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MEMBRANE POTENTIAL OSCILLATIONS IN HOMOKARYONS

An Endogenous Signal for Detecting Intercellular Communication

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SUMMARY

Fusion of cells by polyethylene glycol results in homokaryons with lower membrane input resistances than their parental cells, but otherwise unchanged membrane properties. With these large cells, long lasting intracellular recordings can be realized which are impossible with single parental cells. Homokaryons often display hyperpolarizing (up to 50 mV) oscillations of their membrane potentials. In electrically non-coupled cell lines (HeLa, L, Cl-1D) the frequencies of these endogenous signals are 3 oscillations per min. Trypsinized homokaryons of electrically coupled cell lines (BICR/M1R-K, 3T3, BT5C2) have frequencies of 0.3 oscillations per min. By recording the membrane potential oscillations of two contacting homokaryons, the formation of low resistance junctions was followed—without applying exogenous signals—by a superposition of the individual oscillations. Our electronmicroscopical investigations revealed that the intercellular coupling through the membranes of homokaryons can be attributed to gap junctions.

Membrane potential measurements by impaling cells with glass microelectrodes are often accompanied by a membrane leakage around the electrode. The recorded potential difference (pd), is therefore smaller than the cell's real membrane potential. The higher the input resistance, the greater is the effect of this shunt pathway. This is one of the reasons why long lasting experiments with impaled electrodes failed with many mammalian cells. Fusion of cultured mammalian cells by polyethylene glycol [15] increases their cell size, resulting in a decrease of their input resistance without changing specific membrane properties. These giant cells allow manipulations and long lasting intracellular recordings which are impossible with the respective parental cells.

Many of these homokaryons show hyperpolarizing oscillations of their membrane potentials, not yet being described for their parental cells. Hyper- and depolarizing oscillations of the membrane potential are known to be signals for the triggering of physiologically relevant processes [see 2, 22]. In some cultured cells, e.g. homokaryons of L [18–20, 28] and BT5C2 cells [9] or in macrophages [4, 5, 7, 21] an underlying regulatory or informational effect is still unknown. The oscillations described in this paper divide the investigated cell lines into two groups which are characterized by their frequencies. Interestingly, the high-frequency group consisted exclusively of electrically non-coupled cells, whereas the low frequency group included only electrically coupled cells. We have used these hyperpolarizing oscillations as an endogenous signal displaying the formation of low resistance junctions between homokaryons. Our electrophysiological and

Table 1. *Frequencies of membrane potential oscillations of homokaryons from different cell lines at 22 and 30°C*

Cell line	At 22°C			At 30°C		
	Oscillations	No. of expts.	Yield (%)	Oscillations	No. of expts.	Yield (%)
HeLa	3.37±0.2	52	76	4.74±0.17	88	63
L	3.73±0.09	163	58	4.49±0.25	47	73
Cl-1D	2.10±0.10	135	62	2.46±0.25	66	65
BICR-M1R-K	0.31±0.01	98	45	0.51±0.001	836	60
3T3	0.32±0.04	40	20	0.38±0.02	43	70
BT5C2	0.42±0.02	43	15	0.39±0.02	36	44

Values given are mean number of oscillations/min ± SE; number of experiments conducted with each cell line, and percentage yield of oscillating homokaryons.

electronmicroscopical investigations have shown that the membrane of homokaryons is unchanged with regard to its ability to form gap junctions.

MATERIALS AND METHODS

Cell cultures

Permanently growing cells were cultured at 37°C in modified [6] Eagle–Dulbecco medium with 5% calf serum and were regulated at pH 7.2 with an atmosphere of 5% CO_2 in air. The investigated cell lines were the electrically non-coupled HeLa (human cervix carcinoma), L (mouse embryo) and Cl-1D (clone of L cells) and the electrically coupled 3T3 (mouse embryo), BICR/M1R-K (rat mammary tumor) and BT5C2 (rat glioma). The cells were grown in plastic Petri dishes (Falcon or Greiner) to confluency and treated with 40% (w/w) polyethylene glycol (PEG; MW 1540, Koch-Light) for 1–4 min to form homokaryons with more than ten nuclei [15]. Four hours later, the medium was replaced by Hepes-buffered Eagle–Dulbecco medium to avoid major pH changes during the electrophysiological experiment. Homokaryons of the electrically coupled cells were treated with 0.25% trypsin in Ca^{2+}-Mg^{2+}-free isotonic phosphate-buffered salt solution for 0.5–2 min at 37°C. The isolated homokaryons were manipulated into close contact and the formation of intercellular junctions was then followed by intracellular recordings of the pd oscillations of the interacting cells at 26–30°C.

Electronmicroscopy

For electronmicroscopy the cells were grown on specimen carriers (gold; Balzers) in plastic Petri dishes and treated with PEG as described above. Trypsinized homokaryons were added 4 h after fusion. After different time intervals the specimen carriers with contacting homokaryons were transferred into liquid nitrogen. This enabled the preparation of replicas of unfixed membranes in a Balzers BAF 301 instrument. Electron micrographs were taken with a Zeiss EM 10 electron microscope.

Electrophysiology

The electrophysiological measurements were made with 3 M KCl-filled glass electrodes (Hilgenberg-Glas with inner filament) which had tip potentials <-5 mV and resistances ranging from 20 to 40 MΩ. Membrane potentials (inside negative), or more exactly, potential differences (pd) were measured with high input-impedance preamplifiers (WPI model M701 or M750). Membrane resistances were determined by compensating the electrode resistance with the bridge circuit of the M701 preamplifier, passing ramp pulses of current through the electrode and analysing the resulting i-v curves [25]. Successful coupling was indicated by simultaneously recording the individual oscillation frequencies in the contacting cells. A communication ratio is given by the ratio of the oscillation amplitudes of the receiving to the oscillation generating cell. The measurements were performed under a Zeiss Standard RA phase contrast microscope with an electrically insulated 40× water immersion objective.

RESULTS

As already shown [9] membrane potential measurements in homokaryons of electrically non-coupled cells resulted in higher pd values than for their parental cells, and electrically coupled cells had similar pd values for both homokaryons and parental cells, e.g. HeLa, 47 mV vs 30 mV; BT5C2, 56 mV vs 62 mV. Numerous homokaryons of all investigated cell lines displayed hy-

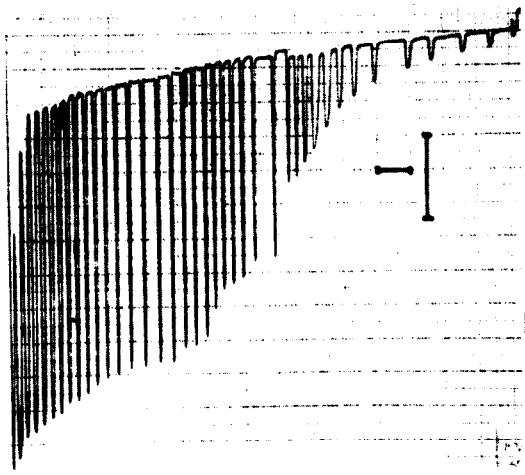

Fig. 1. Membrane potential oscillations and damping in a Cl-1D homokaryon. Figs 1–4, 6. Bars, horizontal, 1 min; vertical, 10 mV.

perpolarizing oscillations of their membrane potentials. The yield of oscillating homokaryons and their mean frequencies are listed in table 1 for two different temperatures.

After impaling homokaryons of non-coupled cells, the initially measured pd values of about 50 mV often decayed to values between 10 and 20 mV, with superimposed hyperpolarizing oscillations of frequencies between 2–3 per minute. In most cases these oscillations were damped and disappeared after 2–5 min; examples are shown in fig. 1 for Cl-1D homokaryons and in fig. 2 for HeLa homokaryons. These homokaryons repolarized without oscillations and stable pd recordings (30–70 mV) were then obtained for 1 h or longer, similar to those measured for non-oscillating homokaryons of non-coupled parental cells.

After impaling homokaryons of electrically coupled cells which formed an electrically coupled 'monolayer', stable pd values of about 60–80 mV were recorded. For the demonstration of gap junction development with electrophysiological measurements, the homokaryons were isolated by trypsinization. After impaling these isolated homokaryons, pd values between 10 and 40 mV were often measured, which were superimposed by hyperpolarizing oscillations with amplitudes of up to 50 mV and stable frequencies of about 0.3–0.5 per minute. The measured pd increased in the same time course as the amplitudes of the hyperpolarizing oscillations decreased, resulting in identical peak values, as is shown in fig. 3 for BICR/M1R-K homokaryons. Sometimes, the pd remained at the initial low level, in these cases the amplitudes of the oscillations remained constant, too. An example of these constant oscillations is shown in fig. 4 for 3T3 homokaryons. Isolated non-oscillating homokaryons had pd values similar to the non-oscillating homokaryons of the respective 'monolayer'.

The different oscillating behaviour of homokaryons of coupled and non-coupled cells is only partially connected with the trypsinization of the electrically coupled homokaryons. When they were allowed to recover from trypsinization for some hours or overnight (but still isolated from each

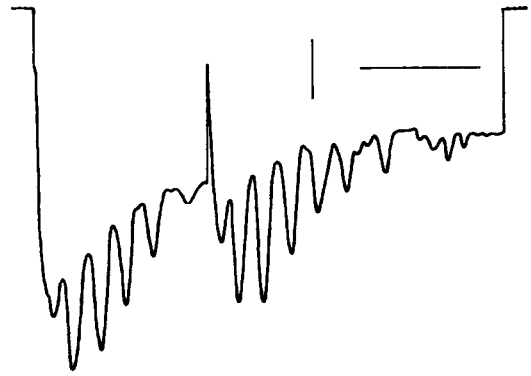

Fig. 2. Membrane potential oscillations and decay in a HeLa homokaryon. The short depolarization after 90 sec is due to a mechanical stimulus followed by another burst of damped oscillations.

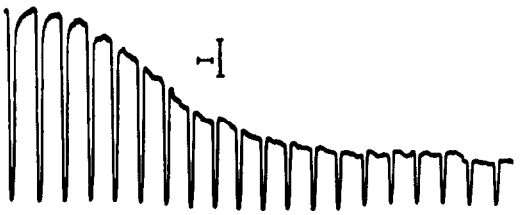

Fig. 3. Section of membrane potential oscillations in a BICR/M1R-K homokaryon starting at a pd of about 40 mV.

other) they oscillated with similar frequencies. The yield of oscillating homokaryons, however, was reduced to less than 10%, the measured pd values were initially up to about 60 mV and remained stable for hours with accordingly smaller amplitudes of their oscillations. Trypsinized homokaryons of non-coupled cells displayed the same oscillation frequencies and the same time course of damping as non-trypsinized homokaryons.

The input resistance of homokaryons was always lower than that of comparable mammalian cells [8, 27]. It varied, however, with the individual size between 1 and 10 MΩ and changed synchronously with the pd oscillations. The measured values were always minimal at the highest hyperpolarization (oscillation peak) and maximal during the 'resting potential' level. The absolute difference (up to 5 MΩ) of the input resistance values thus varied with the amount of the oscillation amplitudes.

Obviously the mechanical insertion of the electrode into a homokaryon of non-coupled cells stimulated an oscillation. As can be seen from fig. 2 the oscillation of the HeLa homokaryon was almost completely damped after 90 sec, when a jolt to the micromanipulator led to a second stimulation. This short mechanical vibration of the electrode led to a transient leakage in the membrane as can be seen from the short depolarization which was followed by another oscillatory burst. Similar stimulations of oscillations in homokaryons of non-coupled cells were induced by short depolarizing or by the off-response of hyperpolarizing current pulses. A mechanical or electrical stimulation of isolated homokaryons of coupled parental cells did neither influence the oscillations of oscillating cells nor trigger stable pd cells to an oscillating behaviour.

Homokaryons of electrically coupled parental cells still have the capability to form gap junctions (fig. 5). The oscillations of these homokaryons, therefore, can be used as an endogenous signal necessary for the electrical demonstration of gap junction formation between contacting cells. The onset of ionic coupling due to gap junction formation was followed in BICR/M1R-K and BT5C2 homokaryons which had been trypsinized 4 h after fusion. Each homokaryon was impaled with a recording electrode, used also to manipulate the cells into close contact. By recording the membrane potential oscillations of two contacting homokaryons, the coupling should be indicated by a simultaneous occurrence of the individual oscillations in each cell. This was the case 20–30 min after first contact when superimposed oscillations could be resolved.

Thirty minutes later the coupling was

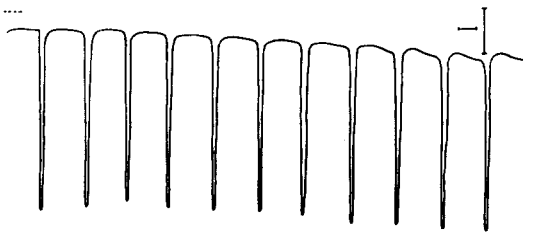

Fig. 4. Section of membrane potential oscillations in a 3T3 homokaryon. Dashed line, zero potential.

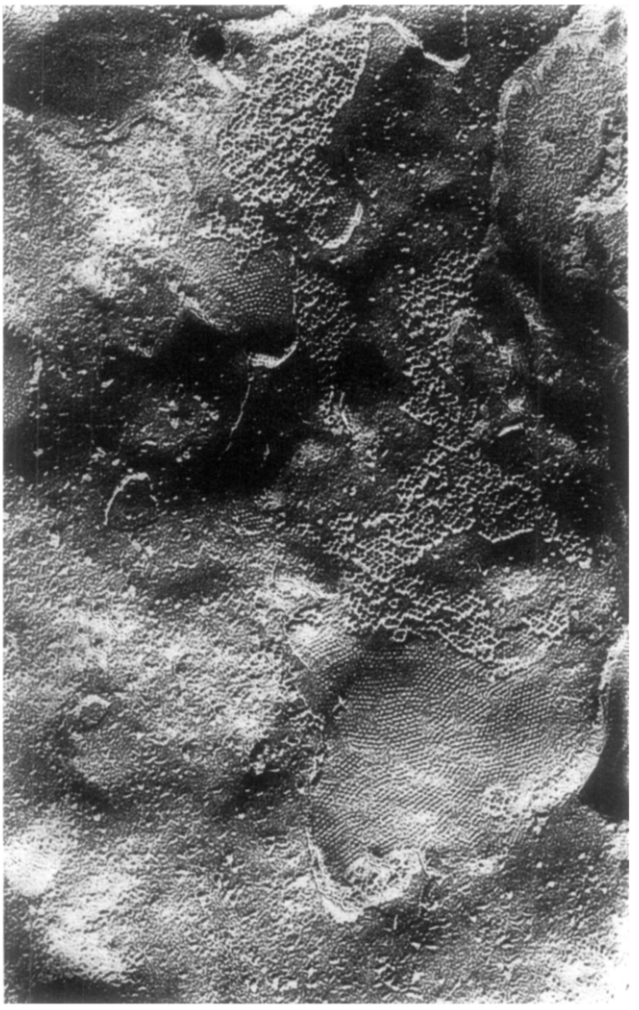

Fig. 5. Gap junctions between BICR/M1R-K homokaryons. ×100 000.

fully established, i.e. the transmitted oscillation amplitude from each cell remained constant. Sections of communication measurements in BICR/M1R-K homokaryons are shown in fig. 6. Since each of the contacting cells provided an endogenous signal, the communication ratio could be determined simultaneously for both directions: it was about 0.5 for BICR/M1R-K and 0.3 for BT5C2 homokaryons. These values are lower than those measured for the parental cells [8, 16]. There was no rectification detectable; the amplitude of the coupling oscillation, however, depended on the surface ratio of the interacting homokaryons. In no case have pacemaker effects been observed: the original frequencies of the interacting homokaryons could always be resolved. After coupling of several homokaryons and superposition of more than three oscillations, the resulting membrane potential oscillations became smoothed out. Three coupled homokaryons are shown as example in fig. 6*d*; after about

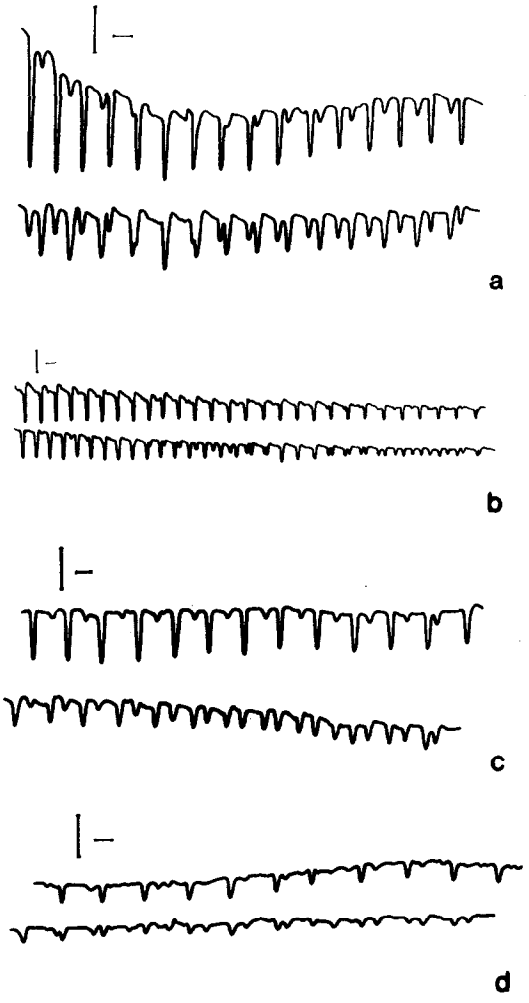

Fig. 6. Superposition of the membrane potential oscillations of contacting BICR/M1R-K homokaryons indicates ionic coupling. Four independent experiments with different times after onset of coupling: (*a*) 60; (*b*) 23; (*c*) 65; (*d*) 197 min. Note the oscillations of a third homokaryon which is not impaled with an electrode in (*d*). Pd levels between 53 and 61 mV.

90 min the oscillations in one of the homokaryons were considerably damped.

DISCUSSION

The physiological relevance of membrane potential oscillations is unquestioned in some cellular systems with control or regulation functions [see 2, 22]. A similar role, however, is unknown for the demonstrated membrane potential oscillations of homokaryons which have been obtained after unnatural fusion induced by PEG treatment.

In a biological system, any oscillatory behaviour (not only electrical oscillations) may occur either as (1) a result of a rapid concentration displacement out of its steady state or as (2) a sustained autonomous process which may be caused by continuously flowing metabolites or secretory products [see 2]. PEG-induced cell fusion is known to be accompanied by excessive secretion processes [15]. This stimulated exocytosis may well be such an intrinsic mechanism as referred to in (2) and thus be a cause for the described oscillations of the membrane potential. If this is the case, the membrane potential should continuously oscillate without external stimulation. For homokaryons of coupled cells this has been shown by registering the oscillation amplitudes of a non-impaled cell via an electrically coupled contiguous cell (see fig. 6*d*). Unfortunately, non-coupled cells cannot be tested in a similar way. However, the damping of their oscillations and the successful restitution of the oscillations by depolarizing manipulations (see fig. 2) point to an extrinsic event of the mechanism (1) mentioned above, i.e. due to an ion concentration displacement induced for instance by electrode impalement. This may indicate that non-coupled homokaryons may still be more sensitive to electrode damage than coupled homokaryons. Assuming a mechanism (1) is responsible for the fast (~3 Hz) and a mechanism (2) for the slow (~0.3 Hz) pd oscillations, then a superposition of both frequencies should be observed in one homokaryon if both mechanisms were simultaneously in operation;

however, this was not the case. The different mechanisms may be the reason why our (unpublished) experiments to inhibit the oscillations by pH shifts, blockage of Na^+, K^+ or Ca^{2+} channels and Na^+-K^+-ATPase led to equivocal results and why we were unable to confirm some experiments where the inhibition of membrane potential oscillations in radiation-induced giant L cells was described [18–20].

Even with expression of these oscillations, membranes of homokaryons still have the same properties as their parental cells, e.g., their sensitivity against ouabain is unchanged [9] and only homokaryons of coupled cells are able to form gap junctions (fig. 5). One to 2 h after treatment with PEG, the fusion process is completely finished and the resulting homocaryons act as a unit. They interact with other cells or homokaryons by normal membrane junctions and do no longer form cytoplasmic bridges [15, 24]. In a 'monolayer' of coupled homokaryons no pd oscillations are resolved and intercellular communication can be determined by applying exogenously generated signals. The measurement of the time course of gap junction formation, however, can only be made with isolated cells. Therefore, homokaryons of coupled cells had to be trypsinized and thus displayed the described pd oscillations.

The time course of gap junction formation between BICR/M1R-K or BT5C2 homokaryons is the same when measured with the endogenously oscillating signals or with exogenously applied signals [9] and is comparable with that obtained from different systems. Until now, similar measurements have only been possible in large non-mammalian embryonic cells [1, 11–13, 17, 26]. In cultured mammalian cells, however, more indirect methods had to be used as measuring the onset of synchronous beating in heart cells [3], by heterocellular signal transfer [10] or by ultrastructural correlates [14, 23]. Since homokaryons regularly survive manipulations with impaled electrodes, they are favorable models for measurements of membrane properties of those cultured cells which normally resist long lasting intracellular recordings.

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