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Cell Junction and Cyclic AMP: III. Promotion of Junctional Membrane Permeability and Junctional Membrane Particles in a Junction-Deficient Cell Type

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Summary. The cyclic nucleotide effect on junction was studied in C1-1D cells, a mouse cancer cell type that fails to make permeable junctions in ordinary confluent culture. Upon administration of cyclic AMP, dibutyryl cyclic AMP, dibutyryl cyclic AMP plus caffeine (db-cAMP-caffeine), or cholera toxin (an adenylate cyclase activator), the cells acquired permeable junctions; they became electrically coupled and transferred fluorescent tracer molecules among each other – a transfer exhibiting the molecular size limit of permeation of normal cell-to-cell channels. The effect took several hours to develop. With the db-cAMP-caffeine treatment, junctional permeability emerged within two hours in one-fifth of the cell population, and within the next few hours in the entire population. This development was not prevented by the cytokinesis inhibitor cytochalasin B. Permeable junctions formed also in two other conditions where the cell-endogenous cyclic AMP level may be expected to increase: serum starvation and low cell density. After three weeks of starving the cells of serum, a junctional permeability arose in confluent cultures, which on feeding with serum disappeared within two to three days. At low cell density, namely below confluency, the cells made permeable junctions, unstarved. In cultures of rather uniform density, the frequency of permeable junctions was inversely related to the average density, over the subconfluent range; at densities of about 1×10^4 cells/cm^2, where the cells had few mutual contacts, 80% of the pairs presumed to be in contact were electrically coupled. In cultures with adjoining territories of high (confluent) and low cell density, there was coupling only in the last, and in this low-density state the cells were also capable of coupling with other mammalian cell types (mouse 3T3-BalbC and human Lesch-Nyhan cells).

Correlated electron microscopy of freeze-fractured cell junctions showed no membrane differentiation in confluent C1-1D cultures. The junctions acquired differentiations, namely particle clusters of gap junction and strands of tight junction, upon cyclic nucleotide application or serum starvation and in the low-density condition. With db-cAMP-caffeine, these differentiations appeared within 4 hr of the treatment (confluent cultures), growing in size over the next hours. Treatment with cycloheximide, but not with cytochalasin B, prevented the development of recognizable gap junction and tight junction in cultures supplied with db-cAMP-caffeine.

Key words: Intercellular communication, cell junction, gap junction, junctional permeability, cell-to-cell membrane channels, promotion of cell-to-cell membrane channels, membrane permeability, cyclic AMP, cancer cell

C1-1D is a cancer cell type that is deficient in cell-to-cell membrane channels in ordinary confluent culture conditions (Azarnia, Larsen & Loewenstein, 1974; Azarnia & Loewenstein, 1977). Here we explore whether this type can be spurred to form channels by manipulations that may elevate the cyclic AMP concentration. We supply the cells with cyclic nucleotides, starve them of serum medium, or subject them to downsteps of density – conditions that, in the preceding papers, were shown to raise the cyclic AMP concentration and junctional permeability in channel-competent cell types (Flagg-Newton, Dahl & Loewenstein, 1981; Flagg-Newton & Loewenstein, 1981). We treat the cells also with cholera toxin that raises cyclic AMP concentration in many kinds of cultured cells (Wolff, Temple & Cook, 1973; O'Keefe & Cuatrecasas, 1974; Brunton & Guerrant, 1974; *cf.* Chlapowski, Kelly, & Butcher, 1975; Schimmer, 1981). We find that a cell-to-cell permeability arises in these conditions.

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Materials and Methods

Mouse C1-1D cells, a malignant thymidine kinase-deficient subline of L cells (Dubbs & Kit, 1964); human Lesch-Nyhan cells, a nonmalignant, inosine pyrophosphorylase-deficient skin fibroblast strain (Fujimoto & Seegmüller, 1970); and nonmalignant mouse 3T3-BalbC cells were grown in Dulbecco-Eagle medium (high glucose) (Vogt & Dulbecco, 1960) supplemented with 10% new-born calf serum (Gibco). The medium was exchanged every 2–3 days. The culture conditions, the medium for cell dissociation, the cyclic nucleotide and caffeine solutions and the procedures for their administration to the cultures, and the methods for freeze-fracture electron microscopy are described in the first paper of this series (Flagg-Newton et al., 1981). In the determinations of the time of cell dislodgement (Table 7), the dissociation medium was used at a fivefold dilution. Freeze-fracture of low-density cultures was performed on centrifuged cell pellets; the cells were harvested after fixation with the aid of a rubber policeman. Cholera toxin (0.1% water stock solution), cycloheximide (freshly dissolved in the medium for each experiment), and cytochalasin B (1% dimethylsulfoxide stock solution) were used at the medium concentrations given in the Results. The dimethylsulfoxide was at a final 0.02% medium concentration (vol/vol) at which it had no junctional effect on its own.

Electrical coupling was measured by passing, with the aid of two microelectrodes, rectangular pulses of current (i, 2.5×10^{-9} A, inward) between the interior of one cell and the grounded medium, and measuring the resulting displacements of membrane potential in this cell (V_1) and a *contiguous* one (V_2). The electrodes were each connected to a balanced bridge circuit and each served to pass current and to measure potential, thus allowing us to determine coupling in both directions across a junction (Fig. 5, *top inset*). The limit of resolution was a transfer resistance (V_2/i_1) of 8×10^4 Ω. The electrodes were filled with 0.5 M K_2SO_4. For tests of junctional transfer of the fluorescent tracer carboxyfluorescein (376 daltons), one of the electrodes was filled with a mixture of this molecule (100 mM) and K_2SO_4 (100 mM) and served to iontophorese the carboxyfluorescein anion into the cell during the coupling measurement. Lissamine Rhodamin B (LRB)-labelled Glu-Glu-Glu OH and fluorescein isothiocianate (FITC)-labelled insulin A were used as accessory permeability probes in the experiments ascertaining channel-mediated cell-to-cell transfer (Table 2) (*see* Simpson, Rose & Loewenstein, 1977; Socolar & Loewenstein, 1979, for properties and preparation of these probes).

Measurements of cell membrane resistance were taken on single cells (not in contact) by means of two microelectrodes, one for passing current and one for recording voltage; the cells were seeded 24 hr before at a density of 1 [10^4 cells/cm^2]. This was also the protocol for the resistance measurements on serum-starved cells; but here, for continuous serum starvation, the medium from a parallel serum-starved culture was used to wash the dissociation medium off and to suspend the test culture in the seedings. During the measurements of coupling and resistance, limited to 15 min, the culture dishes were at 30–35 °C, in a warmed room air atmosphere outside the incubator.

The method and criteria for scoring of incidence of fluorescent-tracer transferring junctions were as in the first paper of this series. But in the present work the acceptance criterion of two first-order transferring junctions introduced no bias; when it occurred at all, transfer was always multijunctional with C1-1D cells. For determination of the incidence of electrically coupled junctions, we scored the proportion of coupled cell pairs among the pairs tested.

For the studies of the effect of local cell-density variation, we scraped, with the aid of a rubber policeman, a 3-mm cell strip in a confluent culture. The medium was then exchanged and junctions tested 24 hr later.

Results

Junctional Transfer

cAMP, db-cAMP, Caffeine

Confluent C1-1D cells cultured in medium with 10% serum show no junctional transfer of molecules within the resolution of the methods, not even transfer of small inorganic ions mediating junctional electrical coupling (Azarnia & Loewenstein, 1977). In these conditions and against this background of zero junctional communication, we supplied the cultures with cyclic nucleotides. The cells developed communication as a result (Fig. 1).

On application of cyclic AMP (cAMP, 1 mM), dibutyryl cyclic AMP (db-cAMP, 1 mM) or the combination of db-cAMP (1 mM) and caffeine (1 mM), the cells became electrically coupled and transferred carboxyfluorescein (c-fluorescein) among each other (Table 1). With db-cAMP-caffeine, for example, typically, 20% of the junctions tested became electrically coupled within 2 hr, and this incidence rose over the next hours to 100% (Fig. 2). In some runs, this maximum was attained within 5–8 hr. C-fluorescein transfer became detectable a few hours later; by 24 hr the incidence of transferring junctions ranged 48–54% (Fig. 2; Table 1).

Comparable effects were produced by application of db-cAMP alone or of cAMP. With the last, the incidence of communicating junctions were lower (24 hr) (Table 1). With caffeine (1 mM) alone, in 10 measurements on one culture, we found no electrical coupling.

The induced permeability appears to be mediated by cell-to-cell channels – channels that exhibit the permeation cutoff typical of mammalian cells (Flagg-Newton, Simpson & Loewenstein, 1979; Schwarzmann et al., 1981): LRB Glu-Glu-Glu OH passed marginally between db-cAMP-caffeine-treated C1-1D cells, but FITC-insulin A (which exceeds the channel permeation limit) did not (Table 2). The permeation failure of the last molecule also afforded a demonstration that the cyclic nucleotides did not promote formation of cytoplasmic intercellular bridges.

The electrical resistance of nonjunctional cell membrane, measured at 24 hr of the db-cAMP-caffeine treatment on single cells not in contact, was not significantly different from that in controls (Table 3).

In medium serum-free, the incidence of electrical coupling in response to db-cAMP or db-cAMP-caffeine was smaller than in medium with serum. At 24 hr of db-cAMP treatment, the electrical coupling incidence in serum-free medium was 40% as against 100% in the controls, and even by 48 hr the incidence was only 75%. This may have been due to a lowered sensitivity for detection

of coupling; the cell membrane resistance in serum-free medium was lower than in control medium (Table 3).

5′-AMP, Na-Butyrate

Control treatments with 5′-AMP (1 mM) or Na-butyrate (1 mM) produced no detectable electrical coupling (Table 1).

Cholera Toxin

Exposure of the cells to the adenylcyclase activator, cholera toxin (1–5 μg/ml) led to development of junctional communication and, among the various junction-promoting agents used, this seemed most potent. Within 24 hr of its administration, all of the cell interfaces were electrically coupled. This agent also produced the highest incidence of fluorescein-transferring junctions, 83% by 48 hr (Table 1) – not far from the mark of cell types that make permeable junction on their own (Flagg-Newton et al., 1981).

Serum Starvation

As in the normal cell types studied in the preceding paper (Flagg-Newton & Loewenstein, 1981), lack of serum in the medium promoted the formation of permeable junctions in the C1-1D cells, but the demonstration of this effect required a somewhat different strategy. In serum-free medium or even in medium with 0.5% serum, the cells did not develop junctional communication over the three-week period they survived in that condition. However, they survived more than six weeks in medium with 10% serum, that was not renewed; and in this condition we found development of communication. The cells then grew for about three weeks, and toward the end of this period the permeable junctions made their appearance. (Like a number of other malignant cells types [Holley & Kiernan, 1971; *see also* Holley, Armour & Baldwin, 1977], the C1-1D cells need little serum for growth.)

Figure 3 illustrates an experiment in which cells were seeded sparsely (2[10^4 cells/cm^2]) at time zero, the time of the last feeding with 10%-serum medium. The cells reached confluency by day 7, and we tested electrical coupling from then on. Up to day 21, the cells were noncoupling, as they were in their confluent condition before time zero. But within the next four days, all cells tested had become coupled, and this state was maintained for at least another 25 days. On feeding the cells with either new 10%-serum medium (arrow *a*) or 10% serum alone (arrow *c*), but not with serum-free medium (arrow *b*), the coupling incidence returned towards zero within the next 2–3 days. Thus, starvation of serum, not starvation of other medium components, seems the stimulant for coupling here.

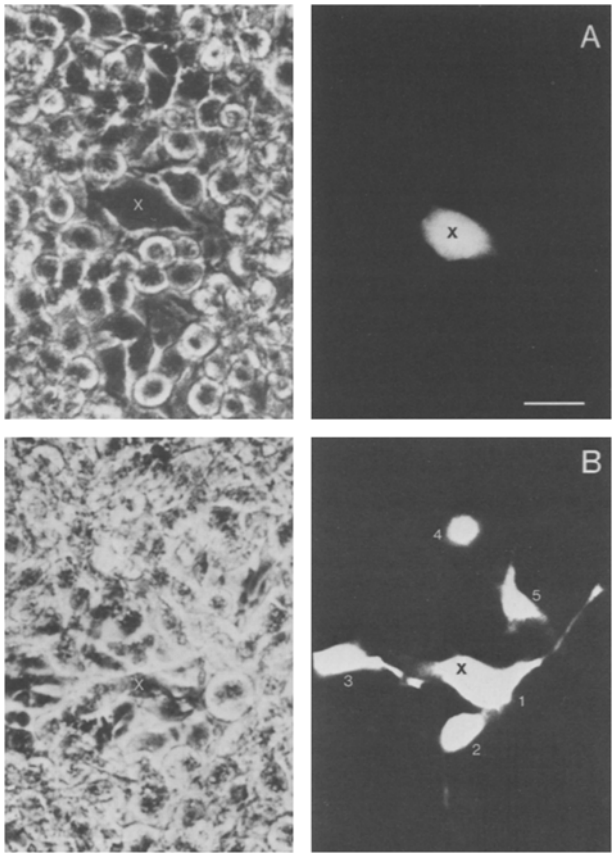


Fig. 1. The emergence of a junctional permeability upon treatment with db-cAMP-caffeine. Carboxyfluorescein is microinjected into a cell (*x*) of a confluent C1-1D culture (*A*) in control medium and (*B*) after a 24-hr exposure to db-cAMP. In *B* the tracer is seen to have passed to several neighbors (*1–5*). The tracer here had also passed to five other neighbors, but the fluorescence was too faint to be seen in the photographic reproduction. Left, phase contrast; right, darkfield. Calibration, 50 μm

In a variant of the preceding experiment, the cells were in 1%-serum medium when the feeding was stopped. Electrical coupling then developed, too, but the incidence was lower. The maximum incidence recorded (day 40) was 75% as against 100% with the preceding protocol. Since the serum remanent was probably smaller in this variant, the lower coupling incidence may reflect a lower detection sensitivity, as already discussed above for the case of db-cAMP stimulation in serum-free medium. It might also conceivably reflect in part a serum requirement for the development of coupling, and so there could be more than one serum factor involved in the promotion of coupling.

We may now compare this effect with that of serum deprivation in the cell types with basal expression of permeable junction (*compare* Fig. 3 with Fig. 2, paper II). Immediately comparable are the declining phases of communication incidence following serum restoration, where the experimental conditions were similar in the two kinds of cells. And indeed, the time courses of these phases are similar, taking into account that the decline goes all the way to zero

Table 1. Effects of externally applied agents on junctional communication in confluent cultures

Agent	Time (hr)	Communication incidence	
		Electrical coupling[a] (%)	c-fluorescein transfer[b] (%)
Control medium		**0** (0:131; 131, 13)	**0** (0: 32; 8, 2)
cAMP	24	**60** (6: 10; 10, 1)	**21** (9:42; 6, 1)
db-cAMP	24	**100** (20: 20; 20, 2)	**53** (41: 77; 11, 3)
db-cAMP-caffine	24	**100** (60: 60; 60, 6)	**48** (93:194; 25, 6)
	24	**100** (10: 10; 10, 1)	**46** (16: 35; 4, 1)
	24	**100** (10: 10; 10, 1)	**54** (32: 59; 7, 1)
	24	**100** (10: 10; 10, 1)	
Cholera toxin			
1 µg/ml	48	**100** (20: 20; 20, 2)	**83** (34: 41; 6, 2)
5 µg/ml	24	**100** (10: 10; 10, 1)	**73** (45: 62; 8, 2)
Na-butyrate	48	**0** (0: 10; 10, 1)	
5′ AMP	24	**0** (0: 10; 10, 1)	

[a] Incidence of electrically coupled cell pairs (microelectrodes were in contiguous cells). In parenthesis in the following order: the number of coupled cell pairs; the number of cell pairs; the number of measurement trials; and the number of culture dishes.

[b] Incidence of first-order c-fluorescein-permeated junctions. In parentheses: the number of permeable first-order cell junctions; the total number of first-order junctions of the injected cells; the number of injection trials; and the number of culture dishes examined.

Each incidence value subsumes the data from the same culture or, where more than one culture dish, it subsumes the data from parallel subcultures. Coupling and fluorescein transfer data are from the same runs, except for db-cAMP and cholera toxin 5 µg/ml where one additional run was made for fluorescein transfer on parallel subcultures. Four separate runs were made in the db-cAMP caffeine condition. Culture densities range 20–50 [10^4 cells/cm^2]. All agents at 1 mM concentration, except cholera toxin. In the db-cAMP-caffeine combination each agent, 1 mM in this and following tables.

Table 3. Nonjunctional cell membrane resistance and potential

Condition	Time	Membrane resistance (MΩ)	Membrane potential[a] (mV)
Control		22±12 (22)	24±7 (22)
db-cAMP-caffeine	24 hr	20±11 (20)	25±8 (20)
Serum starvation	24 hr	21±10 (20)	25±6 (20)
	19 days	20± 8 (20)	14±6 (20)
	24 days	21± 9 (20)	15±5 (20)
Serum free	24 hr	15± 7 (12)	21±6 (12)
Cycloheximide	24 hr	19± 6 (18)	18±6 (18)

Mean values ±SE; in parentheses, the number of measurements. Measurements on single cells not in contact. Cells had been seeded 24 hr before the measurements at a density of 1 [10^4 cells/cm^2]. Cycloheximide, 10 µg/ml.

[a] Potential at zero current.

Table 2. Controls against transfer not mediated by cell-to-cell channels

Condition	Permeability probe	Permeable-junction incidence (%)
Control	LRB Glu-Glu-Glu OH	**0** (0:74; 10, 3)
db-cAMP-caffeine	LRB Glu-Glu-Glu OH	**5** (2:43; 5, 1)
db-cAMP-caffeine	FITC insulin A	**0** (0:61; 8, 3)

All treatments 24 hr. Cycloheximide at 10 µg/ml; all other agents, 1 mM. Confluent cultures. For properties and preparation of permeability probes *see* Simpson et al., 1977, and Socolar & Loewenstein, 1979.

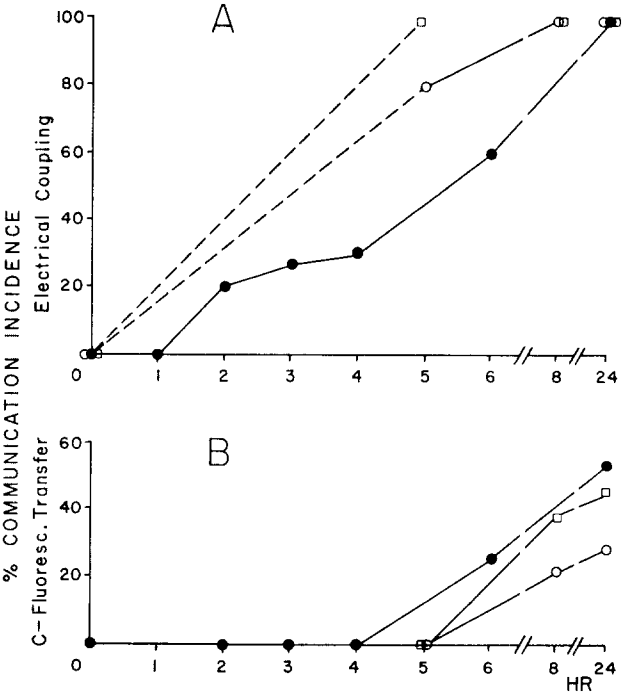


Fig. 2. Time course of the db-cAMP-caffeine effect, as indexed by the incidence of communicating junctions. (*A*): Incidence of electrically coupled junctions. (*B*): Incidence of c-fluorescein transferring junctions. Three representative series of experiments. The data for each series are from parallel subcultures (data detail in Appendix Table F-2); electrical coupling and c-fluorescein transfer were determined in the same runs

in the C1-1D cells. For the rising phases, the experimental conditions were different. But if one may discount the 21-day lag period when no detectable junctional change occurred in the C1-1D cells (attributing it to the medium's higher initial serum concentration and to the C1-1D's lower serum requirement), these phases, too, would appear not to be very different.

The declining phase also resembles that seen in another cancer cell type, the mouse sarcoma lines S-180 I and II. These cells were found to lose coupling within two days of renewing their nutrient medium

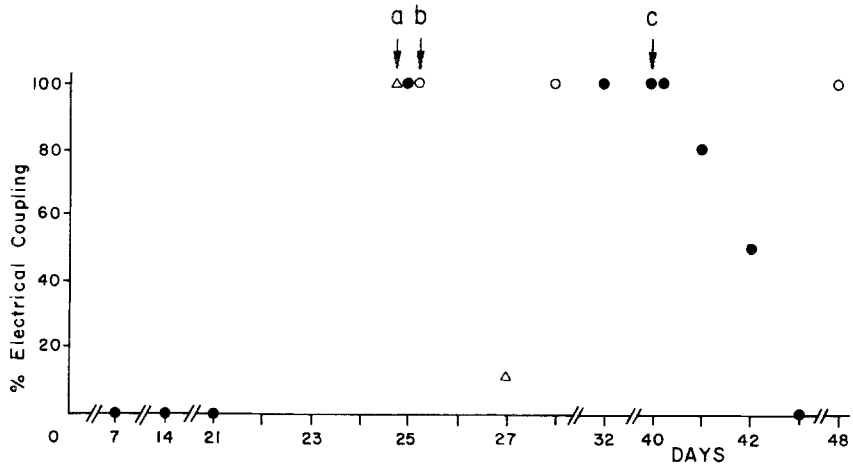


Fig. 3. The emergence of a junctional permeability during serum starvation and its abolition after serum restoration. At time zero, C1-1D cells were seeded in fresh 10%-serum medium and from then on the medium was not changed until the times indicated by the arrows. ●, ○, △ are the incidences of electrically coupled junctions from three different series of experiments (○ and △ are from parallel cultures). The cultures became confluent at time 7 days, when the electrical coupling tests were begun in the series ●. Arrows indicate the application of (*a*) fresh 10% serum-medium (series △), (*b*) fresh serum-free medium (series ○), and (*c*) fresh 10% serum only (series ●). Each data point corresponds to 10 coupling measurements taken on one culture dish

(Furshpan & Potter, 1968). Since the medium contained serum, it is possible that also that effect was serum-dependent and hence ruled by cAMP-related mechanisms. The recovery of coupling in unrenewed medium took only 4–5 days in the S-180 cells, but this could be a matter of serum requirements or basal endogenous cAMP levels (*see* Discussion and Flagg-Newton & Loewenstein, 1981).

The nonjunctional membrane resistance, measured at days 19, and 24 of serum starvation, was not different from that in the control condition (Table 3). Thus the sensitivities for detecting electrical coupling were entirely comparable.

Cell Density

C1-1D cells are smaller than the cell types dealt with in the two preceding papers, and so reach confluency at a higher density, ~20 [10^4 cells/cm^2]. It is in this confluent state, i.e., in the density range of 20–50 [10^4 cells/cm^2], where the aforegoing junctional tests were carried out and where (in control conditions) the incidence of coupling was zero.[1] At lower densities, there turned out to be coupling, especially about 1–2 [10^4 cells/cm^2] where only a small fraction of the cell surfaces were in contact as seen in phase contrast (Fig. 4).

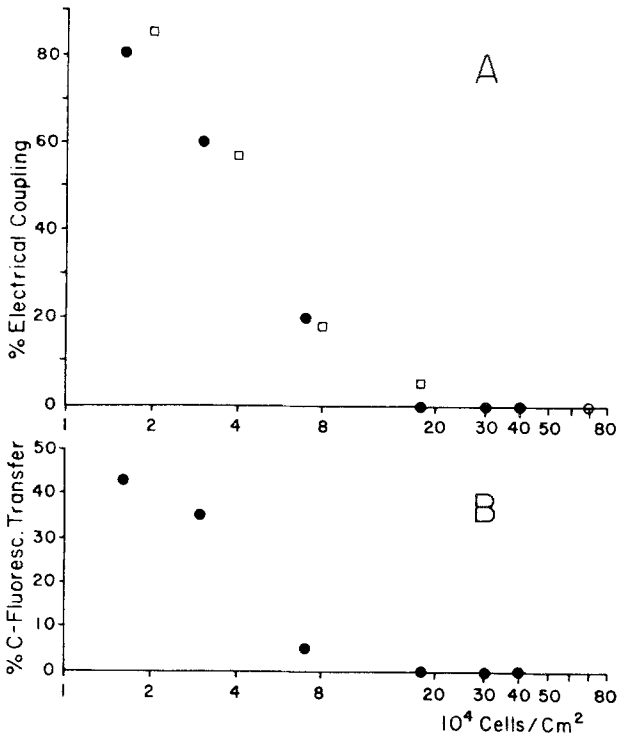


Fig. 4. Communication incidence at various C1-1D cell densities. Cultures were seeded to various densities and the communication incidence was tested 24 hr thereafter. The abscissae (logarithmic) give the density values at the times of the tests. (*A*): Incidence of electrically coupled junctions. (*B*): Incidence of fluorescein-transferring junctions. Each symbol represents the data from a series of experiments performed on parallel subcultures (data detail in Appendix Table F-4)

[1] 'Confluency' here has the usual meaning of tissue-culture terminology: a continuous cell layer without coarse spaces between cells as seen in the light microscope. Over the 20–50 [10^4 cells/cm^2] range there is yet no appreciable piling up of cells. Our earlier work had been done in this condition, too (Azarnia et al., 1974; Azarnia & Loewenstein, 1977; Larsen et al., 1977).

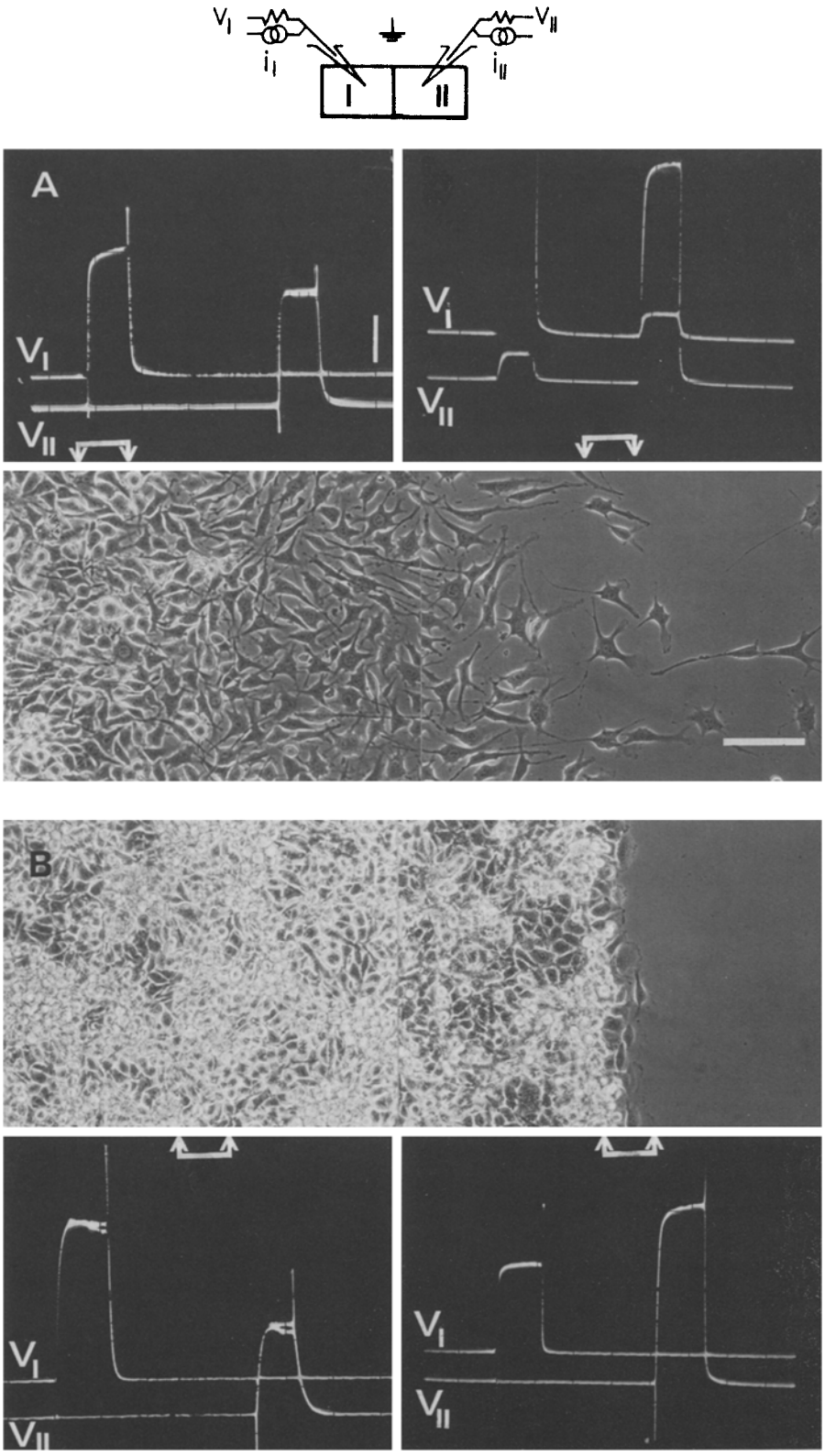


Fig. 5. (*A*): Electrical coupling at different local densities in a culture. A strip of a confluent culture was scraped. Cells at the wound edge have migrated into the denuded territory. The micrograph shows the situation 24 hr after the wounding at the time when electrical coupling was measured. Top right and left are sample recordings of coupling measurements taken in the low- and high-density cell regions, respectively. These regions are bracketed between arrows on the micrographs. The top inset shows the electrode arrangement: current is pulsed into cell *I* and, alternatively, into cell *II*, and the resulting displacements of membrane potential are measured (Methods). (*B*): A similar experiment, but the culture here was exposed to 2 μg/ml cytochalasin B throughout, preventing cell migration. Sample recordings from the edge and the center of the culture, respectively, 24 hr after the scraping. Calibration 100 μm; 50 mV. For the complete data of the experiments of this kind, *see* Table 4

Table 4. Electrical-coupling incidence in regions of locally different densities (wound experiment)

Local density	Electrical coupling incidence (%)
High	**0** (0:14; 14, 2)
Low	**39** (29:75; 75, 6)
High, cytochalasin-B-treated	**0** (0:23; 23, 3)

High and *low* refer to local densities, such as those in the bracketed regions on the photomicrographs of Fig. 5. Measurements, 24 hr after wounding.

Table 5. Promotion of junctional communication by cyclic nucleotide in the presence of cytochalasin B

Condition	Time (hr)	Electrical coupling incidence (%)
db-cAMP-caffeine	6	**100** (20:20; 20, 2)
	24	**100** (10:10; 10, 1)
db-cAMP-caffeine +cytochalasin B[a]	6	**95** (19:20; 20, 2)
	24	**100** (10:10; 10, 1)
Cytochalasin B	6	**0** (0:10; 10, 1)
	24	**0** (0:10; 10, 1)

Confluent cultures. Cytochalasin B, 1 μg/ml.

[a] Cytochalasin B was applied together with db-cAMP-caffeine in one 6-hr series, and 1 hr before the db-cAMP-caffeine in the other 6-hr series.

We examined the influence of cell density in conditions of relatively uniform density and in conditions where the densities differed locally. Figure 4 illustrates an experiment exploring conditions of the first kind. Cultures were seeded to various densities and the incidences of electrical coupling and c-fluorescein transfer were determined 24 hr thereafter. (The abscissa values are the densities at the time of the junctional testing.) Electrical coupling was present over the range of 1–16 [10^4 cells/cm^2], the coupling incidence rising steeply with decreasing density. At the low end of this density range, 85% of the junctions tested were electrically coupled; 43% passed c-fluorescein.

The trend was similar in conditions of locally varying density. We made use here of Todaro, Lazar and Green's (1965) classical wound experiment where cells in a quiescent confluent culture are stimulated to proliferate and to migrate by scoring the culture. Thus, in the experiment of Fig. 5, cells had moved out at the edge of a wound inflicted to a confluent C1-1D culture, establishing a territory of low cell density close to one where the high density of the original confluent culture had been maintained. The cells in the low-density region were coupling; those in the high-density region were not (Fig. 5, Table 4).

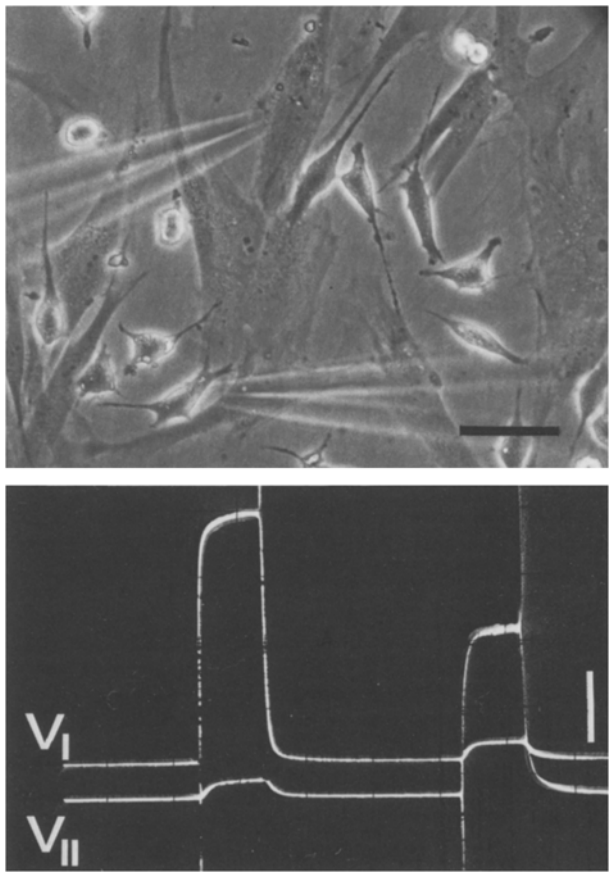


Fig. 6. Heterologous coupling between C1-1D and Lesch-Nyhan cells at low C1-1D cell densities. The C1-1D cells came from a confluent culture; after dissociation, they were seeded sparsely onto subconfluent Lesch-Nyhan cultures. Electrical coupling was measured 24 hr thereafter. *Top*: phase contrast photomicrograph of the coculture. The Lesch-Nyhan cells are the large cells with distinct nucleoli. The C1-1D cells are the small, refractile cells. The microelectrodes are also seen: the top electrode is on the Lesch-Nyhan cell and the bottom electrode, on the C1-1D cell, on which the measurements were taken; another Lesch-Nyhan cell here mediates between the two. (This is an exceptional arrangement; in all our other measurements, the electrodes were in contiguous cells.) *Bottom:* a sample record of the electrical coupling: V_I, Lesch-Nyhan; V_{II}, C1-1D. Current is pulsed alternately into Lesch-Nyhan and C1-1D, as diagrammed in top inset of Fig. 5. Calibrations 50 μm; 50 mV

A variant of this experiment, wounding in the presence of cytochalasin B, showed that the promotion of coupling was not due to the wounding itself. In the presence of this inhibitor of cytokinesis (2 μg/ml), there was no cell migration from the wound edge and the cell density in the culture was uniformly high. The cells near the wound edge were then noncoupling as were the cells anywhere else in the culture (Fig. 5*B*). We show further on that cytochalasin B *per se* does not prevent coupling.

Heterologous Junctions. In the low-density state, the C1-1D cells are also capable of coupling with other

Table 6. 'Artifactual' intercellular coupling produced by cycloheximide treatment.[a]

Intercellular communication	Communication incidence (%)
Electrical coupling	**21** (10: 48; 48, 3)
c-fluorescein	**3** (4:124; 16, 3)
LRB Glu-Glu-Glu OH	**0** (0: 47; 6, 2)

Confluent C1-1D cultures treated with cycloheximide, 10 μg/ml, 24 hr.

[a] Artifactual is used here in the sense of a nonphysiological coupling not mediated by cell-to-cell channels.

cell types. This point was examined in mixed cell cultures where Lesch-Nyhan cells or 3T3-BalbC cells, two cell types with basal expression of permeable junction, had been seeded sparsely onto sparse C1-1D cultures. 3T3-BalbC cells can make permeable junctions with other mammalian cell types, including at high densities (Michalke & Loewenstein, 1971), and we found here that Lesch-Nyhan cells can do that, too. We chose these cell types also because they are easily distinguishable from C1-1D, by their size, shape, and optical refraction.

At low density, the C1-1D cells coupled with either cell type. Figure 6 gives an example of such a heterologous coupling for the combination with Lesch-Nyhan cells. In the high-density state, on the other hand, the C1-1D cells did not establish heterologous coupling (Lesch-Nyhan cells seeded onto high-density C1-1D cultures). Nor did such coupling ensue when Lesch-Nyhan cells were seeded, in the presence of cytochalasin B, onto the wound edge of a confluent C1-1D culture (but Lesch-Nyhan coupled to Lesch-Nyhan in these conditions).

The Junctional Cyclic Nucleotide Effect in Cytochalasin B-Treated Cells

Like a number of other cancer cell types, the C1-1D cells underwent changes in shape upon cyclic nucleotide stimulation (*cf.* Willingham, 1976). During the treatment with cyclic nucleotides or cholera toxin, serum starvation and the downsteps of cell density, the C1-1D cells became flatter and emitted fine, often long, processes which were best seen after c-fluorescein injection (e.g., Fig. 1). These morphological reactions differed in magnitude – the flattening was most pronounced in serum starvation and the processes longest in the cyclic nucleotide treatments – but they were conspicuous in all four conditions.

We treated the cells with the cytokinesis inhibitor cytochalasin B (1 μg/ml). In one series, the agent was applied together with db-cAMP to confluent cultures; in another it was applied 1 hr before. In either case, the morphological changes did not sensibly develop; yet, electrical coupling developed just the same (Table 5). [By itself, cytochalasin B did not promote coupling (Table 5).]

Consequently, the morphological changes – at least those at the light-microscope scale – are not necessary for the promotion of cell-to-cell channel formation. This is in line with the results obtained with the normal cell types described in the preceding papers, where enhancement of channel formation is not accompanied by conspicuous morphological changes. The outcome is also in line with earlier work on 'unenhanced' channel formation between normal cells, where cytochalasin B (or colchicine) neither abolishes existing communication nor prevents its development (*see* Loewenstein, 1979, for an account of the published and unpublished results).

Effects of the Protein-Synthesis Inhibitor Cycloheximide. 'Artifactual' Intercellular Coupling

We show below that cycloheximide treatment prevents the cyclic nucleotide-promoted development of differentiated junctional structures. In a correlated attempt to find out whether protein synthesis is required for the development of junctional permeability, we treated the cells with cycloheximide (5–20 μg/ml) together with db-cAMP-caffeine for periods up to 24 hr. A proportion of the cells were electrically coupled after such treatments, but this provided no useful information for the question at hand; treatment with cycloheximide (10 μg/ml) on its own (in the absence of db-cAMP-caffeine) turned out to promote an electrical coupling. After a 24-hr treatment, 21% of the cell pairs tested were coupled (Table 6).

Although this result throws no light on the present problem, we thought it worth reporting because the drug is so commonly used, including – as it now turns out, riskily – in trying to answer questions about formation of cell-to-cell channels. Our preliminary results indicate that the cycloheximide-induced electrical coupling is not mediated by cell-to-cell channels: the electrical coupling was generally weak (weaker than the cyclic nucleotide-stimulated coupling) in cells with discrete boundaries in phase contrast – the only kind of cells on which coupling was determined – and such weakly coupled cells invariably failed to pass c-fluorescein. A small fraction of the cells (3%) were more strongly electrically coupled and these exhibited fluorescein passage – a slow seepage restricted to one cell neighbor of the injected cell (unlike the channel-mediated flow in the cyclic nucleotide-stimulated coupling which was typically rapid and multijunctional) – but no LRB Glu-Glu-Glu OH passage (Table 6). We have the impression that cyclo-

heximide promotes plasma membrane fusion; there were blebs and intimate membrane contacts, if not fusion, visible in the electron microscope, and an inordinate number of multinucleated cells, in the phase contrast microscope – and all this within 24 hr of the cycloheximide treatment. We suppose that the intercellular coupling observed reflects early stages of membrane fusion: the weak electrical coupling might reflect some form of tight (nonphysiological) membrane-membrane apposition (protein-depleted regions?) and the stronger coupling with fluorescein passage, the first molecular defects in membrane matrix, before final membrane dissolution.

Cellular Growth

C1-1D cells grow to high density (10^6 cells/cm^2) and eventually pile up on each other – the common pattern of cancer cells in culture. This pattern changed as the cells acquired permeable junctions on stimulation with db-cAMP or db-cAMP-caffeine: they grew more slowly (their doubling time fell from 1 to 1.8–2.5 days) plateauing at a density of 4–10 [10^4 cells/cm^2]. Such an apparent normalization of the *in vitro* growth has been seen in several other cancer cell types in response to exogenous cyclic nucleotides (*cf.* Chlapowski et al., 1975; Willingham, 1976).

Junctional Structure

A correlated electron microscopic study of the cell-junction structure was performed for several of the aforegoing experimental conditions. In confluent culture, the base line condition where the C1-1D cells are not electrically coupled, the freeze-fractured cell junctions showed no membrane differentiations of any sort (*see also* Larsen, Azarnia & Loewenstein, 1977). The junctions acquired differentiations, namely the membrane-particle clusters of gap junction and the membrane strands of tight junction in the various experimental conditions where they acquired electrical coupling.

Db-cAMP-Caffeine. With db-cAMP-caffeine, junctional differentiations became apparent 4 hr after the treatment of confluent cultures. At that time, the gap junctions comprised some 10 to 100 particles and the tight junctions, single, short strands (Fig. 7, *top row*). By 8 hr, these junctional structures were, on the average, larger and more frequent, and more so by 24 hr; some of the gap junctions then contained hundreds of particles, and the tight junctions contained several strands, often forming elaborate networks (Fig. 7).

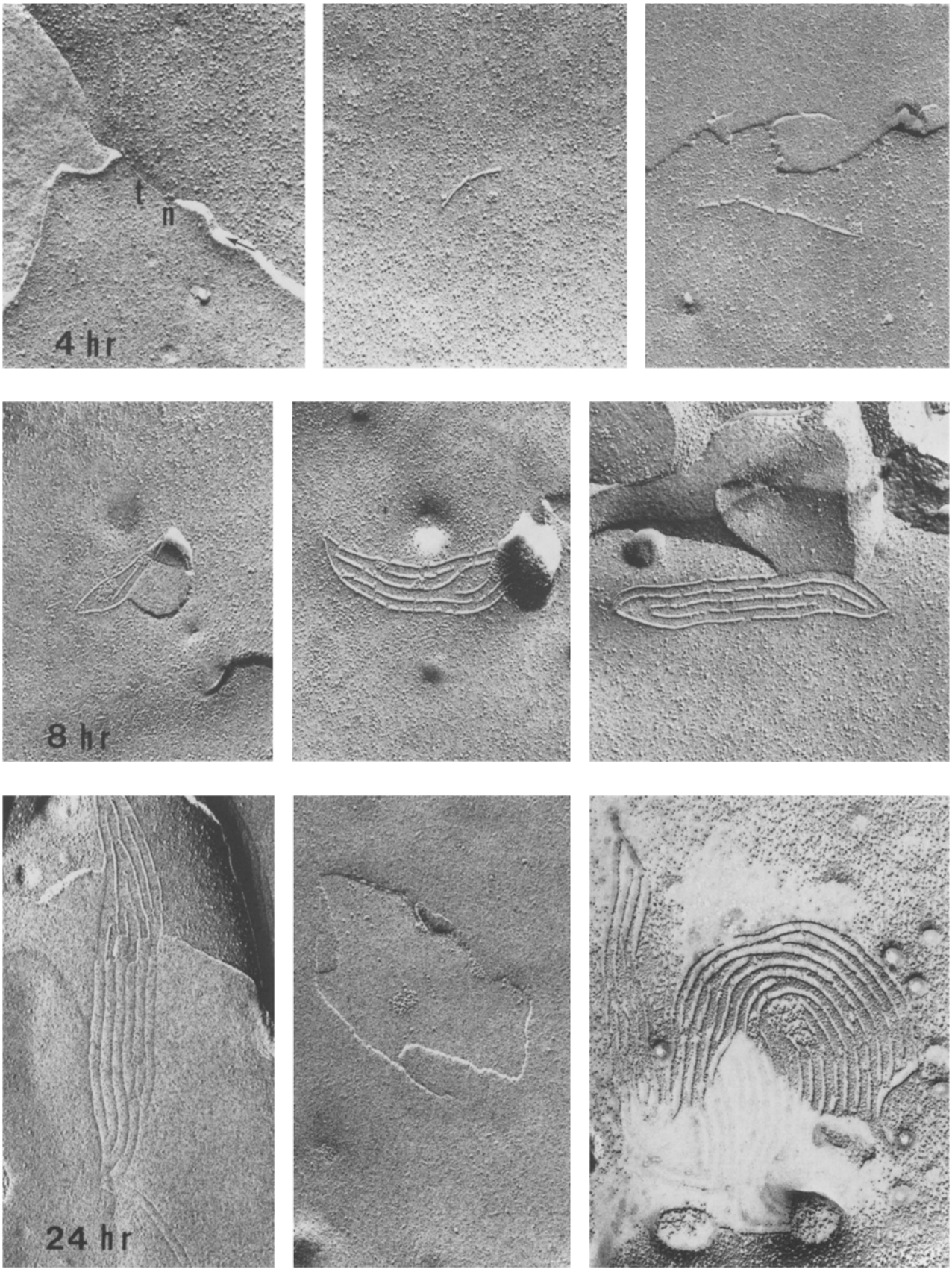
Before differentiation, the closest C1-1D membrane approximation was about 100 Å in confluent culture, similar to that at nondifferentiated regions in the junction-competent 3T3-BalbC cultures. Upon differentiation, the intermembrane distance at the junctional regions reduced sharply to that typical for gap junction and tight junction in mammalian cells (Fig. 7, *top left*) (Revel & Karnovsky, 1967; Caspar, Goodenough, Makowski & Phillips, 1977; Staehelin, 1974).

Serum Starvation. Confluent cells, serum-starved for 40 days, developed both types of junctional structures, too, and these were particularly large. Figure 8 gives an example belonging to the series of electrical measurements of Fig. 3 (series ●).

Cell Density. At low cell density, there is less chance, of course, for cell-cell contact by random encounter than in confluent culture. Nevertheless – and in striking contrast to the confluent condition – the few contacts there were, exhibited both gap junctions and tight junctions. Figure 9 illustrates this for a culture at a density of 1 [10^4 cells/cm^2], a culture parallel to one of Fig. 4 (series ●) on which junctional transfer was determined. (We examined only the condition of relatively uniform culture cell density; freeze-fracture of identified cell regions would have been difficult in conditions of locally different cell density.)

Protein Synthesis Inhibition. Treatment with cycloheximide (10 μg/ml) prevented the cyclic nucleotide-stimulated formation of junctional differentiation. We saw no gap junctions or tight junctions in cells treated for 4, 8 and 24 hr with db-cAMP-caffeine that had been exposed (for the same time plus 1 hr before) to the protein synthesis inhibitor. This structural result, of course, is not hampered by the problem of the cycloheximide-induced artifactual coupling described above, and the cycloheximide treatment seems to cause no major change in conductance of nonjunctional cell membrane even after 24 hr (Table 3). Thus, the promotion of the junctional differentiation, like that of gap junction in the 3T3-BalbC cells dealt with in the first paper of this series, appears to require protein synthesis.

Cytochalasin B. Treatment with cytochalasin B (1 μg/ml, 6 hr), prevented neither the development of gap junction nor of tight junction. These observations paralleled the electrical measurements of the 6-hr series of Table 5.


4 hr
8 hr
24 hr

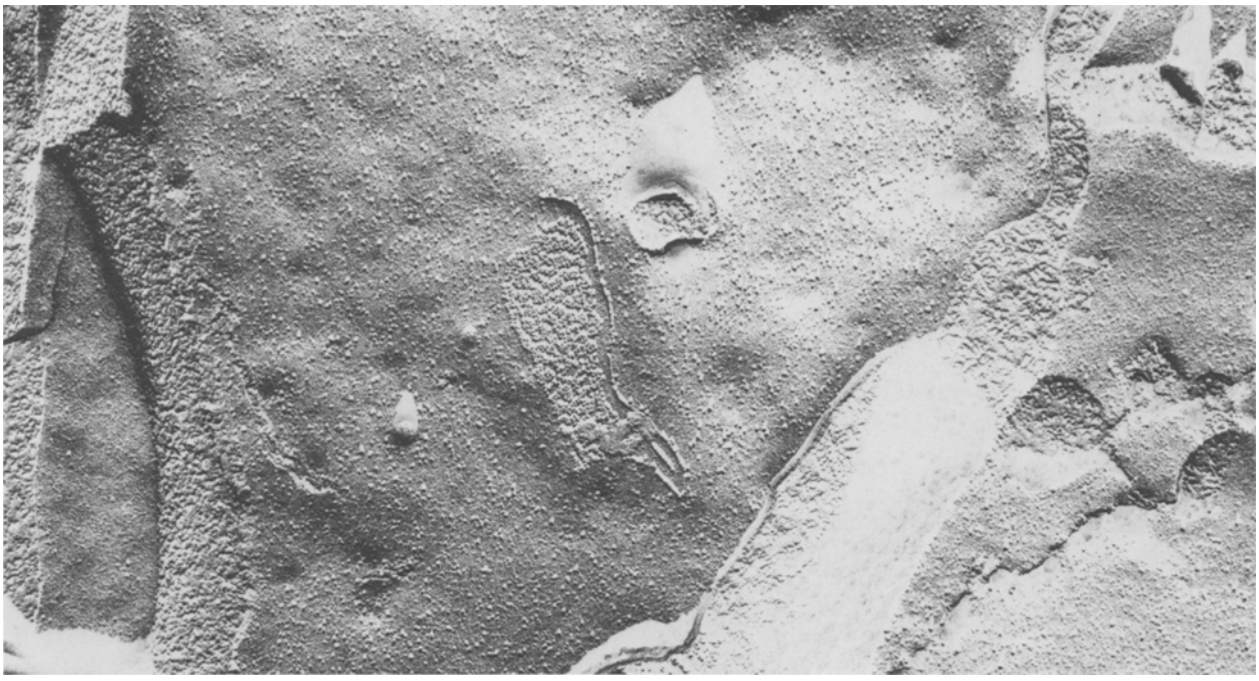


Fig. 8. The emergence of gap junction and tight junction during serum starvation. Electron micrograph of (confluent) starved C1-1D cells. Large gap junctions and tight junctions are present. The cells were in the 40th day of starvation and are from a culture parallel to that of Fig. 3 (series ●) on which electrical coupling was measured. Magnification 48,000 ×

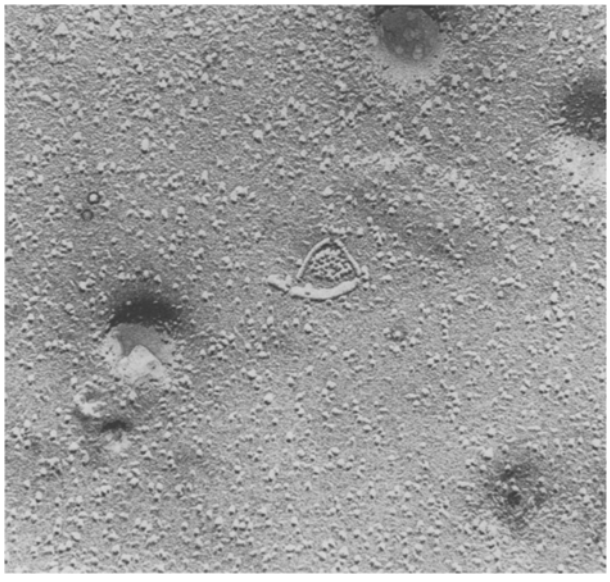


Fig. 9. Gap junction and tight junction form at low cell density. Electron micrograph of a C1-1D cell contact in a culture with a density of 1 [10^4 cell/cm^2] belonging to series ● of Fig. 4. Magnification 81,000 ×

Cell Adhesiveness

Various cancer cell types become more resistant to being dislodged from the dishes by the common cell dissociation procedures, when they are exposed to cyclic nucleotides (e.g., Johnson & Pastan, 1972; Grinnell, Miran & Srere, 1973). This also turned out be so for the C1-1D cells. The time required for getting $\geqq 95\%$ of the cells off the dish by exposing confluent cultures to dissociation medium (Methods), increased from 135 to 200 min after 24-hr db-cAMP-caffeine treatment (Table 7). A similar tendency was noticed in low density cultures.

As they came off the dishes, the cells separated also from each other (although not always singly). Evidently, the cells became more adhesive to the dishes and probably also to each other by the cyclic nucleotide treatment – an effect that may well be related to the aforedescribed development of junctional structures. As in that development, applications of cytochalasin B (1 μg/ml, 24 hr) together with db-

Fig. 7 (facing page). The emergence of gap junctions and tight junctions upon treatment with db-cAMP-caffeine. Freeze-fracture electron micrograph of confluent C1-1D cultures treated with db-cAMP-caffeine. Representative views of junctions of cells treated for 4, 8, and 24 hr. Gap-junctional particle clusters (*n*) and tight-junctional strands (*t*) occur separately or in close proximity to each other, occasionally, the last enclosing the former. At 4 hr (*top row*) these junctional structures are still small; some gap junctions have less than 10 particles, our acceptance criterion for gap junction (*see* the first paper of this series). However, when they are in close proximity to junctional strands (*top, left* and *right*), they are identifiable as junctional particles without undue risk; they presumably represent early stages of gap junction formation. By 8 hr both differentiations are larger, and more so by 24 hr. Top left shows a typical transition from nonjunctional to close membrane apposition. Part of the adjoined membranes are differentiated as tight junction (*t*) and gap junction (*n*); the rest are at a distance of more than 100 Å (*arrow*), as is typical for nondifferentiated membrane. Magnification 49,500 ×

Table 7. Time for cell dislodgement

Condition	Time[a] (min)
Control	135
db-cAMP-caffeine	200
db-cAMP-caffeine + cytochalasin B[b]	> > 200
Cytochalasin B	80

[a] Time required for dislodging ≧95% of the cells of confluent cultures from the dishes by a dissociation medium consisting of Ca, Mg-free saline with 0.1 g/liter trypsin and 0.1 g/liter EDTA.

[b] Cytochalasin B (1 μg/ml) applied together with db-cAMP-caffeine.

The cultures had been for 24 hr in each given condition at the time of the dislodgment tests.

cAMP-caffeine did not prevent the enhancement of adhesiveness. In fact, the time for cell dislodgement was then even longer than with db-cAMP-caffeine alone (but cytochalasin B on its own did not prolong the dislodgement time) (Table 7).

Discussion

The C1-1D cells acquired cell-to-cell channels in response to the various experimental conditions expected to elevate their cAMP concentration. This resembles the junctional reaction of the normal cells described in the two preceding papers of this series, but the C1-1D response is more dramatic, arising, as it were, from a zero base. The resemblance is in both, junctional permeability and gap-junctional structure, leading us to believe that the responses are ruled by the same cAMP-related mechanisms already outlined (paper I, pp. 117, 118; paper II, p. 131). Then, only one new aspect would need to be accounted for: the very low (or zero) base level of cell-to-cell channels in the C1-1D cells in the confluent condition. A simple possibility is that these cells have a low cAMP base level; or, to state this in terms of the proposed regulatory mechanism, that the junctional upregulation in C1-1D started from a threshold or subthreshold level of endogenous cAMP, whereas in the normal cells it started above threshold. The possibility of a lower cAMP level in Cl-1D is not farfetched; a variety of cancer cell types are low in cAMP (Otten, Johnson & Pastan, 1971; Sheppard 1972; Ryan & Curtis, 1973). This point is now under investigation.

As a part of their junctional response, the C1-1D cells underwent a change in their approximation. In their noncommunicating state, the cell membranes, as seen in the electron microscope, get to be as close as about 100 Å, and this is also as close as the (junctionally) nondifferentiated membrane regions of normal communicating cells get to be. This distance shortens to about 20 Å at the differentiated regions in the communicating state, and this is like the distance of differentiated-membrane apposition in normal communicating cells. Thus, we face once again the question of what causes the critical membrane approximation in the cAMP-stimulated state, but with one more piece of information: cytochalasin B does not prevent the development of cell-to-cell channels. This renders the possibility of an approximation by cytoskeletic forces less likely, and the possibility of a direct molecular interaction between the membrane surfaces comes to the fore. However, it does not take us much farther in the question of where cAMP is acting, whether on cell membrane approximation or, more directly, on channel biosynthesis and assemblage.

Apart from gap junctions, the development of a junctional permeability correlated with the development of tight junctions in the C1-1D cells. We have no reason to believe that this structure was in a major way a mediator of the junctional permeability, at least not of the permeability to c-fluorescein: In 3T3-BalbC cells – which had no detectable tight junctions before or after the db-cAMP treatment – the increase in junctional permeability was accompanied by increase of gap junction alone (Flagg-Newton et al., 1981).

C1-1D is one of several cancer cell types that are junction-deficient (Loewenstein, 1979). The deficiency behaves genetically like a recessive trait; it is corrected by (somatic) hybridization of the cell with a junction-competent cell (Azarnia & Loewenstein, 1977; Larsen et al., 1977). It can also be corrected by supplying the cell with RNA from a competent cell (Dahl, Azarnia & Werner, 1981). From the present results it is clear that the defect does not lie in the basic cell-to-cell channel structure: properly stimulated, the C1-1D cell formed channels; it did so even with other cell types, the junction-competent Lesch-Nyhan and 3T3-BalbC cells, and hence its protochannels or channel components in general are presumably compatible with those of junction-competent cells.

The results touch also on the cancer problem, in general. The finding that the emergence of junctional permeability was associated with normalization of the C1-1D growth pattern and was an early event (≦2 hr after db-cAMP administration) bears on the question of cancer etiology. It is consistent with the hypothesis ascribing the cell-to-cell channel a role in cellular growth regulation (Loewenstein, 1979). But, given the multiplicity of cellular reactions to cAMP elevation (e.g., Kram, Mamont & Tomkins, 1973; Temin, 1967; Rozengurt & Pardee, 1972), it would

be unwise to consider it anything more than a consistency, at this stage.

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Appendix

Appendix Table F-2. Data for Fig. 2

Experiment	Time (hr)	Communication incidence	
		Electrical coupling (%)	c-fluorescein transfer (%)
●	0	**0** (0:10; 10, 1)	
	1	**0** (0:10; 10, 1)	
	2	**20** (2:10; 10, 1)	**0** (0:55; 7, 1)
	3	**27** (3:11; 11, 1)	**0** (0:63: 9, 1)
	4	**30** (3:10; 10, 1)	**0** (0:58; 8, 1)
	6	**60** (12:20; 20, 1)	**26** (11:43; 6, 1)
	24	**100** (10:10; 10, 1)	**54** (32:59; 7, 1)
○	5	**80** (8:10; 10, 1)	**0** (0:35; 5, 1)
	8	**100** (10:10; 10, 1)	**22** (13:59; 6, 1)
	24	**100** (10:10; 10, 1)	**28** (17:60; 6, 1)
□	5	**100** (10:10; 10, 1)	**0** (0:48; 6, 1)
	8	**100** (10:10; 10, 1)	**43** (23:54; 7, 1)
	24	**100** (10:10; 10, 1)	**46** (16:35; 4, 1)

Appendix Table F-4. Data for Fig. 4

Experiment	Density [10^4 cells/cm^2]	Communication incidence	
		Electrical coupling (%)	c-fluorescein transfer (%)
●	1.6	**80** (8:10; 10, 1)	**43** (21:49; 12, 1)
	3	**60** (6:10; 10, 1)	**35** (15:43; 10, 1)
	7	**20** (2:10; 10, 1)	**5** (2:38; 8, 1)
	18	**0** (0:10; 10, 1)	**0** (0:52; 8, 1)
	30	**0** (0:10; 10, 1)	**0** (0:41; 6, 1)
	40	**0** (0:10; 10, 1)	**0** (0:35; 5, 1)
□	2	**85** (6: 7; 7, 1)	
	4	**57** (69:120; 120, 6)	
	8	**18** (7: 39; 39, 1)	
	16	**5** (4: 80; 80, 1)	
○	50	**0** (0: 88; 88, 9)	