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Electronmicroscopical and electrophysiological investigations on polyethylene glycol induced cell fusion

Elektronenmikroskopische und elektrophysiologische Untersuchungen zur polyäthylenglycolinduzierten Zellfusion

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Abstract

Cell fusion – polyethylene glycol – secretion – pseudopodia

Cells of monolayer cultures are fused by high concentrations of polyethylene glycol (PEG) with a molecular weight of approximately 1500. This process is independent of extracellular ca^{++}ions. PEG changes transiently the surface membrane and leads to fusion only after replacing it by normal medium. Before the final fusion of two cells, the onset of ionic coupling via longer lasting pseudopodial contact can be measured. Only cells that are synchronous in the secretory and pseudopodial response to PEG may fuse with each other.

Introduction

Since membrane fusion of plant protoplasts and animal cells is induced by several different agents such as lyso compounds [10, 16], Ca^{++}ions [2, 16, 17], virus [11], and polyethylene glycol (PEG) [1, 8, 13], there may well be a universal endogenous mechanism which is switched on after addition of these substances. Several hypotheses on this mechanism of fusion have been published (for review see [5, 15]), but there are still many unresolved questions to answer, before a final theory may be developed.

Because of its low cytotoxicity, its high fusion rate and its relatively clear defined chemical structure, PEG offered some advantages for the investigation of the fusion mechanism. In this paper we report electrophysiological and electronmicroscopical investigations on fusions of cultured mammalian cells. Our experiments show that

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fusion of membranes is at least a three-step process: initiation of secretion and thus membrane synthesis, transient alteration of the surface membrane properties, and contact of susceptible membranes.

Materials and methods

Cell lines and fusion conditions

The L cell derived mouse fibroblasts Cl-1 D were obtained from DR. PETERS (GENETIC Institute, University Köln) whereas the HeLa cells and the rat brain tumor cells BT 5 C 2 were the same as already described [9]. The cells were cultured in plastic dishes (Falcon) using modified [4] Eagle-Dulbecco's (Ea-Du) medium supplemented with 10 % calf serum.

Normal grade PEG from Hoechst, Koch-Light, Polysciences, and Roth had been used; highly purified PEG for gaschromatography was obtained from Merck. PEG was liquified by warming in a water bath at approximately 70° C, so that v/v mixtures with Ea-Du medium without calf serum were possible.

Monolayers of the cells were incubated with PEG at room temperature for different times as indicated in Table I. The fusion process was observed under phase contrast microscopes (Leitz Diavert and Zeiss Standard) and recorded with a TV time-lapse recorder (National). The fusion index was determined 3 hours after PEG by counting the nuclei per 100 cells in May-Grünwald-Giemsa stained preparations.

Electrophysiology

Membrane potentials (PD) and ionic coupling were measured with KCl-filled glass microelectrodes by operational amplifiers (WPI 701 and WPI 750). For details see [7].

Electron microscopy

The cells were fixed in situ with 3 % glutaraldehyde in phosphate buffer for 30 minutes at room temperature, 60 minutes with 1 % osmium tetroxide in phosphate buffer and 60 minutes with 1 % uranyl acetate in aqueous solution. With a rubber policeman the cells were removed from the plastic dishes, pelleted and mixed with equal volumes of 2 % agar in physiological saline. Small cubes of this mixture were stepwise dehydrated in ethanol and embedded in Epikote (Roth). Ultrathin sections were made with a Reichert Om U 3 microtome, poststained with lead citrate and uranyl acetate and viewed under a Zeiss EM 10 microscope.

Results

Fusion index and cytotoxicity

The yield of cell fusions depends on cell density of the treated culture; the highest fusion index was counted when the cells were still in the late log phase of growth and formed already a monolayer. This was evident especially for the epithelioid HeLa cells, whereas the fibroblastoid cells Cl-1 D and BT 5 C 2 gave reasonable fusion yields also in subconfluent regions. The highest yield of cell fusion was given by PEG with a molecular weight of about 1500, which we had tested with different concentrations on more than 10 cell lines, 3 of which are listed with their optimal PEG concentration in Table I. The PEG preparations from different manufactures gave similar fusion indices, but were different, however in their cytotoxicity levels. Because of its high fusion index and low

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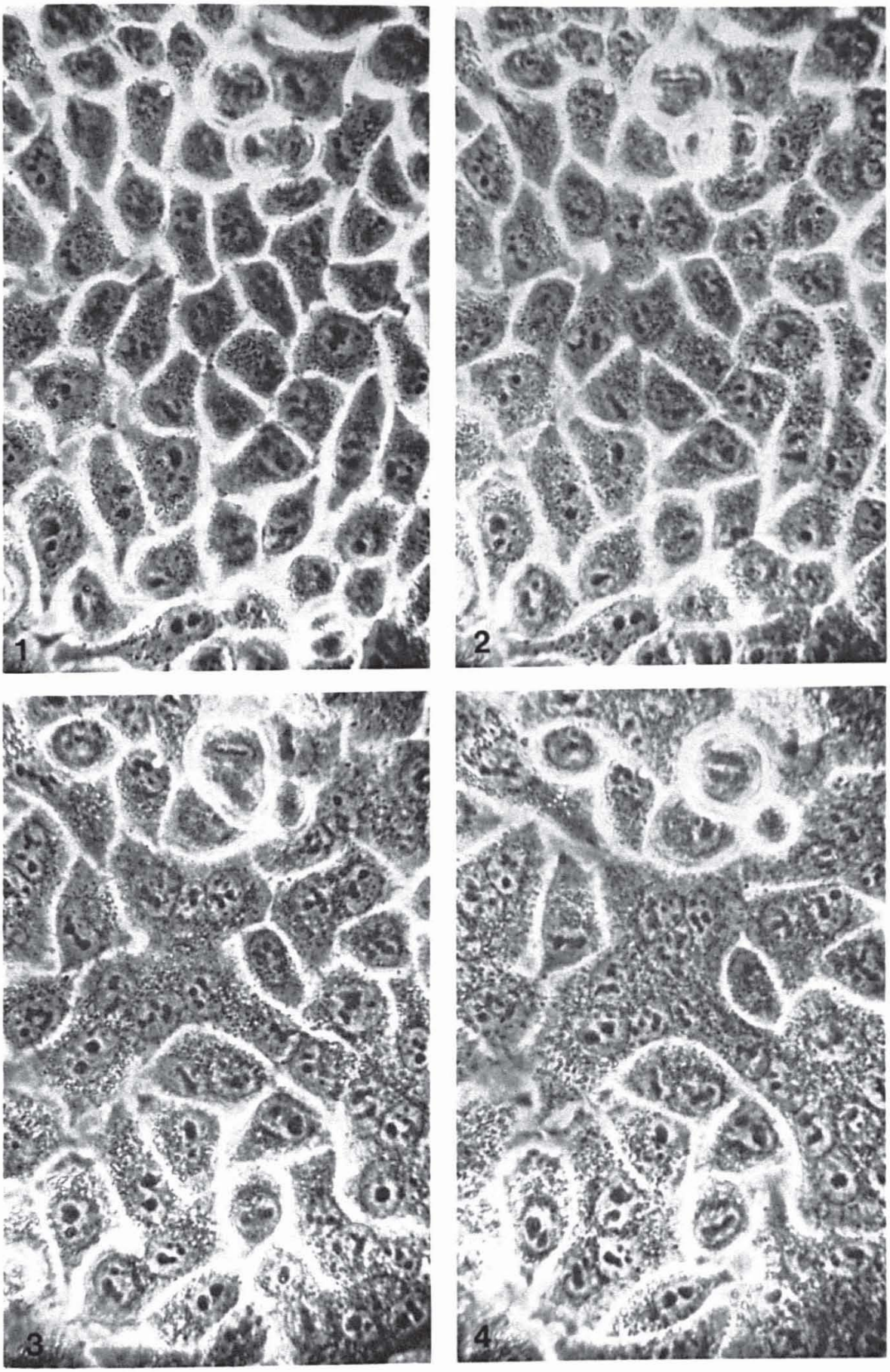
Figs. 1 to 4. HeLa cells at 3, 12, 18, and 41 minutes after PEG treatment. Note that some cells remain separated, e. g. the mitosis cell (top right).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cytotoxicity, we used PEG 1540 from Koch-Light for the experiments presented in this paper. PEG 1500 from Polyscience gave similar results, whereas PEG 1500 from Roth had a slightly higher cytotoxicity, and almost no cells survived for more than 3 hours the treatment with PEG 1500 from Hoechst.

Obviously these PEG grades were not quite pure; however, this impurity had no effect on fusion, only on cytotoxicity. To exclude the possibility of a fusion caused by impurities, especially from divalent cations which may be introduced during the preparation of the substance, we tested also very pure PEG grades (purchased for gaschromatography) and complexed eventually present divalent cations by addition of EDTA. PEG 1000 for gaschromatography fused the cells with the same high fusion index and with the same low cytotoxicity as PEG 1540. Gaschromatography PEG 4000, however, gave only a fusion index of 122 for HeLa cells under the same conditions and was also not cytotoxic. Addition of 2 mM EDTA to the PEG 1540 solution did not affect the fusion index, 3 mM EDTA caused cell death within 1 hour.

Tab. I. Optimal fusion conditions for treatment with 45 % PEG (molecular weight 1540, Koch-Light) at room temperature. Fusion index (number of nuclei/100 cells) was determined after 3 hours in May-Grünwald-Giemsa stained cultures.

Cell line	Incubation period minutes	Fusion index	Fusion index of untreated control
HeLa	5	380	109
Cl-1D	25	216	112
BT5C2	15	266	103

Light microscopy

Approximately 30 seconds after addition of PEG-medium the cells produced spike-like protrusions and about 5 minutes later the nucleus could no longer be seen. During this PEG treatment no fusion could be observed, not even within a 30 minutes incubation period. Replacing PEG by normal Ea-Du medium, the nucleus reappeared within 2 minutes and the cells produced numerous pseudopodia. These pseudopodia enabled closer contact between the cells and led to fusions of neighbouring cells (Figs. 1 to 4) within 15 minutes. The fusion process was finished after about 1 hour of PEG replacement. Numerous polynuclear cells could be observed at this time, but also many cells with pseudopodia, which at this stage no longer led to fusions (Fig. 5). Often polynuclear cells separated some hours later forming smaller polynuclear cells. The higher the number of nuclei in one cell, the earlier it separated. The fate of these cells, however, had not been studied in this context.

Electrophysiology

The PD of PEG incubated cells was reduced from different control levels to about 15 to 20 mV within 2 minutes (Tab. II) and an almost constant zero potential indicated no marked change of electrode tip-potentials. Under PEG treatment the cell membrane and cytoplasm became very viscous. Upon insertion of an electrode into a cell, the membrane did not show its normal elastic behaviour but appeared very soft; the electrode penetrating it without any noticeable hindrance. After withdrawal of the

electrodes the insertion channels remained visible for several minutes indicating a high viscosity of the cytoplasm and the membrane. After washing PEG off the cells, the PD increased in all cases and the control PD was reached after about 1 hour. Several hours later, the PD was in most cases higher than the control level, especially in multinucleated cells. Furthermore, the stability of the PD measurements was considerably better.

Tab. II. Membrane potentials (mean ± standard deviation) of 3 cell lines in different solutions. 1. Control: Ea-Du medium + 10% calf serum; 2. 45% PEG in Ea-Du medium without calf serum; 3. 60 minutes after PEG: Ea-Du medium + 10% calf serum; 4. Ouabain: HeLa 10^{-7} M, Cl-1D and BT 5 C2 10^{-3} M in Ea-Du medium + calf serum, incubation period 18 hours. 5. Ouabain + PEG: addition of 45% PEG into cultures treated as described in column 4, immediate measurement.

Cell line	Control	45 % PEG	60 minutes after PEG	Ouabain	Ouabain + PEG
	mV	mV	mV	mV	mV
HeLa	29.0 ± 0.6	20.6 ± 0.5	35.6 ± 1.3	7.3 ± 0.7	14.2 ± 0.3
Cl-1D	24.4 ± 0.6	13.0 ± 0.4	31.3 ± 0.7	7.9 ± 0.2	15.4 ± 0.4
BT5C2	50.1 ± 0.4	15.1 ± 0.3	46.6 ± 0.7	8.4 ± 1.1	18.6 ± 0.5

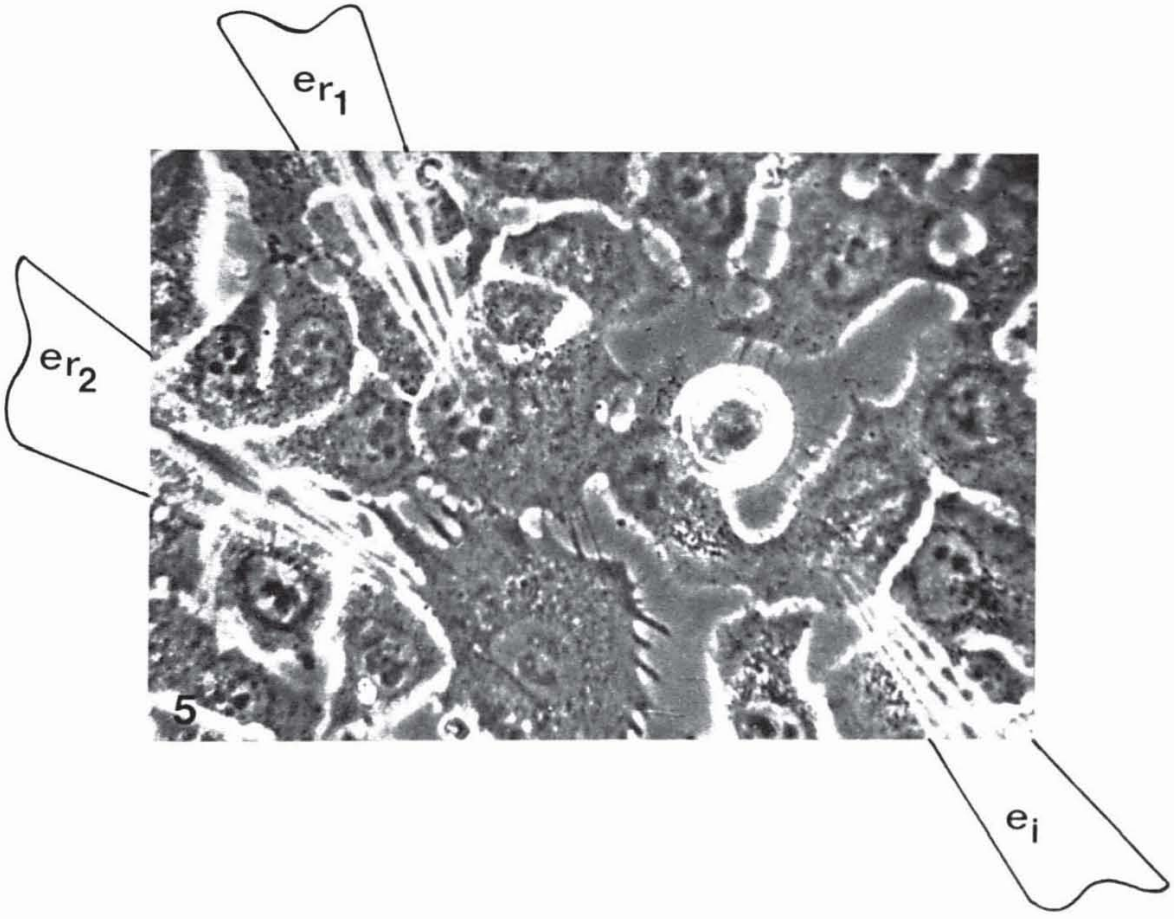

Fig. 5. HeLa cells 40 minutes after PEG treatment. The cell indicated by the stimulating electrode e_i is ionically coupled with the cell indicated by recording electrode e_{r1}. In spite of numerous pseudopodial contacts the cell indicated by the recording electrode e_{r2} is not coupled to the others.

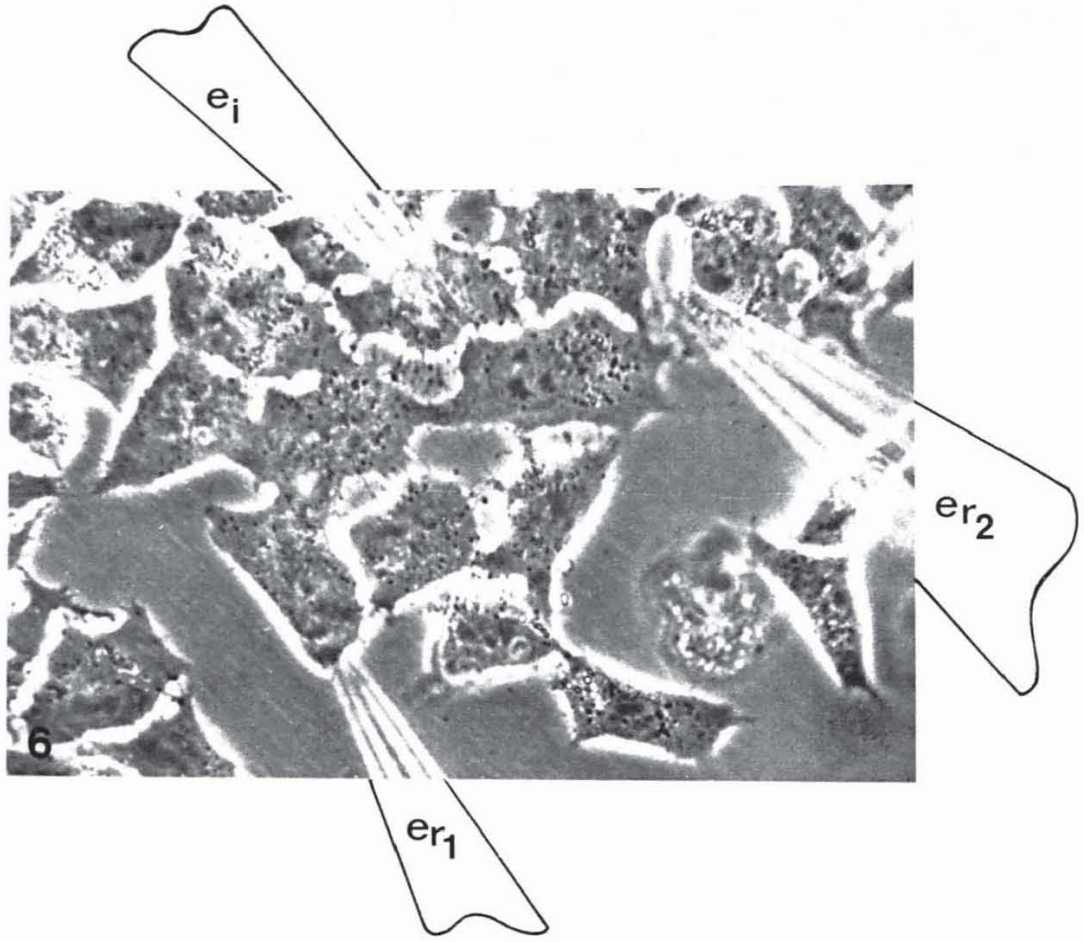

Fig. 6. HeLa cells 20 minutes after PEG treatment. The cells indicated by the electrodes e_i and e_{r1} are already ionically coupled with each other, the cell indicated by the electrode e_{r2} is not coupled with the others.

A 20 hours inhibition of the Na-K pump by ouabain decreased the PD of control cells to about 8 mV. This value was instantly raised to about 15 mV, when PEG was added to the ouabain-treated cells (Tab. II), which again showed this high viscosity behaviour. After replacing the PEG-ouabain solution against Ea-Du medium, the PD recovered and the cells fused in the same way as the non-ouabain treated cells.

For the demonstration of the onset of fusion, ionically non-coupled cells (e. g. HeLa) are of special interest. By measuring the intercellular communication it was found, that cell contact by pseudopodia does not necessarily indicate electrical coupling. In spite of its numerous pseudopodia, the mononuclear HeLa cell in Figure 5 was not ionically coupled with the elongated polynuclear cell, 40 minutes after PEG. Even at earlier stages when all cells showed pseudopodia, the electrical contact, and therefore

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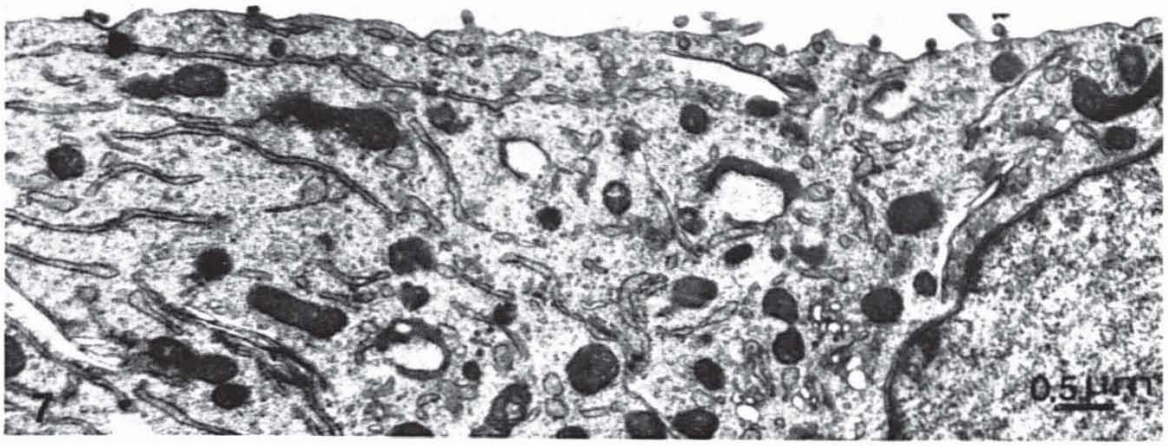
Fig. 7. Cl-1 D cell, untreated control: heavily stained mitochondria and filamentous ER. – Bar 0.5 μm.

Fig. 8. Cl-1 D cell immediately after a 20 minutes PEG treatment: swollen mitochondria and partially vesicular ER. – Bar 0.5 μm.

Figs. 9 and 10. Cl-1 D cells 30 minutes after a 20 minutes PEG treatment. Numerous vesicles fused with the surface membrane and dense bodies indicate a secretion process. – Bar 0.5 μm.



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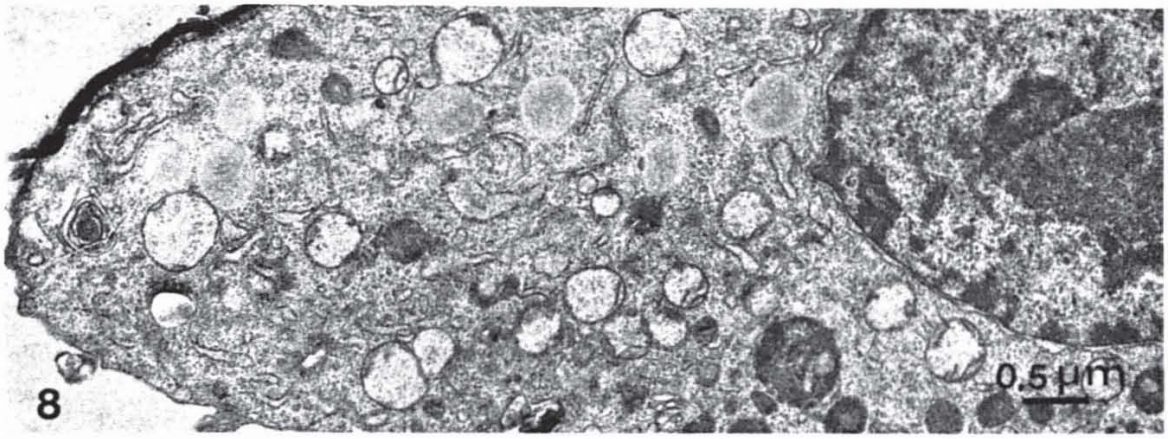

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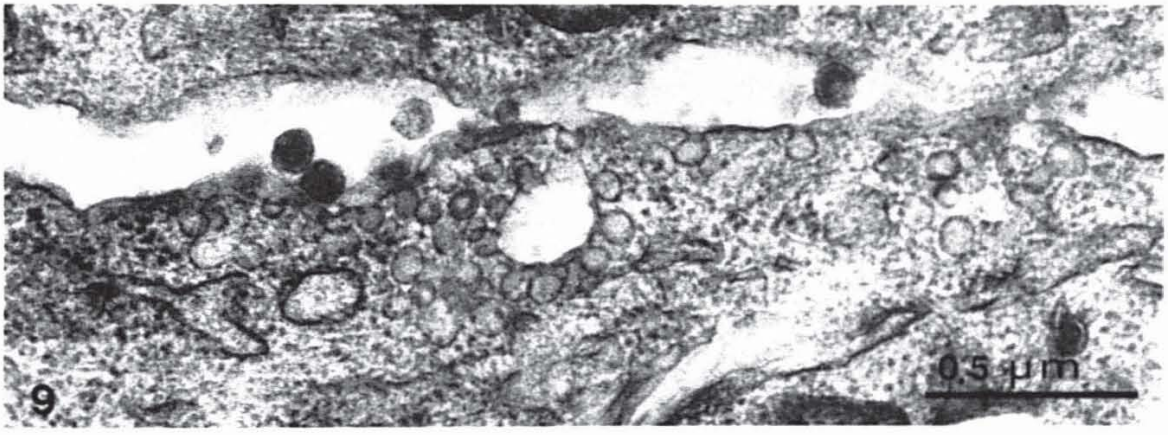

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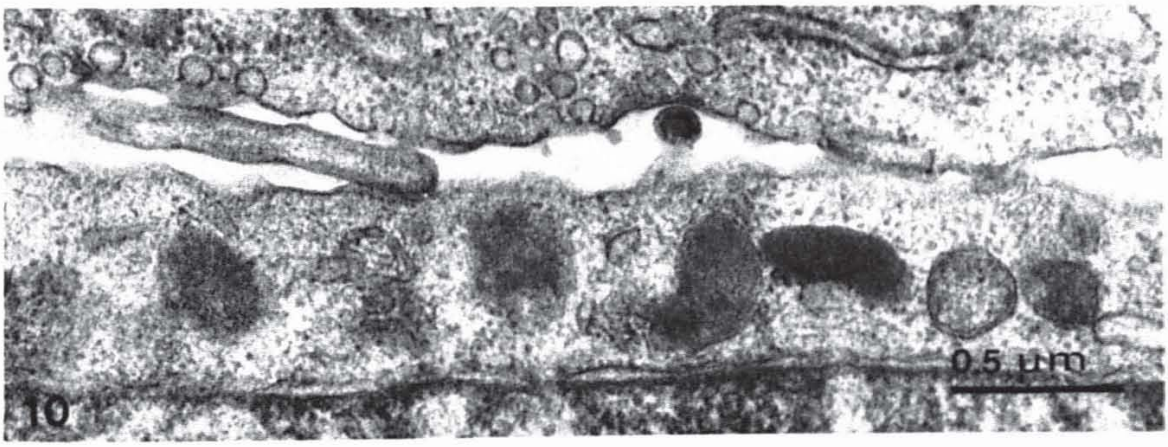

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the fusion, occurred only when larger parts of the membrane had contact for several minutes. The HeLa cells in Figure 6 were 20 minutes after PEG already connected by cytoplasmic bridges; the cell indicated by the current injecting electrode e_i is coupled via another cell with the cell indicated by the recording electrode e_{r1}. No current spread into the cell indicated by the recording electrode e_{r2}. In contrast to the situation in Figure 5, where only one cell still had pseudopodia, the cells may well fuse some time later at this stage.

Electron microscopy

The electronmicroscopical observations are demonstrated here with pictures taken from Cl-1D cells; similar results have been obtained, however, for HeLa and BT5C2 cells. Untreated culture cells showed the known inner structures, such as the laminar filamentous ER, prominently stained mitochondria, sometimes Golgi apparatus and various intracellular organelles (Fig. 7). With up to 5 minutes incubation in PEG, no drastic alterations of the inner structures could be seen, except that the prominent staining of mitochondria was no longer evident. After 10 minutes incubation in PEG, significant alterations of inner structures were observed: the filamentous ER changed to a vesicular form, mitochondria appeared swollen and empty, only with few cristae (Fig. 8). This state did not change with up to 30 minutes treatment with PEG. 10 minutes after washing off PEG, the inner structures appeared almost normal again; ER was laminar, mitochondria were no longer swollen and were once again prominently stained. Besides these normal structures, numerous dense bodies with or without membranes, coated vesicles and an increased number of Golgi fields were then seen. The vesicles became fused with the plasma membranes and the dense bodies were budded into the intercellular space (Figs. 9, 10). About 30 minutes after PEG most cells had built up these structures and often fused cells could be detected (Fig. 11). Membrane fusion occurred preferentially at regions where numerous vesicles and microfilaments led to a spotlike fusion between attached cells. 90 minutes after PEG many fused cells were detected and the appearance of inner structures was quite normal again.

Discussion

Monolayer cells in culture were preferentially fused by PEG with a molecular weight of 1540. Since the cytotoxic effect of PEG with a molecular weight of about 1500 was different for grades from different manufacturers, impurities of these substances were also taken into account. The unknown impurity responsible for cell death had obviously no effect on the fusion, but there could be another common impurity which caused cell fusion. Since during the manufacturing process of PEG, divalent cations are used as a catalytic substance and such ions have been reported as a necessary additive for fusion [2, 16, 17], they could have been such an impurity. Addition of 2 mM EDTA to the PEG medium, however, did not influence the yield of fusions and even 2 mM EDTA in the incubation medium could not reduce the yield of fusion but led to cell death after 20 hours. This does not exclude that Ca^{++} ions are released within the cells after PEG treatment.

High grade purity PEG (for gaschromatography) of a molecular weight of 1000 had the same fusion yield and a similar low cytotoxic effect as the best PEG 1540 batch. Such a pure PEG with a molecular weight of 4000 proved to be almost incapable of inducing such fusions under the described conditions.

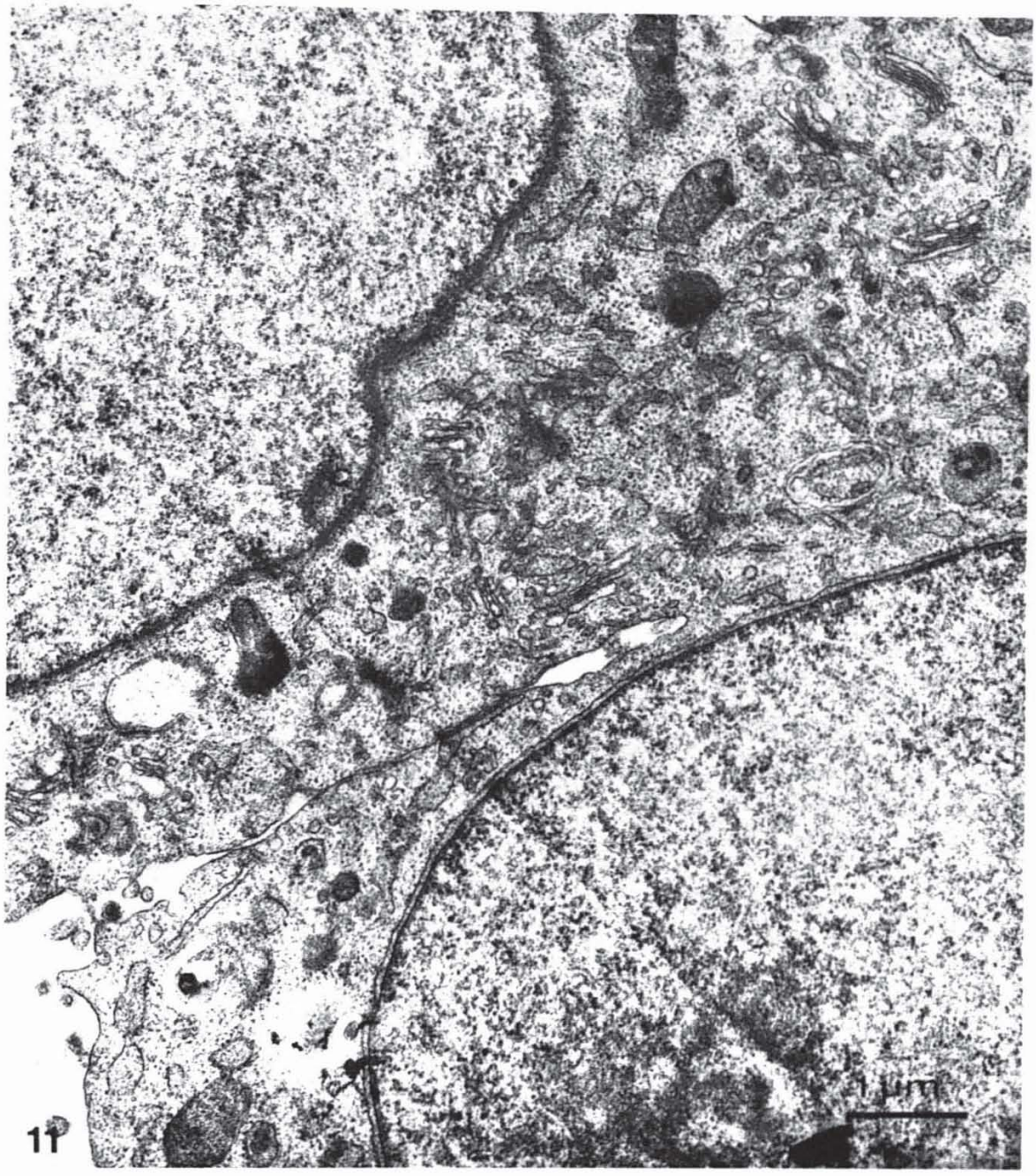

Fig. 11. Cl-1D cells 30 minutes after a 20 minutes PEG treatment. The cells are partially fused. Note the numerous Golgi fields, coated and uncoated vesicles and the reappearing of heavily stained mitochondria. – Bar 1 µm.

These experiments showed that only PEG of a certain molecular weight was responsible for the induction of cell fusion, but did not indicate whether the substance interacted with the outer plasma membrane (fusion from outside), or penetrated into the cells (fusion from inside), or if both steps were necessary.

As long as the cells were kept in PEG medium, no cell fusions occurred, which of course does not completely rule out fusion from outside, because many metabolic processes are inhibited in this solution. The microelectrode measurements revealed drastic alterations of the electrical properties and the viscosity of the PEG treated cells, so that a penetration of PEG into the cells can be assumed which can also be concluded

by interference measurements (our unpublished results). Because of its high viscosity the hydrophilic polymer PEG penetrated into the cells with a lower velocity than water coming out. This hypertonic medium led to the spiked appearance of the cells. The penetration of PEG into the cells resulted in a gradual change of phase differences, so that inner structures could no longer be observed under phase contrast. They were still present, however, as shown by the electronmicroscopical observations (Fig. 6). The penetrated PEG influenced intracellular molecules and the plasma membrane, so that a completely different diffusion potential, a different membrane permeability and a very viscous behaviour of the whole cell resulted. The inner milieu of the cytoplasm probably became hypotonic with regard to the mitochondria and the vesicular ER.

Washing off the extracellular PEG led to a diffusion of free intracellular PEG out of the cells, resulting in the reappearance of inner cell structures under phase contrast observations. This was always accompanied by plasma membrane ruffling and pseudopodial contact, that has also been described for other monolayer cultures [12, 14] and virus-induced fusion of ascites cells [6]. Our electronmicroscopical investigations revealed that a secretion process starts a few minutes after washing off PEG. This secretion process was indicated by the increased appearance of Golgi fields, dense bodies, coated and uncoated vesicles, some of which could also have been due to membrane recycling (Figs. 9 to 11). Electrophysiological measurements have shown that fusion of two active cells is preceded by ionic coupling via pseudopodia.

Cell fusion occurring after PEG treatment, however, is no unspecific event starting from inside which could result after other cell treatments leading to such severe secretions. Preliminary experiments revealed that the initiation of secretion by addition of non-metabolised substances to cell cultures, resulted always in secretion processes but did not end necessarily with cell fusion (our unpublished observations). PEG with a higher molecular weight than 6000 had no effect on cell fusion, but obviously interacted with the surface membrane of e. g. lymphocytes, in such a way that a better cell contact was obtained which resulted in a potentiation of a mitogen-induced lymphocyte stimulation [3]. Taking these results into account, one can assume that the PEG fusion process must at least be subdivided into three steps: 1. An initiation of secretion processes, resulting in membrane synthesis and pseudopodia formation; 2. a transient transformation of the surface membrane; 3. spotlike contacts of such susceptible cell membranes leading to fusion. Only those cells fuse with each other that are more or less synchronous in their secretion process.

Using this working hypothesis, investigations are in progress on combinations of substances which do not fuse per se, but which may allow to differentiate between essential and non-essential processes for the fusion.

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