J. Membrane Biol. 29, 373–382 (1976)

Electrophysiological Study of Coupling between Cultured Cells of the Mouse Mammary Gland in Five Distinct Physiological States

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Received 26 January 1976; revised 6 May 1976

Summary. Electrical coupling has been observed between cultured cells of the mouse mammary gland in five distinct physiological or pathological states. We have employed young primary cultures of cells dissociated from the following tissues: normal glands from young virgin or midpregnant females, hyperplastic alveolar nodules (believed to be precancerous) transplanted in gland-free mammary fat pads, and spontaneous mammary adenocarcinomas and their pulmonary metastases. All successfully impaled pairs of cells (a total of 97 pairs) were found to be ionically coupled. Furthermore, in normal and tumor cell cultures, electrical coupling was observed between dome-dome and dome-nondome cell pairs. This study correlates with electronmicroscopic studies of fresh normal, hyperplastic, and tumor samples, which show the presence of gap junctions in all three.

Low-resistance junctions are specialized membrane sites that permit direct exchange of ions and small molecules between cytoplasms of adjacent cells. Their presence is detectable experimentally, as electrical coupling, metabolic cooperation, or the passage of dyes, between both excitable and nonexcitable cells of a variety of tissues and even between different cell types [3, 6, 11, 12, 15, 16, 19, 35]. Evidence from electronmicroscopy suggests that the gap junction [32] serves as the low-resistance pathway [12, 25].

Despite abundant proof of its existence, the function of low-resistance coupling of nonexcitable cells remains unknown. A widely discussed possibility is the exchange of signals regulating the coordination or integration of populations of cells [11, 19, 35], as in the growth and function of normal tissues. On the assumption that some abnormal forms of growth may reflect a failure of such exchange [20], a number of studies have compared electrical or morphological coupling in tumors or transformed cells in culture and in their nearest available normal counterparts; retention of coupling has been reported in some cases [4, 10,

34, 36], its loss or reduction in others [1, 2, 13, 14, 17, 24, 21]. Most of these studies have used transplanted tumor lines or established cell lines, in which cases the modification of junctional competence with time is a possibility, or human surgical specimens, for which the selection of normal control samples may pose problems. In no case has it been possible to compare multiple examples of a spontaneous tumor of known malignancy with the tissue of origin sampled in various physiological states.

As a strongly cyclic, hormone-sensitive tissue, normal mammary epithelium shows synchronous or integrated behavior in many ways. Mice infected with mammary tumor virus provide a spectrum of normal, hyperplastic, and neoplastic mammary tissues. In electronmicroscopic studies of thin-sectioned and freeze-fractured material ([30, 31] and *unpublished*), we have demonstrated gap junctions in normal and neoplastic mammary epithelial cells, both *in vivo* and in confluent cell cultures. We report here the results of tests of electrical coupling of epithelial cells from mammary tissue in a series of physiological and pathological states. Because the complex architecture of the mammary gland tissues makes accurate measurement *in vivo* prohibitively difficult, we have employed young primary cultures of cells dissociated from the following tissues: normal glands from young virgin or midpregnant females, hyperplastic alveolar nodules (presumably precancerous, [8]) transplanted in gland-free mammary fat pads, spontaneous mammary adenocarcinomas, and their spontaneous pulmonary metastases.

Materials and Methods

Source of Tissues

Normal glands were taken from virgin or mid-pregnant BALB/cCrgl, C3H/Crgl, or BALB/cfC3H/Crgl mice. Hyperplastic tissue was obtained from primary outgrowths of hyperactive alveolar nodules, transplanted to gland-free mammary fat pads or from nodule transplant lines D1 and D2 carried in BALB/c hosts [26]. Spontaneous mammary tumors and metastases in the lungs were obtained from C3H or BALB/cfC3H mice. Metastatic tumors were transplanted subcutaneously in isogenic mice and allowed to grow large enough to provide sufficient cells for culture; all metastatic tumors used were histologically identified as of mammary origin.

Cell Culture

Methods of cell dissociation and preparation for culture have been described elsewhere [30]. Dissociated cell suspensions were plated at densities of $2–5 \times 10^5$ cells/cm^2 of substrate

in 35 mm Falcon plastic petri dishes and cultivated in Waymouth's medium supplemented with 10% calf serum, insulin 10 μg/ml, and cortisol 5 μg/ml, reaching confluency in two days. On the 3rd day, the medium was replaced with the same mixture except that 0.5% bovine serum albumin was substituted for the calf serum. Electrophysiological measurements were made 1–2 days after this change. Shortly before the cultures were used, the medium was replaced with Hank's balanced salt solution (HBSS), pH 7.2, which had been gassed with 95% O_2 and 5% CO_2. The culture dish was then layered with mineral oil to prevent evaporation loss and kept at 35–37 °C by a heated microscope stage.

The use of serum-free medium after confluency of the cultures and HBSS prior to electrophysiology were to facilitate corollary studies on ion permeabilities ([33] and *unpublished*). Control observations showed the presence of electrical coupling between cell pairs of both normal and tumor cultures in Waymouth's medium supplemented with calf serum or bovine serum albumin.

Electrical Measurements

All coupling records were obtained with two intracellular microelectrodes in adjacent cells. The microelectrodes were filled with 4M K-acetate and had an initial resistance of 100–150 MΩ; they were then beveled [5] to a resistance of 40–60 MΩ. The membrane potentials were measured with conventional hi-impedance preamplifiers, which had bridge circuits for simultaneous recording and current stimulation. Currents of less than 5 nA were applied by a Grass S-4 stimulator and isolation unit; and were monitored across the microelectrode. Permanent records were made with Polaroid photographs of the oscilloscope screen and with a Gould Brush recorder. The recording setup is illustrated in Fig. 1.

For coupling measurements, the microelectrodes were either simultaneously or consecutively introduced into the cells. Entry into a cell was signalled by a negative resting potential

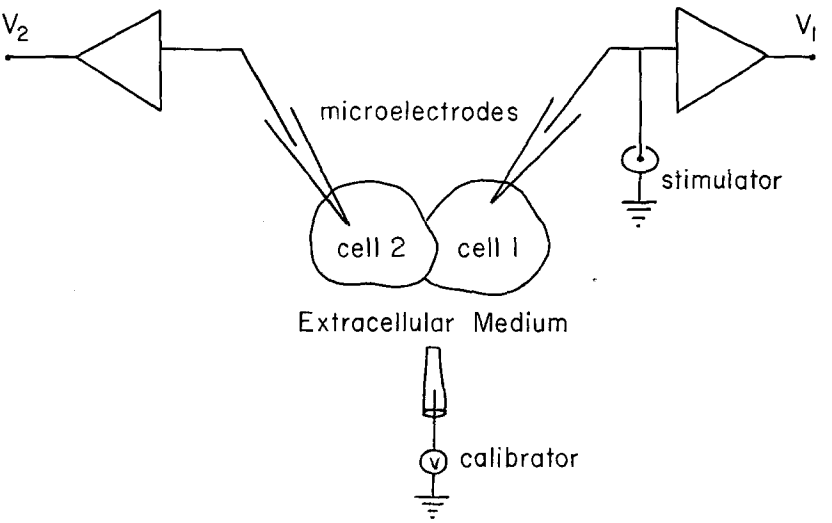

Fig. 1. The recording setup used for electrical coupling measurements on cells in tissue culture. The recording preamplifiers have bridge circuits for simultaneous recording and current stimulation. The calibrator is a low impedance voltage source connected to the reference electrode to introduce calibrating voltages. The preamplifier voltage output was displayed on a Textronix 564B storage oscilloscope and a Gould Brush recorder

with respect to the bathing medium. If both resting potentials remained approximately constant, a pulse of current was passed across cell 1, and the voltage responses of both cells were recorded. Afterwards, one microelectrode was withdrawn to just outside the cell, a current was passed across the impaled cell, and the voltage responses were recorded. No voltage response was recorded by circuit 2 to an applied current pulse in circuit 1 when both microelectrodes were in the bath or when only one microlectrode was within a cell. The balanced bridge circuit allowed monitoring of input resistance in each cell. This permitted a check of membrane damage due to poor cell impalement, which was usually characterized by an unstable resting membrane potential.

Coupling coefficients are used to express the amount of ionic coupling between two cells and were determined by the method of Azarnia *et al.* [1]. Due to the intrinsic limitations of bridge circuits, coupling coefficients were variable and found to range from 0.2–0.95 for normal and 0.1–0.9 for tumor cell pairs.

Results

Electrical coupling was found between all successful impalements of a pair of cells. Table 1 lists, for each of the cell types employed, the number of different dissociations, the number of successful cell pairs penetrated, and the mean ± standard error of the mean (SEM) of the recorded resting membrane potentials. Fig. 2*a* is a phase contrast photograph of a coupling experiment with neoplastic cells. Fig. 2*b* is the electrical coupling record. The estimated coupling coefficient was 0.5 and the cellular membrane potentials were −27 and −28 mV. The time course of a coupling experiment is illustrated in Fig. 3*b* with a pair of midpregnant normal cells. The applied current pulse was 0.3 nA, the estimated coupling coefficient was 0.95, and the resting membrane potentials were −24 and −25 mV. The presence of electrical coupling between cultured primary tumor cells is in agreement with the preliminary results of McGrath [22].

Table 1.

Type of cell cultured	Number of tissue dissociations	Number of coupled cell pairs	Resting membrane potentials (mean ± SEM; mV)
Virgin normal	1	1	−28.5 ± 2.5
Midpregnant normal	14	22	−20.0 ± 1.0
Primary hyperplastic outgrowth	2	12	−32.0 ± 2.2
Hyperplastic outgrowth line: D1	1	4	−28.0 ± 2.0
Hyperplastic outgrowth line: D2	2	6	−17.4 ± 1.5
Mammary tumor	14	38	−21.5 ± 0.8
Metastasis in lung	2	14	−33.5 ± 1.3

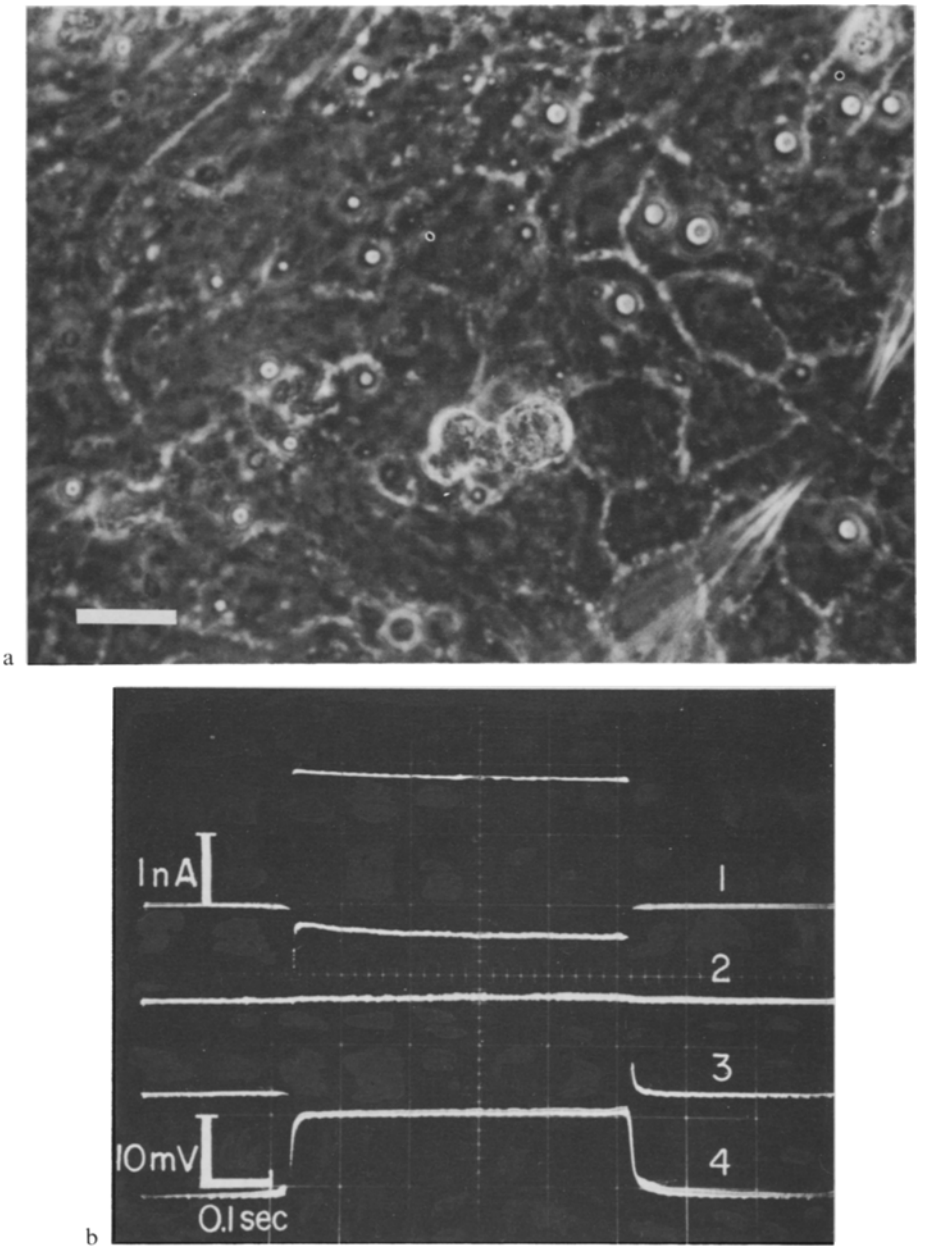

Fig. 2. (*a*) Phase contrast photograph of neoplastic cells; adjacent cells in the lower right are impaled by microelectrodes. Calibration bar is 20 μm. (*b*) From a Polaroid photograph of a coupling experiment for a pair of neoplastic cells. Trace 1 is the applied current pulse monitored across the microelectrode in cell 1. Trace 2 is the voltage response measured by circuit 2 to an applied current pulse across cell 1 with microelectrode 2 in the bathing medium. Trace 3 is the voltage response recorded across cell 1 to the applied current pulse. Trace 4 is the voltage response measured by circuit 2 to an applied current pulse across cell 1 with microelectrode 2 in cell 2. The recorded resting potentials of cells 1 and 2 were −27 and −28 mV (inside negative), respectively

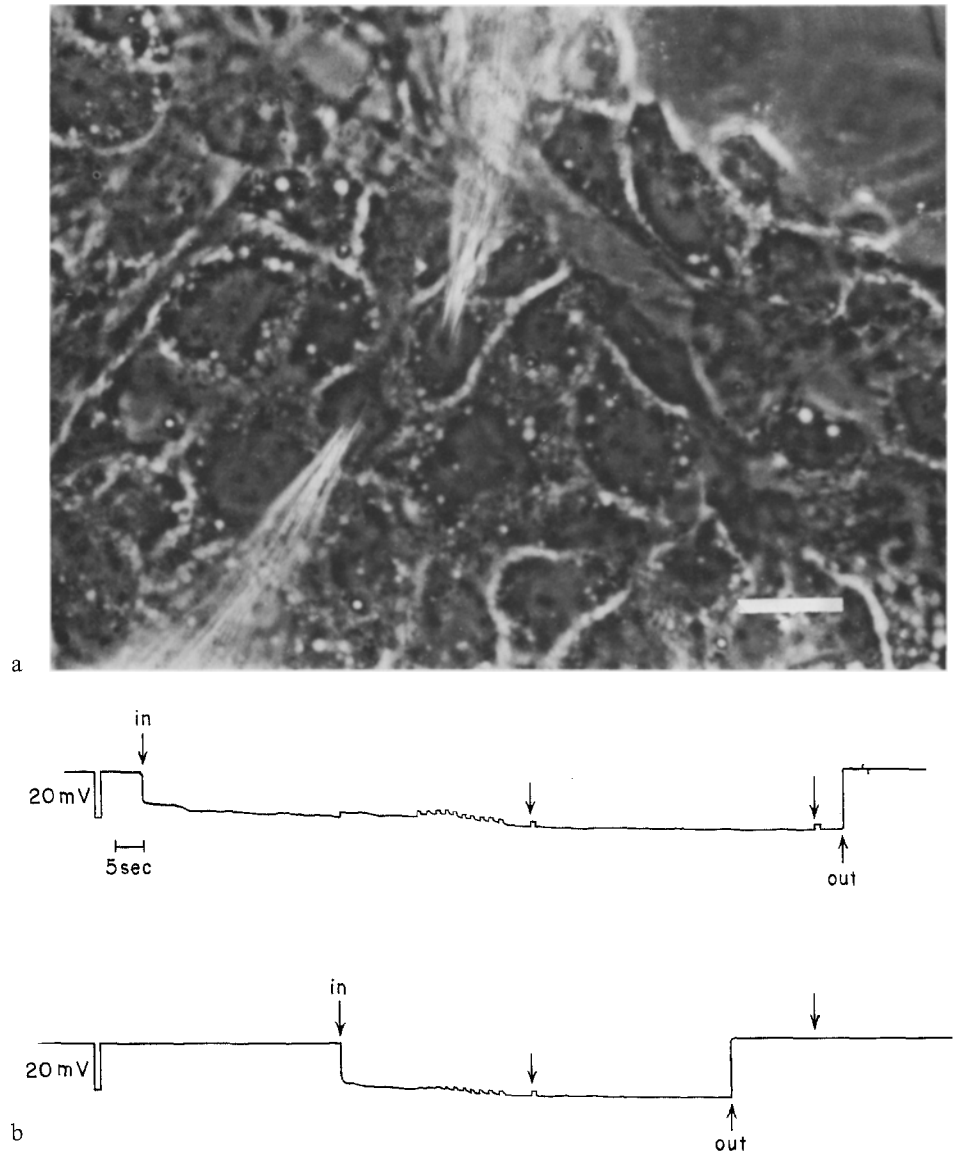

Fig. 3. (*a*) Phase contrast photograph of midpregnant cells; microelectrodes impale two cells in the center. Calibration bar is 20 μm. (*b*) From a Gould Brush record of a coupling experiment for a pair of midpregnant cells. The top trace is the voltage record across cell 1 and the bottom trace is the voltage record across cell 2. The applied current pulse was 0.3 nA. There was no voltage response recorded by circuit 2 to an applied current pulse across cell 1 after microelectrode 2 had been withdrawn from cell 2

The mouse mammary epithelium forms a confluent epithelial sheet, within which turgid, multicellular, blister-like domes develop in both normal and tumor cell cultures [7, 23, 37]. Morphologic observation

shows no structural differences between dome and nondome epithelial cells [30], and the evidence suggests that dome formation is the combined effect of transepithelial transport and transitory variations in adhesiveness [28, 30]. Coupling between dome-dome cell pairs and dome-nondome cell pairs was examined to see whether development of these structures might be related qualitatively to the presence of low-resistance junctions. Electrical coupling exists between dome-dome ($n=4$) and dome-nondome ($n=4$) cell pairs in both normal and tumor cell cultures.

Attempts were made to examine the extent of electrical coupling between a cell and its adjacent neighbors. Due to the size of the cells, slight mechanical disturbances often caused disruption of the penetrated cell. However, in a few cases (normal, tumor, and metastases) a cell was found to be coupled to all of its neighbors. Typically, cell pairs separated by greater than two cell distances showed strong attenuation of the voltage response to an applied current pulse. Electrical coupling, however, was found between cell pairs separated by as many as four other cells.

Discussion

Electrical coupling has been observed between cultured cells of the mouse mammary gland in five physiological or pathological states. Furthermore, in normal and tumor cell cultures, electrical coupling was observed between dome-dome and dome-nondome cell pairs. Estimated coupling coefficients were found to range from 0.2–0.95 for normal and from 0.1–0.9 for tumor cell pairs. Accurate determination of coupling coefficients requires the utilization of three microelectrodes. Attempts were made to penetrate cells with double-barrelled microelectrodes. This was considered unsuccessful, since in all cases, the recorded transmembrane potential was less than -5 mV and the double-barrelled microelectrode penetration caused visible cellular disruption. Injury to a cell apparently caused uncoupling from neighboring cells, but electrical coupling between neighboring uninjured cells was unaffected. The loss of coupling with injury may be a result of the influx of the extracellular medium into the injured cell [29].

The present study suggests that extensive coupling exists between cells in culture, but its existence *in vivo* is untested. However, electronmicroscopic studies of fresh normal, hyperplastic, and tumor samples show the presence of gap junctions in all three ([30, 31] and *unpublished observations*). The presence of electrical coupling does not necessarily imply

freedom of movement of larger molecules which may be necessary for cell interaction. There could exist a difference between normal and tumor cells in the exchange of metabolites while ionic movements are unimpaired. Solution of this problem necessitates the use of tracers.

The role of coupling, in mammary epithelium as in other tissues, is unknown. Cyclic growth, differentiation, and regression might be assisted by the spread of informational substances from cell to cell. Furthermore, lactating epithelium must perform transepithelial transport of selected components from blood to milk [18]. The presence of Na^+–K^+ ATPase on the basal and lateral membrane has been inferred [27] and its responsiveness to prolactin has been postulated [9]. Electrical coupling inevitably distributes the work of pumping ions, so that the ionic activity performed by any one cell would be modified by the activities of its coupled neighbors. The maintenance of electrical coupling between mammary adenocarcinoma cells is perhaps unsurprising, in view of the transport capability indicated by dome formation in culture. Furthermore, both dome formation and electronmicroscopy demonstrate the presence of intercellular occluding junctions providing a transepithelial permeability barrier. These membrane specializations in the cultured tumor cells are morphologically intact and are functional within the limits of the tests thus far applied.

The authors gratefully acknowledge Dr. R.A. Steinhardt for the use of his electrophysiological laboratory and equipment and Dr. H.A. Bern for valuable discussions. S. Shen is a NIH postdoctoral fellow. U.S. Public Health Service grant CA-05388 to Pitelka and Bern and NSF grant GB-42547 to Steinhardt provided the financial support.

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