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Cell-to-Cell Communication in Monolayers of Epithelioid Cells (MDCK) as a Function of the Age of the Monolayer

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Summary. We explore the existence of cell-to-cell communication in monolayers of MDCK cells plated at high densities so that they form a continuous monolayer in a few minutes. Lucifer Yellow CH is injected in the cytoplasm of a given cell by using a glass microelectrode with a fine tip (*ca.* 100 MΩ) and passing square pulses of current of 1.0 nA that last 10 msec, every 20 msec, during 1 to 3 min. We then examine the monolayer with fluorescence microscopy. In 27 out of 111 cells injected during the first 4 to 15 hr after plating, the dye was transferred to neighboring cells. Electron micrographs of freeze-fracture replicas prepared at this time, show that 20 to 25% of the lateral surfaces present the aggregates of intramembrane particles typical of gap junctions. These early hours correspond to the formation of occluding junctions and polarization into an apical and a basolateral domain of the plasma membrane (Cereijido, Meza & Martínez-Palomo, 1981). Cell-to-cell coupling then decreases sharply and, in the period between the 1st and 3rd day (mature monolayers), only 4 out of 49 injected cells were able to transfer the dye to their neighbors in the monolayers. No image of gap junctions was found in freeze-fracture replicas of mature monolayers. The degree of coupling between cells, as well as the number of cells coupled to the injected one, were highly variable. The lack of coupling between cells in mature monolayers observed in this article with Lucifer Yellow CH and electron microscopy is in keeping with the absence of electrical coupling observed in a previous work (Stefani & Cereijido, 1983). The transient existence of communicating junctions observed in monolayers of MDCK cells is similar to that described in the literature for embryo tissues during development.

Key Words cell-to-cell communication · Lucifer Yellow · freeze fracture · membrane resistance · epithelial monolayers · MDCK cells

Introduction

Epithelial cells establish between them low resistance junctions (for a review *see* Loewenstein, 1981) that may play a role in growth, differentiation (Loewenstein, 1968, 1979; Burton, 1971; Sheridan, 1971) and in the homeostasis of the tissue (Loewenstein, 1966, 1981). These intercellular communications have also been observed in cultured tissues (Furshpan & Potter, 1968; Borek, Higashino & Loewenstein, 1969; Azarnia & Loewenstein, 1971; Gilula, Reeves & Steinbach, 1972; Sheridan et al., 1978; Flagg-Newton & Loewenstein, 1979). MDCK cells, an epithelioid line derived from the kidney of a normal dog (Madin & Darby, 1958) retain a considerable degree of differentiation and form continuous monolayers that resemble natural epithelia (Misfeldt, Hamamoto & Pitelka, 1976; Cereijido et al., 1978*a*,*b*). Yet in a previous work (Stefani & Cereijido, 1983) we impaled with microelectrodes pairs of neighboring cells, and were unable to detect in one of them the square pulses of current injected in the other. Furthermore, the voltage response of each of those cells was described by a single exponential term, indicating that current was not leaking to other cells. That study was performed with mature monolayers, i.e. plated at confluence for more than one day.

However, recent experiments from this laboratory, using a mutant of MDCK cells that is resistant to relatively high concentrations of ouabain (A. Lázaro et al., *unpublished results*) demonstrated that it can rescue the wild type from the action of the drug when both types of cells are co-cultured in the same monolayer. Since this type of protection has been generally associated to the existence of cell-to-cell communications (Ledbetter & Lubin, 1979, 1980) and in our experiments we discarded the idea that the protection was due to humoral mechanisms (e.g. the secretion of a ouabain-lysin) we decided to reinvestigate the existence of intercellular coupling as a function of time, including early events in recently plated monolayers, using Lucifer Yellow CH. Fluorescent dyes constitute a valuable tool to explore relatively large numbers of cells (Loewenstein & Kanno, 1964; Azarnia & Loewenstein, 1971; Flagg-Newton & Loewenstein, 1979; Jeansonne et al., 1979) and, in particular, Lucifer Yellow was used with embryo cells (Bennett, Spira &

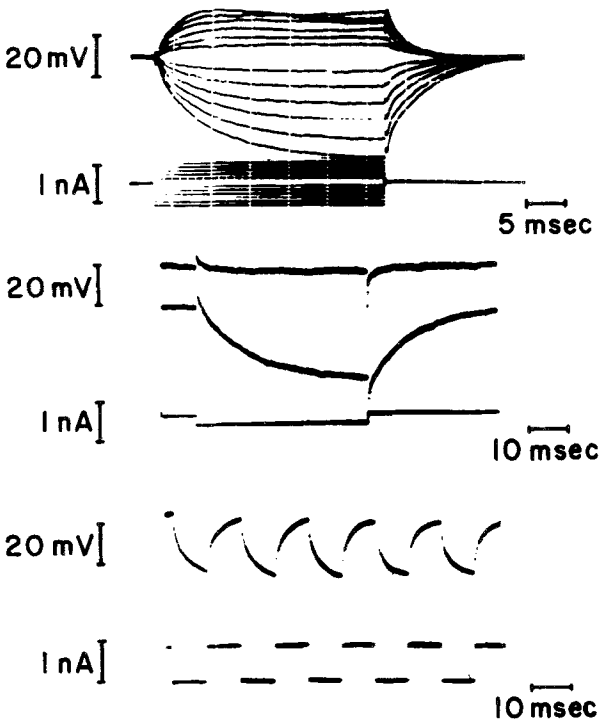


Fig. 1. Electrical characteristics of the MDCK cells and of the procedure used to inject Lucifer Yellow. *Above:* voltage/current relationship obtained with current pulses of opposite polarity and various intensities, as registered with a microelectrode of *ca.* 100 MΩ filled with 4 M K-acetate. Notice the asymmetry of the voltage response for large current pulses. *Middle:* Impalement of and MDCK cell with a microelectrode of 160 MΩ filled with 3% Lucifer Yellow CH in 1.0 M LiCl. The first two recordings correspond to the voltage response to a square pulse of negative current (*third recording*) when the microelectrode is outside (*first recording*) and inside (*second recording*) the cell, respectively. The change in the steady-state voltage corresponds to the membrane potential. *Bottom:* Voltage response to the train of current pulses of 1.0 nA, a duration of 10 msec and an interval of 20 msec used to inject the dye. The injection lasted 1 to 3 min

Spray, 1978; De Laat et al., 1980), retinal cells (Stewart, 1978), aortical venous endothelia (Larson & Sheridan, 1982), rat islet cells of the pancreas (Kohen, Kohen & Rabinovitch, 1983), etc. Furthermore, since the structural basis of cell-to-cell communication is associated with gap junctions, we also resorted to electron-micrograph analysis of freeze-fracture replicas of MDCK monolayers as a function of time, starting 1.5 hr after plating at confluence.

Materials and Methods

Cell Culture

Starter MDCK cultures were obtained from the American Type Culture Collection (MDCK, CCL-34) (Madin & Darby, 1958). In most experiments cells were between 56th to 68th passages. Cells were grown at 36.5°C in T150 Costar bottle (Costar, Cambridge, Mass.) with an air-5% CO_2 atmosphere and 20 ml of Complete Dulbecco's Minimal Essential Medium (CMEM) with Earle's salts (Grand Island Biological Co. (GIBCO), 430-1,600, Grand Island, N.Y.), 100 U/ml of penicillin and 100 μg/ml of streptomycin, and 10% calf serum (GIBCO 617). Cells were harvested with trypsin-EDTA (GIBCO 540), and plated at confluence on glass coverslips contained in petri dishes. They were kept at 36.5°C in an air-5% CO_2 atmosphere with constant humidity (V.I.P. CO_2 incubator 417, Lab Line Instruments, Inc., New Brunswick, N.J.).

Electrophysiology

Microelectrode recordings of electrical parameters and injection of Lucifer Yellow were made in monolayers cultured on glass coverslips and placed on the stage of a Leitz phase-contrast microscope (Leitz Laborlux, Wetzlar, Germany). The recording solution was CMEM. Experiments were carried out at 36.5°C. To avoid evaporation the saline was covered with a thin layer of oil (Cannon Instruments Co.). Microelectrodes were pulled with a two-stage puller (P77 Brown Flaming, Sutter Inst. Co.), from microfiber borosilicate glass capillary tubes (Hilgenberg, OD 1.0 mm; ID 0.5 mm). To measure electrical parameters only, they were filled with 4 M K-acetate. In most cases we used microelectrodes with resistance of around 100 MΩ which gave stable impalements and showed little rectification properties with the currents we used (1 nA). The microelectrodes used had a small tip potential (0 to 10 mV) and if their properties checked after the impalements were altered, the recording was discarded. Microelectrodes were connected to standard high impedance amplifiers (WPI KS 700) which allowed us to record the membrane potential and inject current simultaneously via a subtracting circuit. The injected current was recorded with a WPI preamplifier (KS 700) or via a current-to-voltage converter (Analog Devices 52J) with 100 MΩ in the negative feedback loop, thus 1 mV ≡ −0.01 nA. The bath was grounded via a chlorided silver wire which was connected to the negative input of the current-to-voltage converter.

Prior to penetration the voltage-subtracting circuit was carefully adjusted delivering current steps (0.1 to 1 nA). In addition, the input capacity corresponding to the microelectrode and the amplifier was adjusted with a feedback circuit built in the amplifier.

Microelectrodes for recording and injection of fluorescent probe were pulled as explained above for the electrodes for recording only, but they were filled with 3% Lucifer Yellow CH (Polysciences, Inc. Warrington, Pa.) in 1.0 M LiCl and had resistances of 40 to 400 MΩ. These microelectrodes were connected to a Dagan 8100 Single Electrode System Amplifier (Dagan Co., Minneapolis, Minn.). A train of pulses of 1 nA and 10 msec were injected with an interval of 20 msec during 1 to 3 min. Since Lucifer Yellow is a naphthalimide dye with two sulfonic acid groups yielding a net negative charge of two, the electric pulses applied to deliver the probe into the cytoplasm of the MDCK cells were also negative. The microelectrodes were mounted on a piezoelectric step drive (Burleigh, Inchworm Controller, PZ-550) mounted on a Huxley type micromanipulator. To impale the cells, 4-μm steps were used.

Microscopy

At different times after injection the glass coverslip with the monolayer attached was washed in CMEM and mounted upside

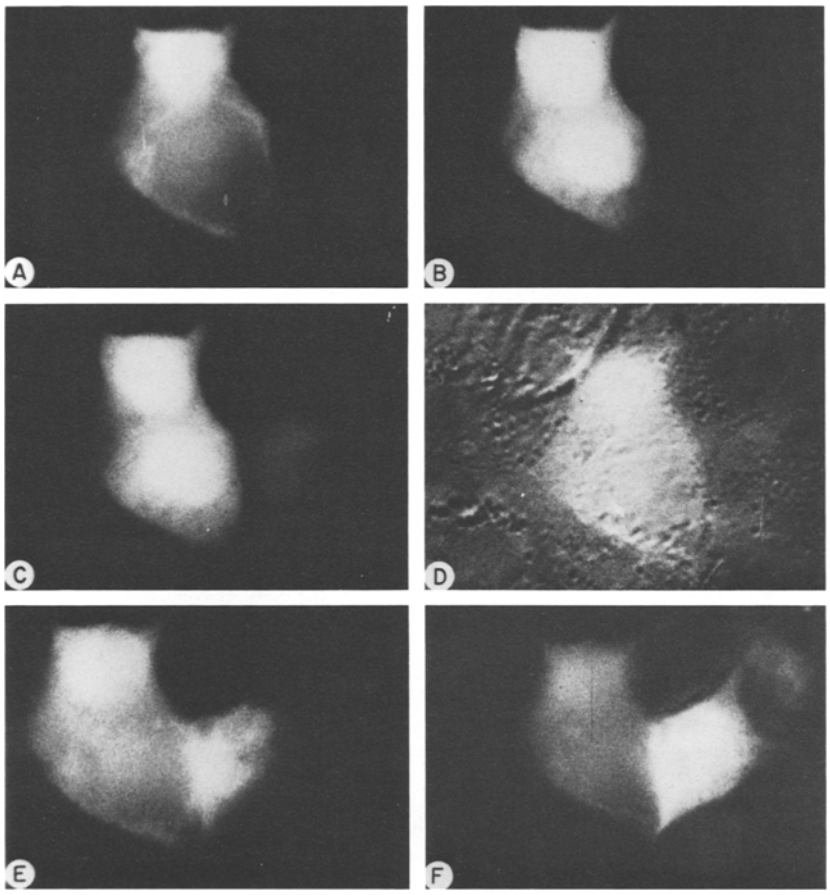


Fig. 2. Transfer of Lucifer Yellow in a group of three MDCK cells. (*A*) Three min after injection of the dye into the cell on the upper left the mark has already diffused to the larger cell on its lower right. Fifteen min (*B*) later the nuclei of both cells appear intensely stained. A very faint image of a third cell starts to show on the right corner (*C*). At this moment, a combined photograph with UV and light shows that other neighboring cells in the monolayer are not connected (*D*). Seventy min post-injection the cell in the lower right corner is clearly stained (*E*) and the image of the three cells have roughly the same intensity. Yet, surprisingly, 10 min later the image of the cell where Lucifer Yellow was originally injected starts to vanish (*F*)

down on a glass slide. The borders of the coverslip were sealed with Humiseal type 1B15 (Columbia Chase Co., Woodside, N.Y.) or nail enamel. The distribution of the probe was observed with an Orthoplan microscope (Leitz) equipped with epifluorescence optics: Filter block I2; BP 450-490; LP 515. Pictures were taken with an automatic Orthomat camera (Leitz) using Tri-X Film. Observations were made from less than a minute to more than two hours post injection.

Freeze-Fracture Electron Microscopy

Monolayers grown in petri dishes coated with collagen were fixed with 2% glutaraldehyde at different times after plating (1.5, 2, 3, 8, 12, 15 and 18 hr and 1, 2, 5 and 7 days). They were then processed for freeze fracture as previously described (Griepp et al., 1983).

Results

The electrical response of the plasma membrane of MDCK cells observed in his study was entirely similar to those observed by Stefani and Cereijido (1983) (Fig. 1). The use of dye-filled microelectrode does not seem to introduce major modifications in the electrical properties of the cells. Thus in the present study the microelectrodes filled with Lucifer Yellow CH in 1.0 M LiCl had a resistance of 94.9 ± 15.8 (23) MΩ and the value of the membrane resistance recorded was 58.2 ± 3.2 (143) MΩ, which compares with 61.6 ± 6.3 (92) MΩ that was the value recorded previously with microelectrodes of 100 to 150 MΩ filled with 4.0 M K-acetate. The fluorescent probe was delivered with a train of pulses lasting 1 to 3 min (Fig. 1, *bottom*) and did not affect the electrical characteristics of the cell.

Figure 2*A* corresponds to a monolayer where a single cell was injected and 5 min later the dye was transferred to its neighbor on the lower right. Fifteen minutes later these two cells were equally stained, their nuclei being more intense than the rest of the cells. In Fig. 2*C* the probe starts to diffuse towards another neighbor on the right. Figure 2*D* is a combined image of epifluorescence and transmitted light, to show that the lack of diffusion of the probe in other directions is not due to the absence of neighbors. By 40 min (Fig. 2*E*) the image of the new stained neighbor is more evident, and the three cells appear equally stained.

The fast diffusion toward the first neighbor and the slower one toward the second indicate that the degree of coupling between cells in the monolayer is heterogeneous. Figure 2*F* illustrates another curi-

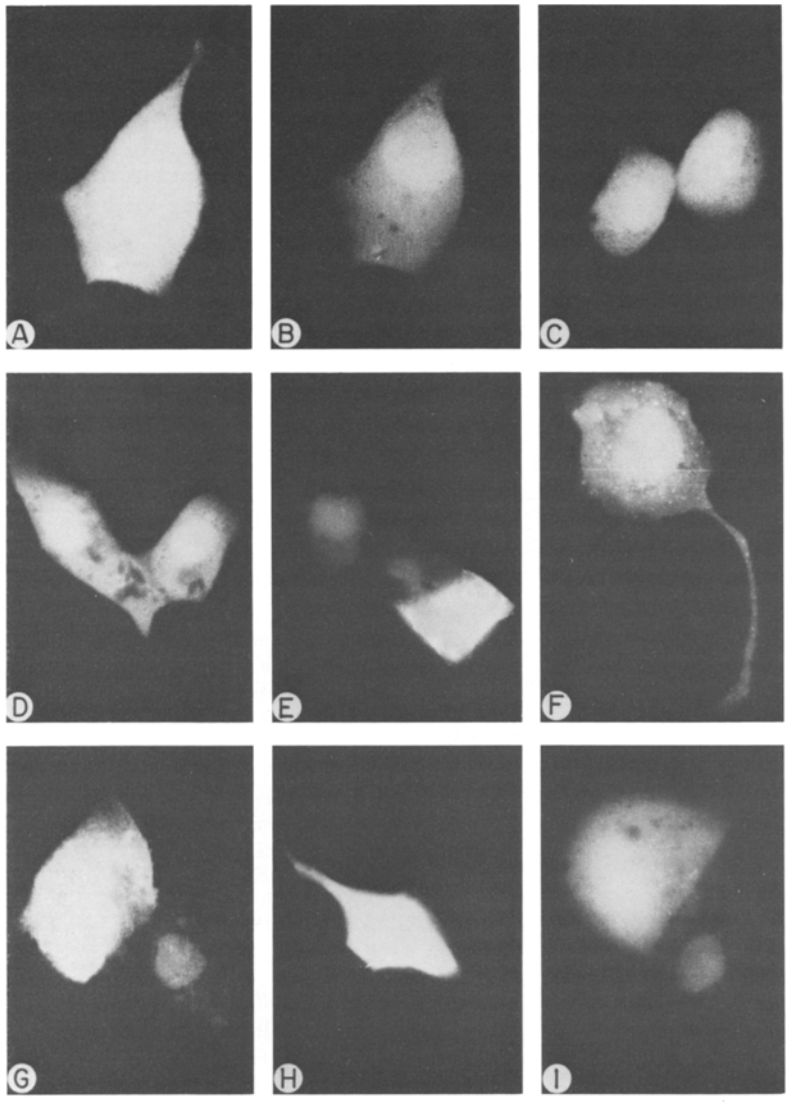


Fig. 3. Injection of Lucifer Yellow CH in MDCK cells plated as monolayers on glass coverslips. The plating density is high enough to achieve confluence in a few minutes. *A* and *B* are pictures of the same cell in a monolayer of 41 hr, taken 38 min apart. *C* and *D* show cells communicated 16 and 52 hr after plating, respectively. *E* corresponds to a cell communicated with a near and a distant neighbor. *F* shows a cell with a particularly long process. *A*, *H* and *I* are cells with a very low degree of coupling

ous characteristic of the transfer of dye: the third cell, that was so slow in staining, now shows the highest intensity of fluorescence. This could not be attributed to bleaching of the dye in the first cell, because the whole group was illuminated each time we inspected the cells. Furthermore, on the basis of a diffusional process, the dye should have achieved the same concentration throughout the cells and the dye in the first cell could never have decreased to a lower level than in other neighbors. Therefore, it is conceivable that cells would react to the presence of the dye by switching on some mechanisms that eliminates it from the cytoplasm. Unpublished studies from this laboratory indicate that MDCK cells retain the ability to extrude fluorescein toward the interspace as some epithelial cells in the normal kidney. However, we have no information yet on the cellular mechanism of this transport.

Figure 3 illustrates other characteristics of the injected cells. Figures 3*A* and 3*B* show the same cell 5 and 70 min post-injection. In the second case the image appears faded because of the repeated exposure to light during this period, yet the probe did not diffuse towards neighboring cells. Figures 3*C* and 3*D* illustrate the case of cells connected within the 3 min of injection. In Fig. 3*E* we injected the cell on the bottom right and it transferred the probe with more intensity to a distant rather than to a closer neighbor. It is not clear whether the distant transfer took place through the nearest neighbor, and this neighbor extruded the probe as suggested above for Fig. 2*F*, or the dye reached the distant cell through long extensions of the type illustrated in Fig. 3*F*. Figures 3*G*, 3*H* and 3*I* correspond to cells that have been injected for more than half an hour. They do show communication with a neighbor, but it is very

poor. A comparison of these three examples with 3*C* and 3*D* supports the opinion mentioned above that the degree of connection varies widely.

Figure 4 summarizes our observations on whether a given cell is connected or not, regardless of the degree and of the number of connected neighbors. The study was carried out as a function of time after plating. Each injected cell is represented by a circle; those that communicated with at least one neighbor are represented with a full circle above the horizontal line. Cells that after 30 min remained as single stained cells are represented with an empty circle. Fractional numbers at the bottom of the Figure indicate the number of connected over nonconnected cells. Communication is observed mainly in recently plated cells. No connection was detected for instance in the second day, and on the third day only four cells (out of 28) had communications. Studies with high intracellular concentration of Ca^{++} (Loewenstein, 1967; Oliveira-Castro & Loewenstein, 1971; Rose & Loewenstein, 1976) and low intracellular pH (Rose & Rick, 1978; Spray et al., 1982*a*,*b*) suggest that a faulty impalement may cause previously connected cells to uncouple, due to the existence of junctional gates. Yet the demonstration that cells injected shortly after plating do have communication, indicates that the lack of connections found after the first day may not be the result of an artefactual impalement. The absence of cell-to-cell coupling in mature monolayers, agrees with the information of our previous study (Stefani & Cereijido, 1983) indicating that 20 pairs of neighboring cells in monolayers in steady state had no electrical coupling, and only in a few cases they presented a very small signal, which was almost completely masked by the noise.

In order to investigate the structural aspect of cell-to-cell communication, we prepared freeze-fracture replicas of the monolayers at different times after plating. Putative gap junctions were first seen in the basolateral plasma membrane 3 hr after plating as aggregates of a small number (less than 10) of intramembranous particles. At 8 to 12 hr larger aggregates (up to 300 intramembranous particles) with the more typical appearance of gap junctions (Fig. 5) were found on 20 to 25% of the lateral surfaces examined. Since only a small area of the basolateral membrane of each cell is represented in a fracture face, this suggests that at this time nearly all cells have established junctions. The frequency of gap junctions and the number of particles within them began to decrease after 15 to 18 hr and at 24 hr or later no gap junctions could be identified. Gap junctions were found both associated with the tight junctions, between tight junction strands (Fig. 5*a*, *b*) or near their ends, as well as at some distance from the tight junction region.

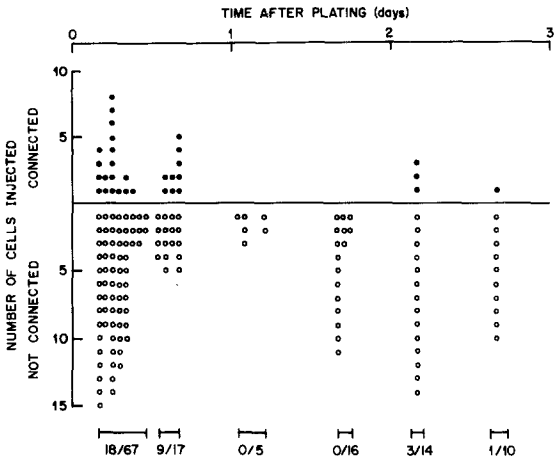
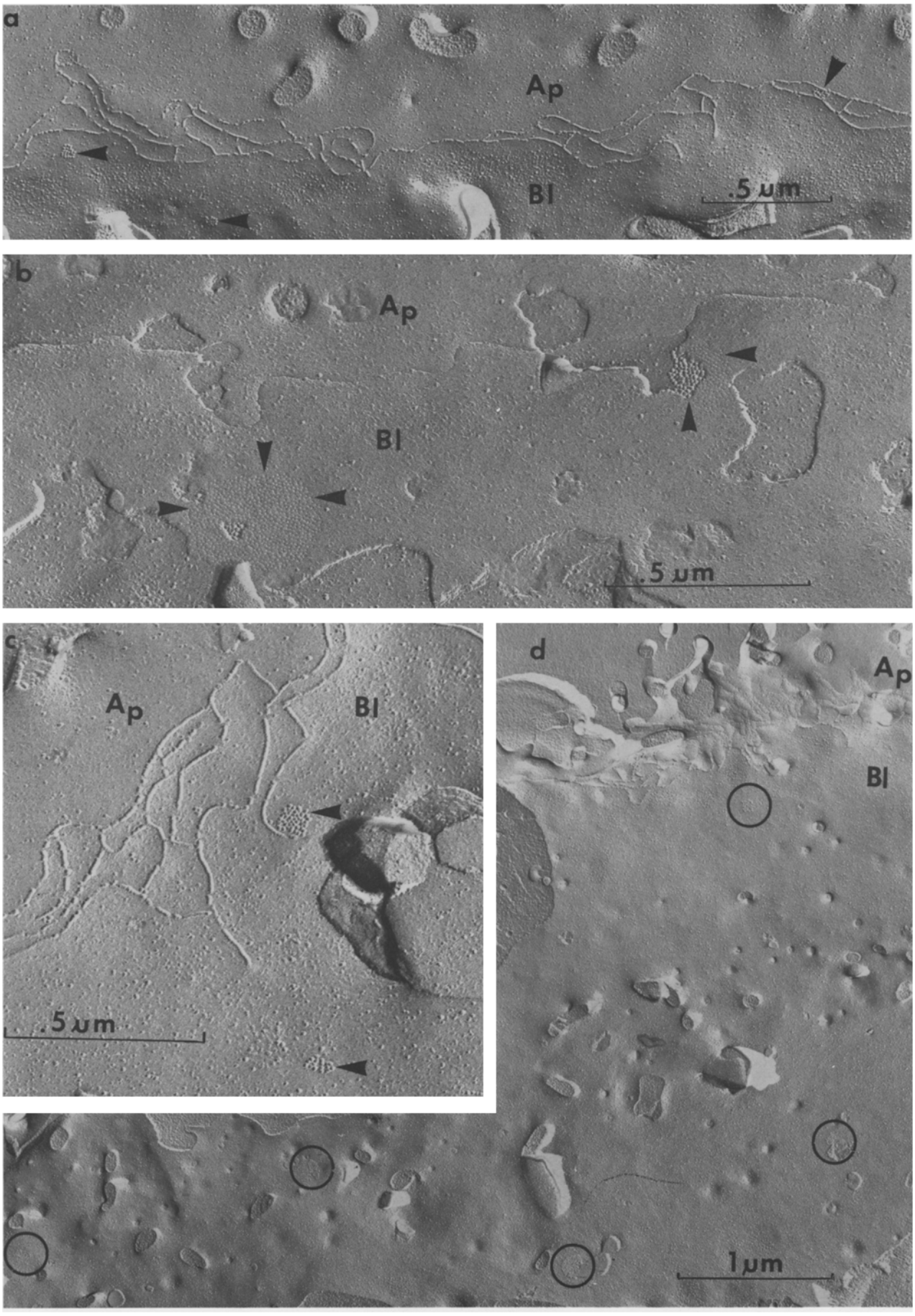


Fig. 4. Connected (*full circles*) and nonconnected MDCK cells (*open circles*) as a function of time after plating. A cell is scored as connected when it communicates with at least one partner. The fractional number at the bottom summarizes the number of connected over nonconnected cells in a given group

Discussion

As mentioned above, in mature monolayers (plated at confluence for more than a day) we could not detect electrical coupling (Stefani & Cereijido, 1983). This agrees with the results of the present study indicating that only 4 out of 49 cells injected transferred the probe to their neighbors. Several authors, following criteria of electric coupling, dye transfer or metabolic cooperation, found that many cell types do not exhibit evidence of communication (Borek et al., 1969; Azarnia & Loewenstein, 1971; Pitts, 1971; Azarnia, Michalke & Loewenstein, 1972; Gilula et al., 1972; Fentiman et al., 1976, 1977). Most of these noncommunicating cells are cancerous. Yet absence of communication was even observed in certain cells of normal tissues (De Laat et al., 1980; Warner & Lawrence, 1982) (for a review *see* Loewenstein, 1979). Yet MDCK cells are not incompetent, as they form cell-to-cell contacts at an early stage of the monolayer. The almost complete lack of communication in cells later on has its morphological counterpart, as we were also unable to find the typical image of gap junctions in freeze-fracture replicas. As observed in other tissues (Larson & Sheridan, 1982) the image of gap junctions appears mixed between the strands of the occluding junctions. Elias and Friend (1976) have pointed out that the discrete particles that form initial strings of the occluding junctions cannot be dis-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tinguished from those of gap junctions, and suspected that the particles of both kinds of junctions may stem from the same pool. Mature monolayers of MDCK cells have occluding junctions (Cereijido et al., 1980*a,b*, 1981; Martínez-Palomo et al., 1980); Griepp et al., 1983) but, apparently the particles forming their strands are unable to serve as connecting entities.

Cell-to-cell communications abound in embryo tissues (Potter, Furshpan & Lennox, 1966; Bennet et al., 1978; Lo & Gilula, 1979; McLachlin, Caveney & Kidder, 1983), and their number is known to vary in accordance with tissular needs such as cell proliferation (Loewenstein, 1968; 1979; Burton, 1971; Sheridan, 1976), coordination of development (Albertini & Anderson, 1976; Gilula et al., 1978; Heller & Schultz, 1980; Dekel et al., 1981; Heller, Cahill & Schultz, 1981; Moore et al., 1981; Brower & Schultz, 1982) and may even disappear and reappear in later stages. Therefore, the observation that newly plated MDCK cells undergo a transient of communication is not surprising. During this early period MDCK cells, that have been previously obtained by trypsination of monolayer grown in plastic bottles, regain their polarization into apical and basolateral regions, form occluding junctions, and adopt the features of a natural transporting epithelium (Cereijido et al., 1978*a,b*; 1980*a,b*; Meza et al., 1980; Martínez-Palomo et al., 1980). In a way, during the early hours after plating at confluence, a monolayer of MDCK cells compares to an epithelium in development, and it is interesting to notice that during this development the cells present junctions that would favor the transfer of organizing signals.

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Fig. 5. (*Facing page*) Freeze-fracture views of gap junctions in MDCK cells 8 hr after plating. *a*) P fracture face with three small gap junctions (*arrowheads*), one between the strands of the developing tight junction and two close to, but not in contact with, the tight junction strands. *Ap* = apical plasma membrane; *Bl* = basolateral plasma membrane (47,500×). *b*) An E fracture face demonstrating two gap junctions (*arrowheads*) associated with (*on right*) and near (*on right*) and near (*on left*) a developing single-stranded tight junction. In both cases the fracture plane travels from the E fracture face to the P face of the adjacent cell plasma membrane exposing in the same gap junction both typical E face pits and P face particles (75,000×). *c*) Two small gap junctions (*arrowheads*) near the tight junction region (75,000×). *d*) A wide area of basolateral membrane where five gap junctions (*circles*) are distributed. Four of these are located at distances greater than 2 μm from the tight junction (27,300×)

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