GAP-JUNCTIONAL COMMUNICATION IN A COMMUNICATION-DEFECTIVE AND IN A COMMUNICATION-COMPETENT TERATOCELLARCOMA CELL LINE

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SUMMARY

We examined the gap-junctional communication properties of a communication-defective cell line R5/3 and its communication-competent revertant H2T12. For these studies, we carried out microelectrode impalements to monitor ionic coupling and dye coupling. Our dye-injection experiments revealed that the H2T12 cells are much more efficient in dye coupling than the R5/3 cells. This latter observation is in agreement with the previous finding that the H2T12 cells are much better metabolically coupled than the R5/3 cells. With ionic coupling measurements, however, both cell lines exhibited similar levels of cell-cell coupling. The R5/3 cells demonstrated an ionic coupling coefficient of 0.19 ± 0.011 (S.E.M.) and H2T12 a coupling coefficient of 0.25 ± 0.009 (S.E.M.). These results in conjunction with observations from other studies indicate that the different experimental approaches for monitoring gap-junctional communication may have different levels of sensitivity for detecting as opposed to measuring the level of cell-cell coupling.

INTRODUCTION

Gap junctions are specialized intercellular junctions that contain membrane channels that allow the direct passive diffusion of low molecular weight molecules between cells. Given this latter property, it has been suggested that gap-junctional communication might play an important role in regulating and coordinating cellular growth (Potter, Furshpan & Lennox, 1966; Pitts, 1978; Loewenstein, 1979) and differentiation (Potter et al., 1966; Sheridan, 1977; Pitts & Finbow, 1977; Wolpert, 1978). To examine this possibility, various approaches have been used to characterize the communication properties of cells undergoing differentiation. The goal in these studies has been to determine whether cell-cell communication is turned on or off in conjunction with specific determination/differentiation events.

An alternative and novel approach, which can also be used to study the possible role of junctions in development, is the isolation and characterization of communication-

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Key words: gap junction, embryonal carcinoma, dye coupling, ionic coupling.
defective cell lines (Wright, Goldfarb & Subak-Sharpe, 1976). For such studies, a system of particular interest is that of the embryonal carcinoma (EC) cells. EC cells are teratocarcinoma stem cells that resemble normal embryonic stem cells in being able to differentiate into derivatives of all three germ layers (Stevens, 1967; Pierce, 1967; Martin, 1980; Gardner, 1983). It is thought that if gap-junctional communication were to play an important role in development, the disruption of communication competency might lead to a disruption in the EC cells' ability to differentiate.

Using the 'kiss of death' selection scheme, Slack, Morgan & Hooper (1978) succeeded in isolating a communication-defective EC cell line, the R5/3 cells. Unfortunately, it was discovered that after selection the R5/3 cells had developed a number of differences from the communication-competent parental cell line, PC13TG8. These differences include changes in ploidy, thioguanine sensitivity and plating efficiency. To compensate for such changes, the communication-competent cell line H2T12 was isolated (Hooper & Morgan, 1979) from the R5/3 cell population and this cell line was then used as a positive control in all subsequent studies. When the R5/3 and H2T12 cells were examined for communication competency using metabolic cooperation assays, the R5/3 cells were found to have a greatly reduced level of gap-junctional communication compared with the H2T12 cells (Hooper & Morgan, 1979). In addition, morphometrically, the R5/3 cells were also observed to have a reduced incidence of gap junctions (Hooper & Parry, 1980).

In this study, we further characterized the communication properties of these two cell lines by monitoring their ionic and dye coupling properties. The results we have obtained indicate that the different methods for monitoring cell–cell communication are not equivalent. In particular, we observed that although dye coupling measurements demonstrated a large difference in communication efficiency as has been observed previously by metabolic cooperation assays, this large difference in coupling was not reflected by the results of the ionic coupling measurements.

MATERIALS AND METHODS

Cells

The embryonal carcinoma cell lines R5/3 and H2T12 were grown at 37 °C in a humidified incubator with 5% CO2 in Dulbecco’s MEM supplemented with 10% foetal calf serum. The cell cultures were passaged every 3–4 days using the following procedure: the cells were washed with phosphate-buffered saline (PBS), dislodged from the Petri dish by gentle pipetting, spun down by brief centrifugation in a clinical tabletop centrifuge, resuspended by repeated pipetting (in and out 20 times) in fresh medium, and then replated at the desired cell density. This method of passaging results in the plating of predominantly single cells. The cells were examined for coupling 3–4 days after plating and before the start of electrophysiological recordings, the culture medium was replaced with DMEM containing 10% foetal calf serum and buffered with HEPES. All the ionic and dye coupling measurements were carried out with colonies that contained 40 to 100 cells. For all the data presented in this paper, the ionic and dye coupling measurements were made over a 4-week interval, and during this time, the cells were propagated continuously as outlined above.

Electrophysiology

The methods and apparatus used for monitoring ionic coupling and for iontophoretic injection have been described previously (Lo & Gilula, 1979). Briefly, ionic coupling measurements were
made with glass microelectrodes with tip resistances of 40–60 MΩ when filled with 3 m-KCl; this corresponds to a tip diameter of about 0.1 μm. Note that the input membrane resistances of both cell lines were found to be similar (data not shown).

For fluorescein injections, microelectrodes with resistances of 15–20 MΩ (as measured when filled with 3 m-KCl) were filled with 2% sodium carboxyfluorescein (w/v) (M, 376). Dye was injected iontophoretically using 0.5 nA hyperpolarizing current pulses of 0.5 s duration at intervals of 1 s. The cell-to-cell spread of fluorescence was visualized with epi-illumination under a Leitz Diavert microscope using a 100 W mercury lamp with standard excitation filters. Fluorescence images were recorded on Kodak Tri-X Pan film with exposures of 90 s. The films were developed in Acufine (Acufine, Chicago).

RESULTS

Using microelectrode impalements, we monitored the ionic and dye coupling properties of the cell lines H2T12 and R5/3. To examine for dye coupling, we injected iontophoretically the fluorescent dye tracer carboxyfluorescein. The extent of dye spread was examined visually as the spread of fluorescence from the site of impalement. These fluorescence images were also recorded on film. To examine their ionic coupling efficiencies, typically two adjacent cells in a colony were each impaled with a microelectrode, and as current was pulsed into one cell the electrotonic potential in both cells was recorded. These measurements were used to measure the degree and the symmetry of the ionic coupling. A detailed description of the ionic and dye coupling measurements obtained with each cell line is given below.

Intercellular spread of dye

For the dye injection studies, we carried out 25 impalements into the H2T12 cells and 26 impalements into the R5/3 cells. Overall, only 1 of the 25 impaled H2T12 cells failed to demonstrate dye coupling compared with 11 of the 26 R5/3 cells (Table 1). With the H2T12 cell line, carboxyfluorescein injected into one cell was usually observed to spread extensively into the surrounding cells. The dye was limited initially to the nearest neighbouring cells, but as time progressed it eventually spread to surrounding cells that were two or three cell diameters away from the site of injection (Fig. 1). In contrast, the metabolic cooperation-defective R5/3 cells showed little or no dye spread even with continuous injection of dye over an extended time (Fig. 2). The extent of dye spread in these cells was limited primarily to the cell at the site of injection and in a few cases to a small number of immediately adjacent cells. These results demonstrate a large difference in dye coupling efficiency between these two cell lines.

To illustrate the total extent of dye spread observed with each cell line, the dye spread data obtained above were plotted onto three-dimensional bar graphs (Fig. 3).

Table 1. Dye coupling between H2T12 cells and between R5/3 cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Total no. of impalements</th>
<th>No. of impalements with no visible dye spread</th>
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<tbody>
<tr>
<td>H2T12</td>
<td>25</td>
<td>1</td>
</tr>
<tr>
<td>R5/3</td>
<td>26</td>
<td>11</td>
</tr>
</tbody>
</table>
Fig. 1. Carboxyfluorescein dye transfer between H2T12 cells. A. Phase-contrast image of an H2T12 cell impaled with a microelectrode. B–E. The cell was injected with carboxyfluorescein using current pulses of 0.5 nA and fluorescence images were recorded at various times after the start of injection: B, 0–5 min; C, 3 min; D, 5 min; and E, 10 min. Bar in A, 44.6 μm.

Fig. 2. Carboxyfluorescein dye transfer between R5/3 cells. A phase-contrast image of an R5/3 cell impaled with a microelectrode. B–D. The cell was injected with carboxyfluorescein using current pulses of 0.5 nA and fluorescence images were recorded at various times after the start of injection: B, 3 min; C, 5 min; and D, 10 min. Bar in A, 44.6 μm.
Fig. 3

H2T12

R5/3

(z)

(y)

(x)

NUMBER OF CELLS FILLED BY INJECTED DYE

1.00  2.00  3.00  4.00  5.00  6.00  7.00  8.00  9.00  10.00

1st ORDER  2nd ORDER  3rd ORDER  4th ORDER

Fig. 3
Cell-cell communication in EC cells

(Fig. 3). Only the results of those impalements that have demonstrated dye spread from the site of impalement are represented in these graphs. On the x axis the number of cells filled by dye injection is indicated; on the y axis the extent of dye spread as revealed by the number of cell diameters that the injected dye has moved from the site of injection; and on the z axis, the total number of impalements demonstrating the extent of dye spread as indicated on the x and y axes. Thus a plot in the graph of six impalements with three dye-filled cells of the second order represents a total of six impalements that have demonstrated dye spread to at least three cells at a minimum distance of 2 cell diameters away from the site of impalement. Such a plot of the dye spread data revealed that for the H2T12 cells the data are clustered mainly towards the middle and to the right-hand side of the graph (Fig. 3A). This indicates that during most impalements a relatively large number of cells are filled by the injected dye and frequently the dye-filled cells are located 1, 2 or 3 cell diameters away from the site of impalement. In comparison, with the R5/3 cells we observed a clustering of data towards the left-hand corner of the graph (Fig. 3B). This indicates that with each impalement fewer cells are filled by the injected dye and those cells that are dye-filled are usually 1 cell diameter away from the site of impalement. This display of the dye spread data clearly demonstrates that the H2T12 cells are much better dye-coupled than the R5/3 cells (compare Fig. 3A and B with C).

Ionic coupling

For the examination of ionic coupling, pairs of adjacent cells near the centre of colonies that consisted of approximately 40–100 cells were impaled (Fig. 4A,c). Ionic coupling was detected in both the H2T12 and the R5/3 cells. This is indicated by the observation that as a current pulse is injected into one cell a simultaneous voltage deflection is detected in that cell (top trace) as well as in the adjacent impaled cell (bottom trace). An example of this is illustrated for each of the two cell lines H2T12 and R5/3 (Fig. 4a,d). These results demonstrate that both the H2T12 and the R5/3 cells are linked by low-resistance channels.

We carried out 24 pairs of impalements with both the H2T12 and the R5/3 cell lines. For each impalement the ionic coupling was monitored alternately from each microelectrode, and in all cases the ionic coupling was observed to be completely symmetrical (Table 2). The efficiency of ionic coupling was estimated by determining the coupling coefficient for each of the two cell lines (defined as the ratio of the voltage deflection detected in the neighbouring cell to the voltage deflection detected in the cell in which current was injected; Bennett, 1966; Socolar, 1977) (Table 2). This analysis revealed that the H2T12 and R5/3 cells have similar ionic coupling efficiencies, but the R5/3 cells demonstrated a perceptibly lower ionic coupling coefficient.

Fig. 3. The distribution of dye spread as characterized by the number of impalements demonstrating dye spread to x number of cells at a distance of y cells removed from the site of impalement. A. Distribution of dye spread in H2T12 cells. B. Distribution of dye spread in R5/3 cells. C. Distribution of dye spread expected for cells that are either well coupled or poorly coupled.
Fig. 4. Ionic coupling between cells. A,C. Each of two directly adjacent cells in an H2T12 colony and a R5/3 colony was impaled with a microelectrode. B,D. Oscilloscope traces revealing the presence of ionic coupling in both H2T12 and R5/3 cells. Thus as a current pulse was injected into one cell, a voltage deflection was detected in that cell (top trace) and also in the second impaled cell (bottom trace). Bar in A, 44-6 μm. In B and D the horizontal calibration bar represents 170 ms and the vertical bar 10 mV.

The R5/3 cells have a coupling coefficient of 0.19 ± 0.011 (S.E.M.) and the H2T12 cells a coupling coefficient of 0.26 ± 0.009 (S.E.M.). The difference between these coupling coefficients is statistically significant at the 99% confidence level.

### Table 2. Ionic coupling between H2T12 cells and between R5/3 cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>No. of pairs of cells impaled</th>
<th>Incidence of ionic coupling (%)</th>
<th>Coupling coefficient* ($V_2/V_1$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H2T12</td>
<td>24</td>
<td>100</td>
<td>0.26 ± 0.009†</td>
</tr>
<tr>
<td>R5/3</td>
<td>24</td>
<td>100</td>
<td>0.19 ± 0.011†</td>
</tr>
</tbody>
</table>

* The ionic coupling is completely symmetrical; there is no difference in the coupling coefficient with respect to the direction of the current pulse.

† The standard error of the mean is indicated.
**DISCUSSION**

We have characterized in detail the ionic and dye coupling properties of the cell lines H2T12 and R5/3. We found that the communication-defective R5/3 cell line is poorly dye-coupled compared with its communication-competent revertant derivative H2T12. This is consistent with the results of previous studies, which demonstrated that the R5/3 cells have a lower incidence of gap junctions (Hooper & Parry, 1980) and exhibit a large reduction in metabolic coupling compared with the H2T12 cells (Hooper & Morgan, 1979). When we examined these cells further for ionic coupling, however, the R5/3 and H2T12 cells were found to be ionically coupled to almost the same extent. These conflicting observations are reminiscent of the results of studies in the insect epidermis in which it was found that at the segment border there is a partial restriction in dye coupling with no detectable difference in the efficiency of ionic coupling (Warner & Lawrence, 1973, 1982; Caveney, 1974; Blennerhassett & Caveney, 1984).

One possible explanation for these disparate observations is that there may be a variable number of two different classes of junctional channels: one class of channels having a large pore size that would allow the ready passage of ions and larger molecules; and a second type of channel with a more restricted pore size that would only allow the diffusion of ions. However, while we cannot exclude such a possibility, it cannot alone account for the properties of R5/3 and H2T12, since when they are compared for their ability to transfer ions by a different assay, i.e. ouabain rescue, a substantial difference is demonstrable (Hooper & Morgan, 1979). Moreover, in thin-section electron micrographs the gap junctions in R5/3 and H2T12 appear indistinguishable (Hooper & Parry, 1980). A second explanation for these observations is that perhaps the permeability of gap-junctional channels can be differentially modulated, but at present there is no evidence to support this possibility.

A third explanation, one that we consider more likely, is that perhaps these disparate observations merely reflect the inherent differences in the sensitivity of each of these three methods for monitoring cell-cell communication. Thus, given that the R5/3 cells have a lower incidence of gap junctions compared with the H2T12 cells, it is expected that the R5/3 cells would have a lower level of gap-junctional exchange compared with the H2T12 cells. This was indeed observed in metabolic and dye coupling studies but not readily apparent by ionic coupling measurements. These results indicate that dye and metabolic coupling assays may be much more sensitive to quantitative changes in the level of cell-cell coupling. Consistent with this possibility is a large number of observations in several developmental systems in which dye coupling was observed to be disrupted with little or no change in ionic coupling. For example, in the mouse embryo, the trophoblast cells have been observed to become uncoupled from the ICM (inner cell mass) cells but only with respect to dye coupling (Lo & Gilula, 1979) and metabolic coupling (Gaunt & Papaioannou, 1979); ionic coupling was found to be maintained between these two cell populations (Lo & Gilula, 1979). Similarly, in the *Fundulus* embryo, dye coupling was lost between the yolk sac cells and the blastoderm but ionic coupling was maintained, albeit at a reduced level.
(Kimmel, Spray & Bennett, 1984). In addition, in the imaginal disk epithelium (Weir & Lo, 1982, 1984) and in the segmental border of the insect hypoderm (Warner & Lawrence, 1982), a reduction in dye coupling has been observed with no apparent break in ionic coupling. Overall, the simplest explanation for these observations is that quantitative changes in coupling can occur during development and that dye and metabolic coupling measurements may be very sensitive assays for detecting these changes.

As ionic coupling has been observed when dye (and metabolic) coupling is reduced in level or completely absent, this would further indicate that in contrast to the dye (and metabolic)-coupling assays, ionic coupling measurements may be very sensitive for detecting the presence of low levels of coupling. Consistent with this latter suggestion are a large number of studies, which reported that ionic coupling was observed in the presence of very few or no detectable junctional plaques (Daniel et al. 1976; Williams & Delhaan, 1981; Meyer, Yancey, Revel & Peskoff, 1981; Preus, Johnson, Sheridan & Meyer, 1981). Moreover, in a hepatoma cell line that formed junctions at a very slow rate it was reported that ionic coupling was detected initially without any dye coupling, but with time as more gap-junctional contacts were established dye coupling was also detected (Azarnia & Loewenstein, 1976).

On the basis of these observations and the results of our study, we suggest that in any study of cell–cell communication, it may be important to use several experimental approaches to monitor gap-junctional exchange. Moreover, as the results of the numerous studies cited above have indicated that quantitative changes in coupling may occur during development, in the future it may be interesting to obtain and examine the differentiation potential of a panel of EC cell lines with varying levels of communication competency. Such studies may provide useful insights into the possible functional role of cell–cell communication in development.

This work was supported by NIH grants CA31042 and GM30461.

REFERENCES


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(Received 11 December 1984 – Accepted 6 February 1985)