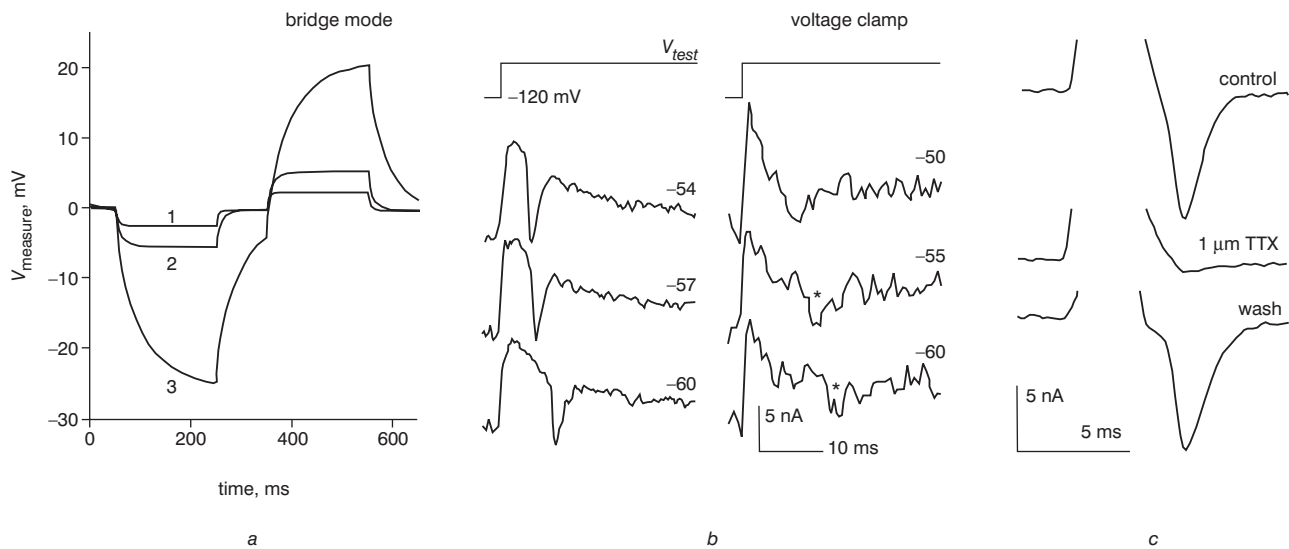


**Fig. 4** Seal formation steps. Maximum resistance measured with different levels of pressure applied to openings (diameter 2  $\mu\text{m}$ ) from eight experiments (abscissa: step 1: opening without cell; step 2: cell placed on opening with positive pressure at opening; step 3: without pressure; step 4: slight negative pressure; step 5: heavy suction; step 6: without pressure; step 7: again heavy suction; step 8: again without suction; step 9: cell removed from opening by means of suction pipette). (a) Formation of megaseals with Purkinje cells. (b) Formation of gigaseals with Purkinje cells (open symbols) and CHO cell (filled symbol). (c) Removal of Purkinje cell from opening by means of suction pipette. Membrane tube (arrow) remained in opening



**Fig. 5** Maximum seal resistances pooled from all experiments after final improvement (PUC cells on openings with 2  $\mu\text{m}$  diameter; CHO cells on 2 and 4  $\mu\text{m}$  openings). (●) Individual results. Horizontal lines of boxes represent first, second (=median value) and third quartiles. Short lines: 10%, 90% level; small square: arithmetic mean

The dependence of the maximum seal resistance on cell type and the diameter of the opening, collected from all experiments, is shown in Fig. 5. The median values obtained with Purkinje cells and CHO cells placed on openings with a diameter of 2  $\mu\text{m}$  were statistically not significantly different ( $25.0 \pm 3.3 \text{ M}\Omega$ , with Purkinje cells, and  $22.8 \pm 12.7 \text{ M}\Omega$ , with CHO cells, SD of the median). A statistically significantly different distribution ( $p \leq 0.1\%$ ; Tukey's quick test) of sample values could be obtained by placing CHO cells on openings with a diameter of 4  $\mu\text{m}$  (median seal resistance  $1.3 \pm 0.5 \text{ M}\Omega$ ).

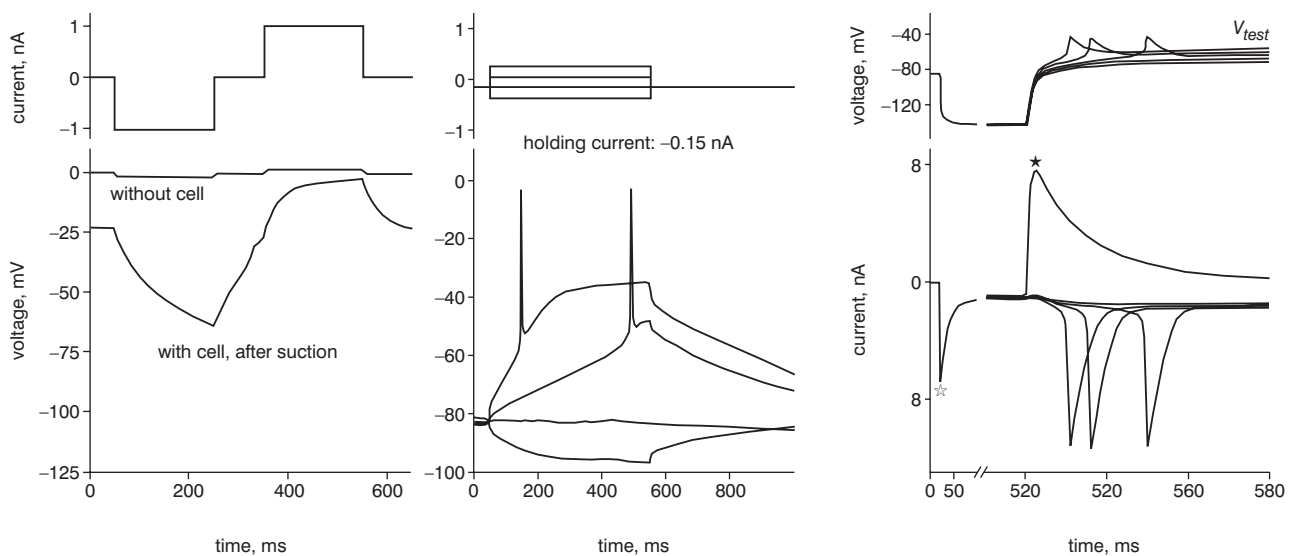


**Fig. 6** Loose-patch recordings from Purkinje cells with micro-openings of  $2\ \mu\text{m}$  diameter: (a) Voltage traces after current injection ( $1\ \text{nA}$ ) in bridge mode. Trace 1: opening alone, trace 2: cell placed on opening, no suction, trace 3: strong suction. (b) Current traces measured in voltage-clamp mode, application of activation protocol described in Fig. 7, test potential as indicated (left: same cell as in (a)). Seal resistance =  $25\ \text{M}\Omega$  and  $22\ \text{M}\Omega$ , respectively. (c) Extracellular recording of TTX-sensitive positive inward currents in voltage-clamp mode, voltage protocol as in (b) (different cell), seal resistance  $27\ \text{M}\Omega$

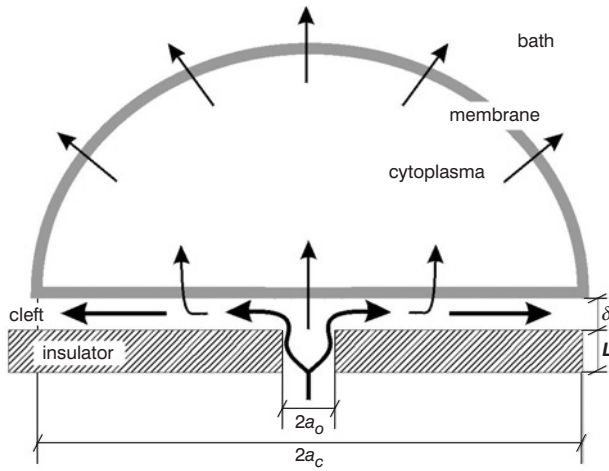
### 3.3 Loose-patch recording

With low resistance configurations, as summarised in Figs 4a and 5, recordings of voltage-dependent currents could be carried out ( $n = 18$  out of 55). In the example shown in Fig. 6a, the resistance increased without alteration of the measured DC potential from  $2.6\ \text{M}\Omega$  (opening alone) to  $5.4\ \text{M}\Omega$ , with a cell attached and without suction, and to  $25\ \text{M}\Omega$ , after strong suction. In the voltage-clamp mode, positive inward currents could be elicited (Fig. 6b) by application of an activation protocol, as

described in Fig. 7. The amplitudes of these TTX-sensitive currents (Fig. 6c) varied from experiment to experiment but could not be related to the value of the seal resistance. In all these experiments, alteration of the resting potential measured in the bridge mode and of the leakage current measured in the voltage-clamp mode could not be observed during suction. Therefore we conclude that these measurements have been carried out extracellularly in the loose-patch configuration (STÜHMER *et al.*, 1983) and, eventually, with an incomplete disrupted patch membrane.



**Fig. 7** Whole-cell recording from Purkinje cell. Diameter of opening =  $2\ \mu\text{m}$ . (a) Current injection in bridge mode immediately after cell positioning with medium suction impulse. Note negative offset with cell. (b) After additional suction impulse action potentials could be elicited by current injection. Latency of action potentials depended on amplitude of current ( $0.2\ \text{nA}$ ,  $0.4\ \text{nA}$ , holding current  $0.15\ \text{nA}$ ). (c) Activation protocol in voltage-clamp mode. Holding potential  $-85\ \text{mV}$ , leakage current corrected. After  $500\ \text{ms}$  hyperpolarising pulse ( $-60\ \text{mV}$ ) depolarising voltage steps to test potentials (top traces, shown are step to holding potential and further with increment of  $4\ \text{mV}$ ) excited positive inward currents (lower traces). Note voltage escape in measured voltage traces. From ( $\star$ ) current resulting from hyperpolarising voltage step, resistance of  $75\ \text{M}\Omega$  could be estimated. This trace was used for eliminating capacitive currents from recordings ( $\star$ ) (current trace reflects uncorrected recording of sub-threshold impulse)



**Fig. 8** Planar cell–substrate contact (radius of contact zone  $a_c$ ). Cell membrane and substrate (thickness  $L$ ) are separated by cleft of electrolytic solution (width  $\delta$ ). Bold arrows indicate spread of current applied via micro-opening (radius  $a_o$ )

### 3.4 Whole-cell recording

In one out of 55 cases where we positioned Purkinje cells on openings with a diameter of  $2\ \mu\text{m}$ , we unambiguously succeeded in the formation of a whole-cell configuration and intracellular recordings in the bridge mode and in the voltage-clamp mode. The transition from a loose-patch contact into a whole-cell configuration (Figs 7a and b) was initiated by a medium suction impulse for cell attraction. The transition was very fast, and so a clear estimation of the seal resistance could not be carried out. Starting from a resistance of  $1.6\ \text{M}\Omega$  of the free opening, the determined resistance increased to  $30\ \text{M}\Omega$  after medium suction and finally to  $75\ \text{M}\Omega$  after additional suction impulses. Unambiguously, action potentials could be elicited in the bridge mode by current injection (Fig. 7b), and positive inward currents were measured in the voltage-clamp mode by application of an activation protocol (Fig. 7c). The micro-opening exhibited a rounded edge at its top end and a very smooth channel-wall surface.

### 3.5 Theoretical estimation of the seal resistance

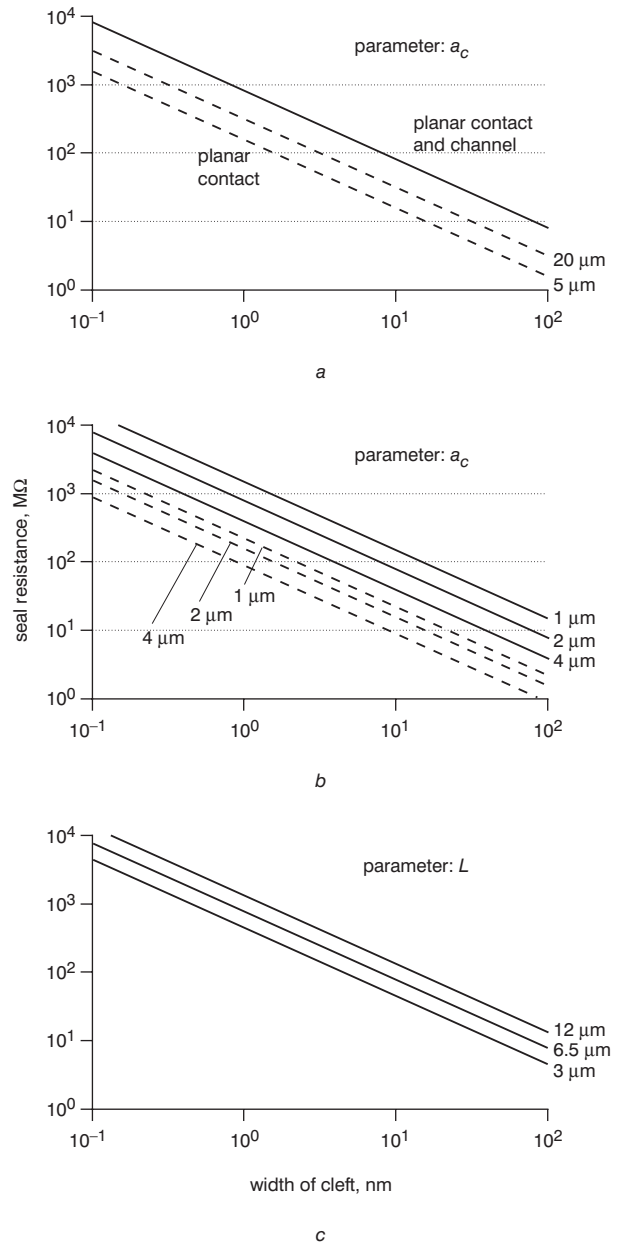
In Fig. 8, the cell–substrate contact is depicted. The attached membrane of a cell with a radius  $a_c$  and the substrate are separated by a cleft with width  $\delta$  of electrolytic solution. A steady current applied via the micro-opening with diameter  $a_o$  spreads along the cleft towards the grounded bath solution surrounding the cell. Owing to the finite resistance of the attached membrane, small amounts of current may also flow into the cell and leave it through the free membrane, as indicated.

Neglecting current passing the contact membrane, the seal resistance  $R_S$  can be estimated as

$$R_S = R_1 + R_2 = \frac{\rho}{2\pi\delta} \left( \ln \frac{a_c}{a_o} + \frac{L}{a_o} \right) \quad (1)$$

where  $R_1$  is the resistance of the electrolytic cleft from the opening to the grounded bath, and  $R_2$  is a serial resistance caused by an electrolytic cleft between a membrane patch drawn into the channel, with length  $L$ , and the wall. The latter is created by the fact of pulling a membrane patch into the channel by suction (Fig. 4c).

In Fig. 9, calculations of the seal resistance depending on various model parameters are given. Equation 1 and the plots indicate that the most crucial parameters that determine the



**Fig. 9** Calculated dependence of seal resistance on distance between membrane and substrate and other model parameters: (a) parameter  $a_c$ ; (b) parameter  $a_o$ ; (c) parameter  $L$ . (---) Planar contact alone; (—) additional contact along wall inside opening. ( $\rho = 60\ \Omega\ \text{cm}$ ;  $L = 6.5\ \mu\text{m}$ ;  $a_o = 1\ \mu\text{m}$ ;  $a_c = 5\ \mu\text{m}$ , and as indicated, respectively)

seal resistance is the distance  $\delta$  between substrate and membrane and whether a membrane patch is sucked into the opening or not (Figs 9a and b). Increasing the diameter of the contact zone alone does not result in a noticeable increase in the seal resistance.

## 4 Discussion

### 4.1 Planar patch approach

Several groups have reported on a planar patch approach for automation of the patch-clamp technique. Different materials and processes have been used to fabricate disposable patch chips. It is also reported that the success rates for gigaseal formation and subsequent whole-cell recording are in the range between 30 and 50% (FERTIG *et al.*, 2002; OWEN and

SILVERTHORNE, 2002; SIGWORTH and KLEMIC, 2002). However, neither a statistical evaluation nor a systematic investigation of the seal formation mechanisms in the planar systems has been published. This may reflect the principal problems that are inherent in this approach.

With planar openings, there are, in principle, two surfaces of the substrate to which the membrane can attach: the planar area surrounding the opening and the wall of the channel penetrating the substrate. From the viewpoint of available standard batch processes for large-volume production, it is preferable to use thin substrates. In this case, seal formation is restricted to the planar adhesion area.

To test whether this membrane–substrate contact is suitable for high-resistance seal formation, we fabricated thin polyimide films with micromachined small-diameter openings. Polyimide offers the advantage that it is transparent, allowing microscopic analysis, and it is known to be cell-culture compatible. We covered the surface with a glass-like  $\text{Si}_3\text{N}_4$  layer, because hydrophilic glass surfaces are proven to be suitable for high-resistance seal formation (COREY and STEVENS, 1983). Because of the fabrication process for the 4  $\mu\text{m}$  openings, the walls had small grooves that made them unsuitable for a tight contact and sharp edges at the rim. The 2  $\mu\text{m}$  openings fabricated with the focused ion beam offered smooth surfaces and a rounded rim.

The experimental results clearly indicate that this planar adhesion approach is inappropriate both for cell positioning by suction and for reliable gigaseal formation. Owing to the contradiction of needing large openings (diameter larger than 4  $\mu\text{m}$ ) for cell attraction and positioning and small openings (diameter lower than 2  $\mu\text{m}$ ) for reliable sealing, additional means, either for bringing cells to the opening or for contacting a membrane patch, are necessary.

The conspicuously low median seal resistance is in good agreement with theoretical considerations on the molecular and electrical nature of the adhesion-mediated contact between a membrane of a native cell and a planar substrate. Assuming distances between 40 nm and 100 nm, as recommended by the work of BRAUN and FROMHERZ (1998) and SORRIBAS *et al.* (2001), we estimated seal resistances ranging from a few megaohms, up to 50 M $\Omega$ . This is in good agreement with our experimental results. SORRIBAS *et al.* (2001) postulate that the glycocalyx coating the cells extracellularly gives rise to minimum cell–surface distances and therefore hinders tight contacts.

Comparing these considerations with the results from our suction experiments, it can be concluded that suction applied via the opening in the centre of the adhesion area results, not in an overall decrease in the distance between the membrane and the substrate, but in the pulling of a membrane patch into the opening. The main contribution to the seal resistance then arises from the adhesion of the membrane along the wall inside the opening. However, it remains unclear how membrane–surface distances below 1 nm, which are necessary for gigaseal formation, can occur inside the channels in planar substrates and inside conventional patch pipettes.

#### 4.2 Loose-seal recording with planar electrodes

Another method for single-cell recording in which the distance of the membrane to a planar surface plays a crucial role is the use of arrays of planar micro-electrodes (micro-electrode arrays (MEAs)) for parallel recording of extracellular potentials in close vicinity of adherent cells (see review by POTTER (2001)). The shape and signal-to-noise ratio of the recorded signal depend strongly on the electrical characteristic of the cell–substrate contact. This contact and the signal

transmission from cell to electrode can be described with an equivalent electrical circuit (BOVE *et al.*, 1997; 1998; BUITENWEG *et al.*, 1998; 2000; 2002; FROMHERZ, 1999; FROMHERZ and STETT, 1995; MAHER *et al.*, 1999; RUTTEN *et al.*, 1999; WEIS *et al.*, 1996). In these models, a sealing resistance represents the electrolytic cleft in the contact, as in the planar patch approach described above. With the equivalent circuits, the resistance can be estimated from measurements of transfer functions and approximation of the geometry of the contact. However, a direct measurement of the seal resistance has not been described yet.

With the micro-openings in contact with reversible Ag/AgCl electrodes, simultaneous current injection into the membrane–surface junction and measurement of the resulting voltage were possible. The results of this direct measurement of the seal resistance are in good agreement with the results from indirect estimations. The attaching of a cell to a planar surface without suction gives rise only to small seal resistances of about 1 M $\Omega$  and below, as can be seen in Fig. 4a, steps 1–3. These values are comparable, for example, with the values described by BUITENWEG *et al.* (1998). With these low resistances, the extracellularly recorded signals are about two orders of magnitude lower than recordings with gigaseals with the patch-clamp method. Thus the signal-to-noise ratio is relatively low and, hence, not suitable for resolving extracellular potentials caused by slow, weak ionic currents. Besides others, this serious disadvantage has stopped the MEA technique becoming a standard tool for cellular drug screening.

Using an array of electrically contacted suction openings, as described in this paper, could be more suitable for this purpose. We have shown that voltage-gated ion currents can be elicited and measured simultaneously: an attribute that is not possible with conventional metallic micro-electrodes. Such a device could be easily used to generate dose/response curves from compounds acting on voltage- and ligand-gated ion channels. Additionally, loose-patch recording offers the advantage that it is non-invasive and therefore preserves the complete functional context of an untouched cytoplasm.

This feature is of importance, for example, in ion-channel safety pharmacology, where the guidelines of the International Conference on Harmonisation<sup>††</sup> dictate the testing of new drugs for their potential to block cardiac ion channels, such as the voltage-gated potassium channel hERG, prior to first use in humans (NUMANN and NEGULESCU, 2001).

## 5 Conclusions

We conclude that the simple planar adhesion approach for single-cell recording with micro-openings is suitable for loose-patch recordings from cells in suspension. It could be developed into a tool for ion-channel drug screening scalable for parallel recording from many cells. However, owing to the limited formation of tight seal contacts between the membrane of primary cells and planar surfaces, this method will hardly be suited for automated, high-throughput whole-cell patch-clamping. For this purpose elongated channels in thicker substrates seem to be more suitable.

*Acknowledgment*—The authors would like to thank Sybille Glock for performing cell preparation and culturing procedures, Dr Thomas Krahn for helpful tips with the Purkinje cells, Dr Thomas Mueller for valuable discussions and ideas and Dr Elke Guenther for reading the manuscript.

They acknowledge the partial funding of the project by Bayer AG.

<sup>††</sup>[www.ifpma.org/ich1.html](http://www.ifpma.org/ich1.html)

## References

- BOVE, M., GRATTAROLA, M., and VERRESCHI, G. (1997): 'In vitro 2-D networks of neurons characterized by processing the signals recorded with a planar microtransducer array', *IEEE Trans. Biomed. Eng.*, **44**, pp. 964–977
- BOVE, M., MARTINOIA, S., VERRESCHI, G., GIUGLIANO, M., and GRATTAROLA, M. (1998): 'Analysis of the signals generated by networks of neurons coupled to planar arrays of microtransducers in simulated experiments', *Biosens. Bioelectron.*, **13**, pp. 601–612
- BRAUN, D., and FROMHERZ, P. (1998): 'Fluorescence interferometry of neuronal cell adhesion on microstructured silicon', *Phys. Rev. Lett.*, **81**, pp. 5241–5244
- BUITENWEG, J. R., RUTTEN, W. L., WILLEMS, W. P., and VAN NIEUWKASTELE, J. W. (1998): 'Measurement of sealing resistance of cell-electrode interfaces in neuronal cultures using impedance spectroscopy', *Med. Biol. Eng. Comput.*, **36**, pp. 630–637
- BUITENWEG, J. R., RUTTEN, W. L., and MARANI, E. (2000): 'Finite element modeling of the neuron-electrode interface', *IEEE Eng. Med. Biol. Mag.*, **19**, pp. 46–52
- BUITENWEG, J. R., RUTTEN, W. L., MARANI, E., POLMAN, S. K., and URSUM, J. (2002): 'Extracellular detection of active membrane currents in the neuron-electrode interface', *J. Neurosci. Methods*, **115**, pp. 211–221
- COREY, D. P., and STEVENS, C. F. (1983): 'Science and technology of patch-recording electrodes', in SAKMANN, B., and NEHER, E. (Eds): 'Single-channel recording' (Plenum Press, New York, London, 1983), pp. 53–68
- DIXON, A. K., RICHARDSON, P. J., PINNOCK, R. D., and LEE, K. (2000): 'Gene-expression analysis at the single-cell level', *TIPS*, **21**, pp. 65–70
- FERTIG, N., BLICK, R. H., and BEHREND, J. C. (2002): 'Whole cell patch clamp recording performed on a planar glass chip', *Biophys. J.*, **82**, pp. 3056–3062
- FROMHERZ, P. (1999): 'Extracellular recording with transistors and the distribution of ionic conductances in a cell membrane', *Eur. Biophys. J.*, **28**, pp. 254–258
- FROMHERZ, P., and STETT, A. (1995): 'Silicon-neuron junction: capacitive stimulation of an individual neuron on a silicon chip', *Phys. Rev. Lett.*, **75**, pp. 1670–1673
- GLITSCH, H. G., KRAHN, T., and PUSCH, H. (1989): 'The dependence of sodium pump current on internal Na concentration and membrane potential in cardioballs from sheep Purkinje fibres', *Pflügers Archiv.*, **414**, pp. 52–58
- GONZÁLEZ, J. E., OADES, K., LEYCHKIS, Y., HAROOTUNIAN, A., and PINNOCK, R. D. (1999): 'Cell-based assays and instrumentation for screening ion-channel targets', *Drug Discov. Today*, **4**, pp. 431–439
- GUIA, A., WANG, X., XU, J., SITHIPHONG, K., YANG, Z., CUI, C., WU, L., HAN, E., and XU, J. (2002): 'Micro-positioning enabled patch clamp recordings on a chip'. Biophysical Society Meeting, San Francisco, CA, USA, pp. Abstract 787-Pos
- HAMIL, O. P., MARTY, A., NEHER, E., SAKMANN, B., and SIGWORTH, F. J. (1981): 'Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches', *Pflügers Archiv.*, **406**, pp. 73–82
- JOHNSTON, P. A., and JOHNSTON, P. A. (2002): 'Cellular platforms for HTS: three case studies', *Drug Discov. Today*, **7**, pp. 353–363
- MAHER, M. P., PINE, J., WRIGHT, J., and TAI, Y. C. (1999): 'The neurochip: a new multielectrode device for stimulating and recording from cultured neurons', *J. Neurosci. Methods*, **87**, pp. 45–56
- MATHES, C., OSIPCHUK, Y., SAVTCHENKO, A., YANG, I., and A., B. (2001): 'Whole-cell recordings from planar patch clamp electrodes: a step towards high-throughput electrophysiology'. Society for Biomolecular Screening 7th Annual Conference (Baltimore MD, USA), Abstract p. 5039
- MONYER, H., and LAMBOLEZ, B. (1995): 'Molecular biology and physiology at the single-cell level', *Curr. Opin. Neurobiol.*, **5**, pp. 382–387
- NEHER, E. (1992): 'Ion channels for communication between and within cells (Nobel Lecture)', *Neuron*, **8**, pp. 605–612
- NUMANN, R., and NEGULESCU, P. A. (2001): 'High-throughput screening strategies for cardiac ion channels', *Trends Cardiovasc. Med.*, **11**, pp. 54–59
- OPSAHL, L. R., and WEBB, W. W. (1994): 'Lipid-glass adhesion in giga-sealed patch-clamped membranes', *Biophys. J.*, **66**, pp. 75–79
- OWEN, D., and SILVERTHORNE, A. (2002): 'Channelling drug discovery. Current trends in ion channel drug discovery research', *Drug Discov. World*, **3**, pp. 48–61
- POTTER, S. M. (2001): 'Distributed processing in cultured neuronal networks', *Prog. Brain Res.*, **130**, pp. 49–62
- RUTTEN, W. L., SMIT, J. P., FRIESWIJK, T. A., BIELEN, J. A., BROUWER, A. L., BUITENWEG, J. R., and HEIDA, C. (1999): 'Neuro-electronic interfacing with multielectrode arrays', *IEEE Eng. Med. Biol. Mag.*, **18**, pp. 47–55
- SAKMANN, B., and NEHER, E. (1983): 'Geometric parameters of pipettes and membrane patches', in SAKMANN, B., and NEHER, E. (Eds): 'Single-channel recording' (Plenum Press, New York, London, 1983), pp. 37–51
- SCHMIDT, C., MAYER, M., and VOGEL, H. (2000): 'A chip-based biosensor for the functional analysis of single ion channels', *Angew. Chem. Int. Ed.*, **39**, pp. 3137–3140
- SIGWORTH, F. J., and KLEMIC, K. G. (2002): 'Patch clamp on a chip', *Biophys. J.*, **82**, pp. 2831–2832
- SORRIBAS, H., BRAUN, D., LEDER, L., SONDEREGGER, P., and TIEFENAUER, L. (2001): 'Adhesion proteins for a tight neuron-electrode contact', *J. Neurosci. Methods*, **104**, pp. 133–141
- STÜHMER, W., ROBERTS, W. M., and ALMERS, W. (1983): 'The loose patch clamp', in SAKMANN, B., and NEHER, E. (Eds): 'Single-channel recording' (Plenum Press, New York, London, 1983), pp. 123–132
- WEIS, R., MULLER, B., and FROMHERZ, P. (1996): 'Neuron adhesion on a silicon chip probed by an array of field-effect transistors', *Phys. Rev. Lett.*, **76**, pp. 327–330
- XU, J., WANG, X., ENSIGN, B., LI, M., WU, L., and GUIA, A. (2001): 'Ion-channel assay technologies: quo vadis?', *Drug Discov. Today*, **6**, pp. 1278–1287

## Author's biography



ALFRED STETT received his Dr.rer.nat. in Physics from the University of Ulm/Germany in 1995. From 1994 to 1996 he participated in the neurochip project at the Max-Planck-Institute for Biochemistry, Dept. Membrane and Neurophysics. In 1996 he joined the University Eye Hospital, Tuebingen and in 1998 the NMI in Reutlingen where he participated in the retina implant project. Currently he heads the Biophysics Group at the NMI. He is co-founder and scientific director of CytoCentrics CCS. His research interests include the interface between chips and cells, functional electrical stimulation, neuroprosthetics and automation of electrophysiology.