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We dedicate this series of papers to the memory of Stephen W. Kuffler, who was with us and shared the joy as the first light shone through the haze.

Cell Junction and Cyclic AMP: I. Upregulation of Junctional Membrane Permeability and Junctional Membrane Particles by Administration of Cyclic Nucleotide or Phosphodiesterase Inhibitor

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Summary. Mammalian cells in culture were exposed to cyclic AMP, dibutyryl cyclic AMP, the phosphodiesterase inhibitor caffeine, or a combination of the last two, while junctional molecular transfer was probed with the series of microinjected, fluorescent-labelled linear molecules Glu, Glu-Glu, Glu-Glu-Glu, and Leu-Leu-Leu-Glu-Glu. The junctional permeability for these molecules increased with each of the agents, most markedly with the dibutyryl cyclic AMP-caffeine combination, as the intracellular cyclic nucleotide concentration rose. The junctional permeability effect developed over several hours. When probed with molecules close to the limit of cell-to-cell channel permeation (the most sensitive setting), the effect was detectable both, as an increase in the (relative) junctional transit rate and as an increase in the number of transferring cell interfaces in the test populations. The number of transferring cell interfaces reached a maximum by 4 hr, when the junctional transit rate, hence the junctional permeability, was still rising. Nonjunctional membrane permeability for the probe molecules, as determined by intracellular fluorescence loss, was not significantly changed (nor was there significant nonjunctional cell-to-cell transfer of molecules before or after the treatments). The rise in junctional permeability was associated with an increase in the number of gap junctional membrane particles, as determined by freeze-fracture electron microscopy: the average size of the particle clusters increased, and the frequency of the clusters increased, particularly that of the smaller (and presumably newer) clusters. This effect was blocked by treatments with the protein synthesis inhibitors cycloheximide or puromycin. These agents caused particle diminution (diminution of cluster frequency but not of average cluster size), with or without cyclic nucleotide. The junctional effects may represent a cyclic AMP-promoted proliferation of cell-to-cell channels. Some physiological implications, in particular, implications for hormone-regulated tissues, are discussed.

Key words: Cell-to-cell junction, gap junction, junctional permeability, membrane permeability, cell-to-cell membrane channels, membrane channel recruitment, cyclic AMP

Cells in organized tissues are commonly connected at their junctions by specialized cell-to-cell membrane channels (Loewenstein, 1966, 1981). These channels are also expressed in cell culture (Furshpan & Potter, 1968; Pitts, 1977; Loewenstein, 1979). In mammalian cells, their permeation-limiting diameter is 16–20 Å and they select noticeably against negatively charged molecules with sizes approaching this diameter (Schwartzmann, Wiegandt, Rose, Zimmerman, Ben-Haim & Loewenstein, 1981; Flagg-Newton, Simpson & Loewenstein, 1979). There are reasons to believe that the channels are contained in membrane particles that are discernible electron microscopically as clusters called 'gap junction' or 'nexus' (Revel, Yee & Hudspeth, 1971; Gilula, Reeves & Steinbach, 1972; Johnson, Hammer, Sheridan & Revel, 1974; Azarnia, Larsen & Loewenstein, 1974; Caspar, Goodenough, Makowski & Phillips, 1977; Unwin & Zampighi, 1980).

In the course of earlier work in this laboratory on mammalian cell cultures, it became apparent that their cell-to-cell channels are affected by the culture density and the serum in the medium (Flagg-Newton et al., 1979). On further examination, these effects turned out to be associated with cellular cyclic AMP. This series of papers describes studies of this junctional action of cAMP, an action manifested at the level of junctional molecular transfer and of junctional

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structure. The junctional transfer is probed with a series of fluorescent linear peptide molecules (Simpson, Rose & Loewenstein, 1977). These molecules, 14–16 Å wide, are close to the cutoff limit for channel permeation and so provide a sensitive means for detecting changes in junctional permeability. In particular, we make use of a glutamic molecular series for the probings – GluOH, Glu-GluOH, Glu-Glu-GluOH – where the variables determining permeation, molecular length, and negative charge increase together. The junctional structure is examined by freeze-fracture electron microscopy, with a technique permitting surveys of large areas of surface membrane.

We show that junctional permeability is increased by several experimental conditions elevating intracellular cAMP concentration, and that this upregulation is associated with an increase in the number of gap junctional particles. The present paper deals with treatments of channel-competent cell types, with exogenous cAMP and phosphodiesterase inhibitor; the second paper examines two culture conditions modulating endogenous cAMP; and the third deals with a channel-incompetent mutant cell type. Preliminary accounts of the results in the present paper have appeared (Flagg-Newton, 1979; 1980).

Materials and Methods

Cell Cultures and Media

The mouse fibroblast lines 3T3-BalbC, the rat fibroblast line B and the rat liver epithelioid line RL (*cf.* Flagg-Newton et al., 1979) were grown in Dulbecco-Eagle medium (high glucose) (Vogt & Dulbecco, 1960) supplemented with serum (10% unless stated otherwise) – calf serum for the 3T3-BalbC and fetal calf serum for B and RL – at 37 °C in an atmosphere of 5–10% CO_2 and air (incubator). The cell culture and the experiments were carried out in plastic dishes (Falcon). 3T3-BalbC cells were routinely passaged before reaching confluency to avoid their undergoing changes in growth properties (Boone, 1975). The dissociation medium for passaging was Ca, Mg-free saline with 0.5 g/liter trypsin and 0.5 g/liter EDTA (Gibco).

10 mM stock solutions of cyclic AMP (cAMP), dibutyryl cAMP plus caffeine (db-cAMP-caffeine), or caffeine in serum-free medium were sterilized by millipore filtration and kept refrigerated for up to 1 week. For the treatments, the growth medium was exchanged for growth medium with the agents at 1 mM concentration.

Junctional Permeability Probing

Junctions were probed with the fluorescent tracer molecules listed in Table 1. The preparation and purification of these tracers are described in Simpson, Rose and Loewenstein (1977) and Socolar and Loewenstein (1979). During the probings, limited to periods of 15–30 min, the culture dishes were at about 25 °C, exposed to room air.

The tracers were iontophoresed into the cells with the aid of a microelectrode (Flagg-Newton & Loewenstein, 1979). The tracers' cell-to-cell diffusion was continuously viewed in a microscope darkfield, and photographed. Usually 3–5 cells were injected in a given culture dish. Injected cells were multijunctional (except for the measurements of junctional transit rates and tracer loss), each injection trial providing information about several junctions. A junction here means two cells in contact as seen in the phase contrast microscope.

Table 1. Junctional probes

Probe	Charge[a]	Mol. wt.
LRB Glu OH	2	688
LRB Glu-Glu OH	3	817
LRB Glu-Glu-Glu OH	4	946
LRB Leu-Leu-Leu-Glu-Glu OH	3	1158

[a] Net negative charge.

LRB = Lissamine rhodamine B (red fluorescent); Glu, glutamic acid; Leu, leucine.

For preparation, characteristics and purity of the probes, *see* Simpson et al., 1977, and Socolar and Loewenstein, 1979. For molecular dimensions, *see* Schwartzmann et al., 1981.

Incidence of Transferring Junctions. We scored the incidence of first-order permeable junctions, that is, the proportion of fluorescent cells among the cells contiguous to the injected one. The fluorescence spread also to higher-order neighbors, but the first-order ones became fluorescent first and their fluorescence intensity was greater, indicating that the tracer transfer between contiguous cells was primarily via the direct junctional route rather than via a more circuitous one mediated by second- or higher-order junctions. In general, when it did at all, a given tracer spread to several of the first-order neighbors of the injected cell. However, occasionally the spread was limited to a single neighbor. Such cases were not scored because of a higher chance that the transfer, instead of junctional, was then via cytoplasmic bridges of incompletely divided cells. This was brought out by a series of control experiments in which LRB (red-fluorescent) was co-injected with FITC-fibrinopeptide (green-fluorescent) in B cultures. The fibrinopeptide molecule exceeds the dimensions for junction permeation (Simpson et al., 1977; Flagg-Newton et al., 1979). While fibrinopeptide never passed in the multijunctional LRB transfers, it passed in 7% of the one-junction transfers. In practice, however, this stringent scoring criterion did not significantly bias the results. As it turned out, one-junction transfer occurred very rarely in the experiments described in the first two papers of this series and not at all in those of the third paper. We used the criterion, nevertheless, not only because it is in principle more precise, but also for consistency with our earlier work (Flagg-Newton & Loewenstein, 1979).

The incidence scores tabulated are cumulative, from several microinjections and often from several culture dishes in the same experimental conditions. For example, the score of 88%, the second entry of Table 2, represents the aggregate of 11 injection trials in two culture dishes where a total of 77 out of 87 first-order junctions were found permeable. Tabulated also are the frequency distributions of the scores of the individual injection trials, arranged in the intervals of 10–49, 50–89, 90–100 and 0 per cent incidence. There were no scores in the 1–9% interval; the maximum number of first-order junctions was 20 at the cell densities of the experiments, and, as already mentioned, the minimum score criterion was 2.

The fluorescence intensity in the injected cell (source cell) was roughly matched in the various trials and was $\gg$ than detection threshold. The source cell tracer concentration was then evidently in the saturation range of the junctional flux-concentration relationship for $LRB(Glu)_2OH$ and $LRB(Glu)_3OH$ (Loewenstein, 1981), where junctional flux is governed by junctional permeability only; the junctional transit times were within 10% in different

control trials in these simple experimental conditions. The method thus offers a simple and reliable index of the state of junctional permeability. This is another advantage of the use of the glutamic series; smaller and less charged molecules, such as carboxyfluorescein or LRB, show less independence of driving force at comparable concentrations (Flagg-Newton & Loewenstein, 1980).

Rate of Junctional Transit. For determination of relative rates of junctional transfer we used two-celled systems (as they occur spontaneously in sparse culture) consisting of one source cell and one sink cell only. Here the source cell fluorescences were exactly matched with the aid of a photodiode system. We measured the *junctional transit time*, the time between the arrival of the tracer at the source cell boundary and its appearance in the sink cell; the reciprocal, the *relative junctional transit speed*, is presented in the Results.

Fluorescence Tracer Loss

Fluorescence loss was determined in one-cell or two-cell systems by means of the photodiode system. The system consisted of an array of low-noise silicone photodiodes (10^{-7} ft-candle, threshold) onto which the cells were projected through the microscope. The amplified photodiode outputs were displayed on a chart recorder. Except for a brief initial phase, presumably dominated by photodecomposition, the fluorescence decay curves could be fitted by single exponentials (Fig. 10). The loss half times were more than 30 min for all tracers, at least two orders of magnitude greater than the tracers' junctional transit times in control conditions (4–21 sec). When measurements were taken on the same cells in control and test conditions (as in Fig. 10), medium at 36° was pumped through the dish at the rate of about 2 ml/min in between the measurements (Flagg-Newton & Loewenstein, 1979). During the measurements themselves, the dishes were at 25 °C.

Transfer via Cytoplasmic Bridges

For estimation of the frequency of cytoplasmic bridges (incompletely divided cells, cell membrane fusions, etc.), we injected fibrinopeptide labelled with fluorescein isothiocyanate (FITC) into the cells. This molecule does not pass between cells in organized tissues that have undergone complete cytokinesis (Simpson et al., 1977). Its cell-to-cell transit in the cultures, a rare occurrence (*see* Results), was taken as an index of such coarse bridging. When it occurred at all, the transit took about a second, faster than the channel-mediated transit of the smaller molecules of the glutamic series.

Intracellular cAMP

Intracellular cAMP was kindly assayed for us by Dr. R.J. Ho, by Gilman's modified protein binding method (*cf.* Robison, Butcher & Sutherland, 1971). 8–16 culture dishes were used for each assay. Cells were harvested with the aid of a rubber policeman, without the use of trypsin. For any given set of experimental conditions the assays were run in parallel at the same time and with the same battery of reagents, to minimize variations. This applies to all assays of the present paper. For the assays of the second paper in this series (which were run separately from the present ones), this applies only for each set of experimental conditions for a given cell type.

Electron Microscopy

The 3T3-BalbC cultures were fixed *in situ* in the dishes with a 2% glutaraldehyde solution buffered with cacodylate (pH 7.4). Glycerol (30%) was added after 1 hr. After a 2-hr fixation, the bottom of the dish (milled previously to a 0.15 mm thickness) was cut into pieces fitting standard Balzer's specimen carriers. The specimens were mounted with polyvinyl alcohol, rapidly frozen in Freon-22, then chilled in a bath of liquid nitrogen, and freeze-fractured (Pauli, Weinstein, Soble & Alroy, 1977) in a Balzer 301 unit. Platinum-carbon replicas were examined in a Phillips electron microscope EM300.

The cells fractured mainly in the plane of their surface membranes. Areas of 2,050 μm^2 of the replicas, as bounded by the grid square mesh, were scanned for gap junctional particle clusters, at a 55,000 magnification. In most cases the grid squares were completely occupied by cell membrane fracture faces; when they were not, the data were normalized for such an area. For statistics calculation, n was the number of grid squares scanned.

Gap junctions were determined on *P* fracture faces where they are identified more readily than they are on *E* faces. The acceptance criterion for a gap junction was a minimum of 10 membrane particles in a cluster, except at a *P-E* transition – a rare occurrence – where the criterion was three particles. The ordinary criterion was set so high, because clusters up to eight membrane particles occurred on membrane areas that were evidently nonjunctional. Gap junction areas and cell membrane areas were integrated, with the aid of a Ladd curve digitizer, on electron micrographs at magnifications of 48,000 and 4,500, respectively. The gap junctional particle spacing, 9.5 nm, was the basis for calculating the number of particles.

For estimation of the equivalent cell area, the surface areas of 39 3T3-BalbC cells were determined, as twice the area occupied by the cell, on top views of phase contrast micrographs at a 175 magnification. The mean area so determined, the *equivalent cell area*, was 3499.7 ± 120 μm^2 (SE). This area estimation neglects cell height and ruffles (which were rare). These factors were also neglected in the membrane area determinations on the electron micrographs, and so the errors of the estimates of gap junction/equivalent cell roughly cancel out. For the gap junction/equivalent cell data given in the Results, the gap junction areas determined on the *P* fracture faces were doubled, on the premise that the probability of gap junction occurrence on *P* and *E* faces was equal. (All other data given are the actual *P*-face values.)

Test Culture Parallelism

All experiments for a given experimental variable in a given table or figure were done on parallel subcultures from the same stock, except when stated otherwise. This close parallelism was important, particularly in the work with 3T3-BalbC cells where junctional transfer and endogenous cAMP not only varied with cell density, but also with the number of cell passages. The cultures were incubated, unpassaged and undisturbed, for at least 15–24 hr before the various experiments were begun.

Results

Junctional Transfer

We determined two parameters of junctional tracer transfer, the incidence of (first-order) transferring junctions and the relative junctional transit rate. Both are indices of junctional permeability, and particularly sensitive ones when the probes are molecules large or charged enough to be close to the channel permeation limit – e.g., LRB$(Glu)_2OH$ or LRB$(Glu)_3OH$. The transit rate is the more direct index, but we use it less extensively as it is technically more difficult to obtain.

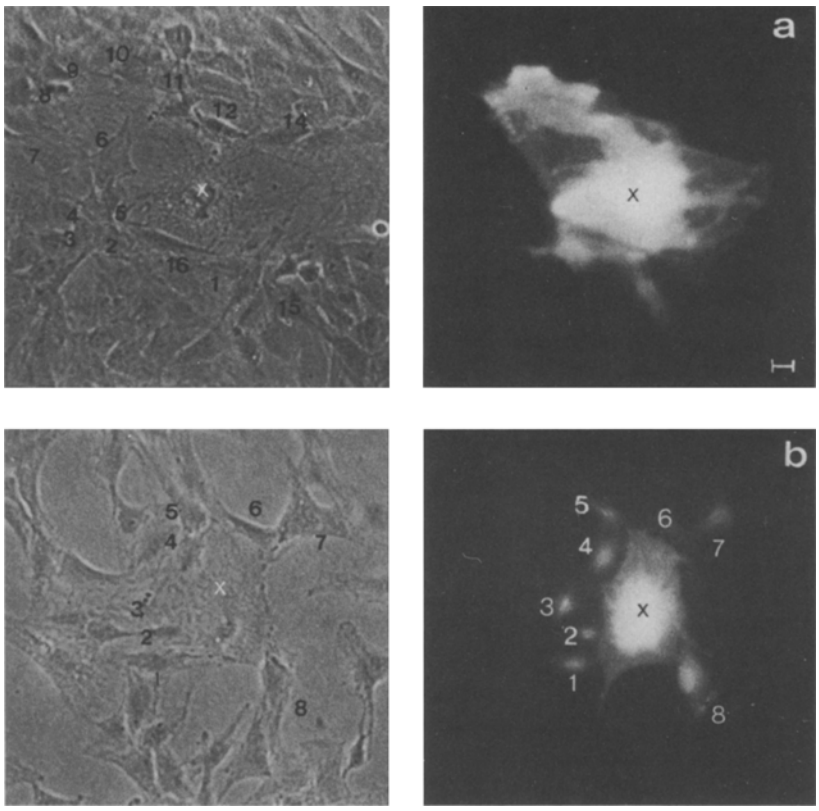


Fig. 1. Improvement of junctional transmission by treatment with db-cAMP-caffeine. B cells. Junctions probed with $LRB(Glu)_3OH$. (*a*): Control. (*b*): About 24 hr after treatment with db-cAMP 1 mM + caffeine 1 mM. Injected cell marked *x*; first-order neighbors, numbered. *Left*, phase contrast; *right*, darkfield. Calibration, 25 μm

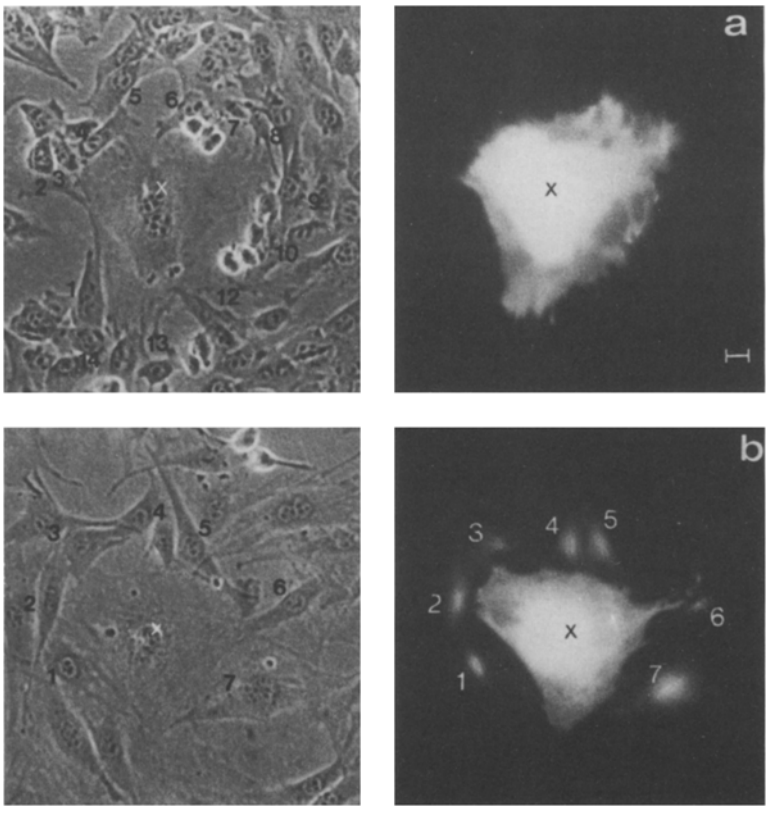


Fig. 2. Improvement of junctional transmission by db-cAMP-caffeine. 3T3-BalbC cells. Junctions probed with $LRB(Glu)_2OH$. (*a*): Control. (*b*): About 4 hr after treatment with db-cAMP-caffeine. Calibration, 25 μm

Table 2. Effects of db-cAMP-caffeine treatment on junctional transfer. B cells

Time after start of treatment (hr)	Permeable first-order junctions											
	$LRB(Glu)_2OH$					$LRB(Glu)_3OH$					$LRB(Leu)_3(Glu)_2OH$				
	Incidence (%)[a]	Frequency[b]				Incidence (%)	Frequency				Incidence (%)	Frequency			
		0	10–49	50–89	90–100		0	10–49	50–89	90–100		0	10–49	50–89	90–100
0	**54** (19:35; 5, 1)	1	1	2	1	**13** (12: 94; 10, 2)	7	2	1	0	**0** (0:114; 10, 3)	10	0	0	0
24	**88** (77:87; 11, 2)	0	0	5	6	**80** (103:130; 15, 4)	0	2	8	5					
48						**74** (59: 84; 9, 2)	0	1	6	2	**22** (14: 61; 8, 2)	4	2	2	0

Application of db-cAMP-caffeine at time zero and repeated every 10–14 hr. All permeability tests on parallel subcultures from the same stock, except the tests with $LRB(LEU)_3(Glu)_2OH$ at time 0.

[a] Incidence (%) of permeable first-order junctions (cumulative scores). In parentheses, in the following order: the number of permeable first-order junctions; the total number of first-order junctions; the number of microinjection trials; and the number of culture dishes examined.

[b] Frequency distribution of the incidence of permeable first-order junctions of the individual trials, arranged in intervals of 10–49, 50–89, 90–100, and 0%. There were no scores in the 1–9% interval; the minimum score criterion was 2, and at the cell densities of the experiments, the maximum number of first-order junctions was 20 for the present cell type as well as for the other cell types in the other tables.

Table 3. Effect of db-cAMP-caffeine on $LRB(Glu)_2OH$ junctional transfer. 3T3-BalbC cells at various densities

Time after treatment (hr)	Cell density (10^4 cells/cm^2)											
	0.843[a]					1–4[b]					6.76[a]				
	Permeable first-order junctions					Permeable first-order junctions					Permeable first-order junctions				
	Incidence (%)	Frequency				Incidence (%)	Frequency				Incidence (%)	Frequency			
		0	10–49	50–89	90–100		0	10–49	50–89	90–100		0	10–49	50–89	90–100
0	**65** (35:54; 8, 2)	0	2	4	2	**13** (7: 57; 9, 2)					**15** (22:154; 16, 4)	9	6	1	0
4	**85** (39:46; 8, 2)	0	0	4	4	**77** (96:124; 19, 3)					**46** (59:128; 12, 3)	2	4	6	0

Application of db-cAMP-caffeine at time 0.

[a] Data from subcultures (32nd passage) of the same stock.

[b] The data for this density range are from a different stock (cultures with >32 passages), representing the combined data at times 0 and 4 hr of Figs. 5*I* and *II* (*see* Appendix-Table F5).

Application of cAMP (1 mM) or of the more permeant dibutyryl cAMP (db-cAMP, 1 mM), or application of the phosphodiesterase inhibitor caffeine (1 mM) caused increase in junctional transfer in the three cell types tested: the incidence of junctions transferring the fluorescent probes rose (Figs. 1 and 2), and the rate of transfer rose. The largest effects were obtained with the combination of db-cAMP (1 mM) and caffeine (1 mM). Therefore, most of the experiments were done with that combination.

Incidence of Transferring Junctions

Table 2 gives the incidence data of the db-cAMP-caffeine-treated B cell cultures, as determined with $LRB(Glu)_2OH$, $LRB(Glu)_3OH$ and $LRB(Leu)_3(Glu)_2OH$. The incidence increased with all three probes, most strikingly with the last two. Thus, for example, the incidence of junctions passing the $LRB(Leu)_3(Glu)_2$, zero in the controls, rose to 22% after the treatment; and the incidence of junctions passing the $LRB(Glu)_3OH$ rose about sixfold.

Comparable effects were obtained with 3T3-BalbC and RL cells when their junctions were probed with molecules close to their control cutoff limit which is somewhat lower than that in B cells. [Here $LRB(Glu)_2OH$ is close to the cutoff in control conditions; *see* Flagg-Newton et al., 1979.] Tables 3 and 4 give the incidence data of $LRB(Glu)_2OH$-permeable junctions for both cell types, corresponding to treat-

Table 4. Effect of db-cAMP on junctional transfer. RL cells

Time after treatment (hr)	$LRB(Glu)_3OH$ permeable first-order junctions				
	Incidence (%)	Frequency			
		0	10–49	50–89	90–100
0	**0** (0:65; 12, 2)	12	0	0	0
2	**0** (0:35; 5, 1)	5	0	0	0
4	**16** (9:55; 7, 2)	4	2	1	0

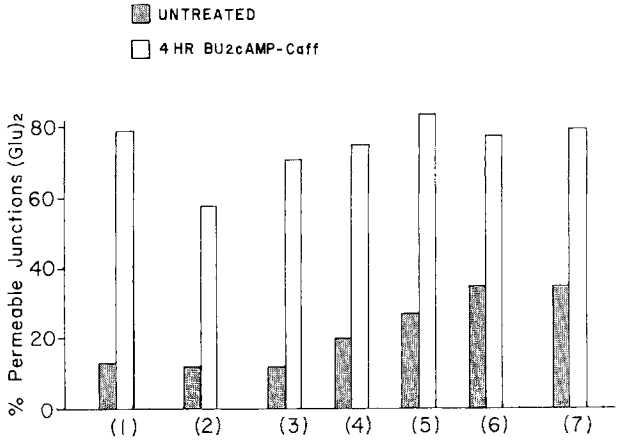


Fig. 3. Junctional effect of db-cAMP in 3T3-BalbC cells. The incidence of junctions permeable to $LRB(Glu)_2OH$ in untreated controls (gray), and 4 hr after application of db-cAMP-caffeine (white). The data represent 7 series of experiments done on cultures from different stocks, but each series subsumes the experiments on parallel subcultures of the same stock. In series 1–5 the cultures had been passaged >32 times; in series 6 and 7 they had been less passaged, and the junctional incidence in the controls was higher. Data detail in Appendix-Table 3

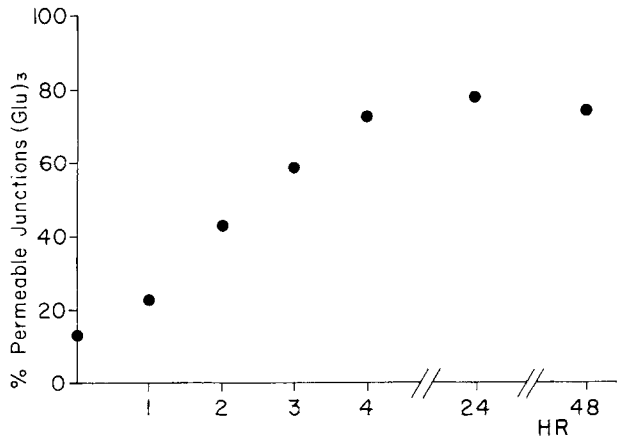


Fig. 4. Time course of the junctional effect in B cells. Incidence of junctions permeable to $LRB(Glu)_3OH$. Application of db-cAMP-caffeine at time 0 and renewed every 10–14 hr. The experiments were done on parallel subcultures from a common stock. This applies also to the curves and graph series in the following figures, except where indicated differently. Data detail, Appendix-Table 4

ment times at which we presumed the rise of the incidence to be maximal (*see* below).

In 3T3-BalbC cultures, where the control incidence of transferring junctions depends on cell density (Flagg-Newton & Loewenstein, 1981), the db-cAMP-caffeine effect was examined at three different densities. The incidence rose at all three, and most above 1×10^4 cells/cm^2 (Table 3).

The incidence levels, following 4-hr treatments, in various runs at similar cell density, were not too far apart. This was so even when the cultures, from different 3T3-BalbC stock, had different control incidences to start with. Figure 3 illustrates this point for seven series of experiments; it also gives an idea of the effect's reproducibility.

Time courses are shown in Figs. 4 and 5. In B cells, typically, the incidence rose over about 4 hr to a maximum. The maximum was maintained for at least 48 hr in experiments in which the db-cAMP-caffeine was renewed every 10–12 hr (Fig. 4, Table 2). 3T3-BalbC cells behaved similarly in regards to incidence rise time to peak (Fig. 5*I*, *II*), but, in regards to the peak maintenance, they behaved in two ways, depending on the stock from which the cells had been derived. With one stock, the maximum was sustained as in B cells; the time course was similar to that in Fig. 4. With another, the incidence declined within 6–10 hr to control or to below control, as shown in Fig. 5*II*.

We do not know why these 3T3-BalbC cell types behaved differently, but depletion of the stimulating agent in the medium evidently was not a reason: When we removed part of the medium from a treated set of subcultures 4–5 hr after the db-cAMP-caffeine application and transferred it to an untreated set, the junctional transfer rose in the latter (Fig. 5*III*). Furthermore, with applications of db-cAMP-caffeine, repeated at 2-hr intervals up to 4 hr, the decline set in just as when the agents had been applied only once at time zero (Fig. 6). With treatments more spaced, the junctional incidence rose repeatedly (Fig. 7).

Treatments with caffeine alone or with cAMP caused the incidence of transferring junctions to increase, too, but less than with db-cAMP-caffeine (Fig. 8).

Rate of Junctional Transit

Relative junctional transit speeds were determined on two-cell preparations of B cultures (Methods). The mean transit speeds of $LRB(Glu)_2OH$ and $LRB(Glu)_3OH$ nearly tripled within 24 hr of the db-cAMP-caffeine treatment (Fig. 9). The speeds were still rising by the time, 4 hr, the incidence of transferring junc-

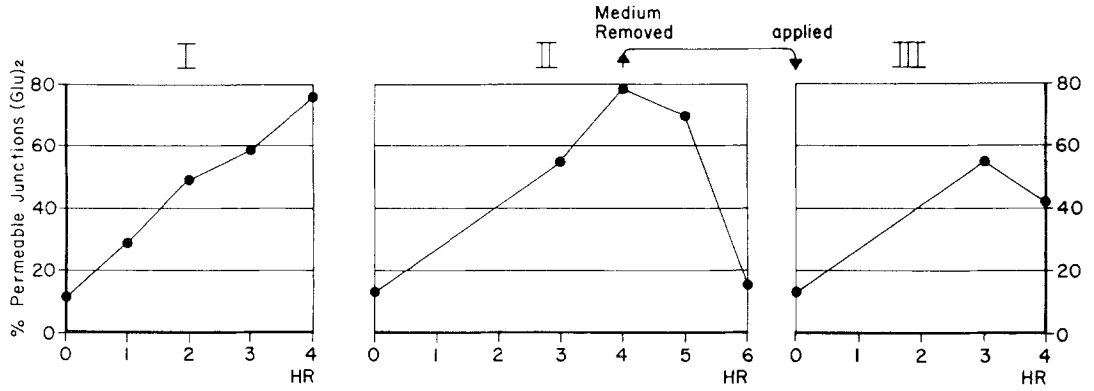


Fig. 5. Time course of the junctional effect in 3T3-BalbC cells. (*I* and *II*): Application (single) of db-cAMP-caffeine (time 0) and its effect on the incidence of junctions permeable to $LRB(Glu)_2OH$ in two independent series of experiments. *I* shows the rising phase of the incidence and *II*, the falling phase, with the same time resolution. (*III*): Application of the medium from a culture of *II* to another set of cultures. Medium is removed from the culture of *II* at time 4 hr (arrow) and applied to the culture set of *III* (the time 0 for this set). Data detail, Appendix-Table 5

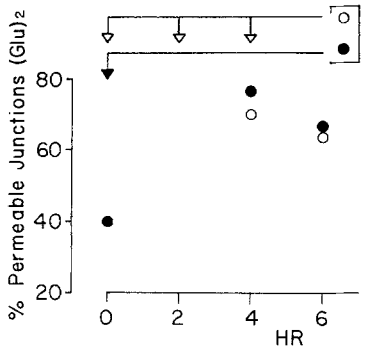


Fig. 6. Repeated db-cAMP-caffeine application does not prevent the decline of the effect in 3T3-BalbC. One set of cultures is treated with db-cAMP-caffeine at times 0, 2 and 4 (○) and another set is treated once only at time 0 (●). Treatment schedule (arrows) on top. Junctions are tested with $LRB(Glu)_2OH$ just before time 0, at 4 and 6 hr. Data detail, Appendix-Table 6

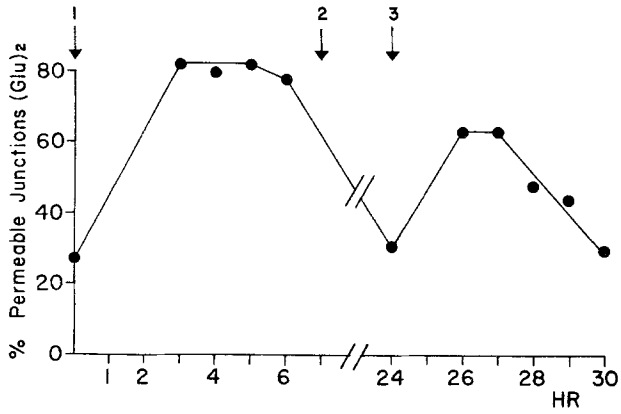


Fig. 7. Two junctional response cycles to db-cAMP-caffeine. The db-cAMP-caffeine is applied three times (arrows) to the same 3T3-BalbC cultures. The third application (*3*) trails the second by 17 hr, but junctional permeability was not tested in the (overnight) period following the second one. $LRB(Glu)_2OH$ is the permeability probe. The cell densities were 0.703 (10^4 cells/cm^2) during the first cycle and 1.35–1.67 (10^4 cells/cm^2) during the second cycle. The cultures had been passaged >32 times at the time of the experiments. Data detail, Appendix-Table 7

tions had peaked, and they continued to rise even beyond 5.5 hr. Since there was no significant change in the tracer loss (*see* below), this means that the junctional permeability was then rising, but by 4 hr the rise already had reached the detection threshold in the maximum number of cell interfaces.

The rising trends of the relative junctional transit speeds of both probe molecules were highly significant. Represented as saturating exponentials, the data of Fig. 9 define a curve with a slope significantly different from zero at a statistical confidence level, P, <0.001. Even the difference between the values at 5.5 and 24 hr (treated as individual points) are different at a $P<0.01$.

Apart from these measurements, the enhanced rate of tracer transfer was qualitatively indicated by the fluorescence intensity in the first-order sink cells of multijunctional preparations. This intensity was almost always increased upon db-cAMP-caffeine treatment and, often, upon cAMP or caffeine treatment; and this applies to 3T3-BalbC and RL as well as to B cells (i.e., to all the junctional incidence data tabulated).

Intracellular cAMP

Intracellular cAMP concentrations were determined in 3T3-BalbC and B cultures before and after treat-

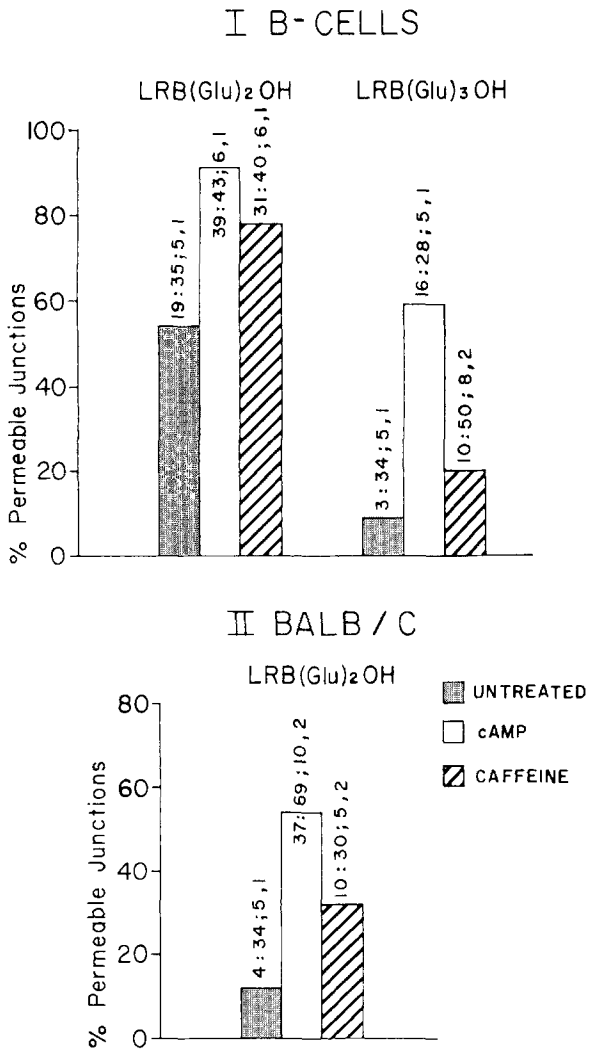


Fig. 8. Junctional effects of cAMP and caffeine. Incidence of first-order permeable junctions in controls (gray) and after treatment with 1 mM cAMP (white), or 1 mM caffeine (hatched). (*I*): B-cell junctions probed with $LRB(Glu)_2OH$ and $LRB(Glu)_3OH$. (*II*): BalbC cell junctions probed with $LRB(Glu)_2OH$. Junctions were tested in *I*, 24 hr, and in *II*, 4 hr after start of the treatment (*see* Figs. 4 and 5 for times of maximum increase of permeable-junction incidence for the two cell types). The numbers on the bar graphs give, in the following order, the number of permeable first-order junctions, the number of first-order junctions tested, the number of trials (microinjections), and the number of culture dishes examined

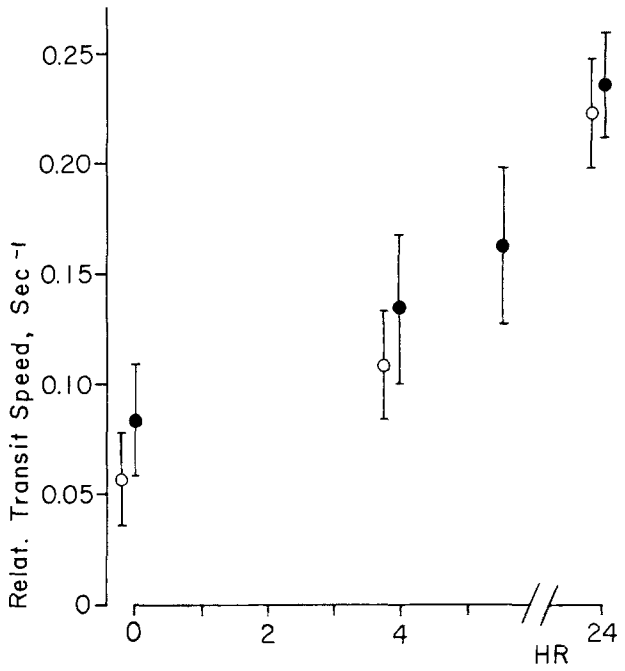


Fig. 9. Time course of the increase of relative junctional transit speed. B cells. db-cAMP-caffeine was applied at time 0; the control data point for each series was obtained just before time 0. The probes were $LRB(Glu)_2OH$ (●) and $LRB(Glu)_3OH$ (○). Plotted are the means (18–26 trials for each data point); the bars subtend the standard errors

Table 5. Intracellular cAMP concentrations

Condition	cAMP (pmol/10^6 cells)	
	3T3 BalbC	B cells
Control	2.0	0.9
Caffeine	3.7	1.4
Control	2.2	0.8
db-cAMP-caffeine	14.6[a]	17.3[a]

Assays in 3T3-BalbC cells were made at 4 hr of treatment and in B cells, at 24 hr, the respective times of maximum junctional transfer. Each datum represents the results obtained on 8–16 culture dishes.

[a] Includes cAMP and monobutyryl cAMP.

ment with caffeine and db-cAMP-caffeine. The assays were run at time zero and at the times of peak incidence of transferring junctions – at 4 and 24 hr, for 3T3-BalbC and B cells, respectively. The intracellular concentrations (which in the case of the db-cAMP-caffeine treatment include, besides cAMP, the more slowly degraded monobutyril cAMP) were markedly increased by the treatments. Table 5 summarizes these results, giving the cAMP concentrations per 10^6 cells. Expressed per mg protein, the cAMP concentration ratios between the various experimental conditions were about the same.

Control Treatments with 5′AMP and Na Butyrate

Treatments with 5′AMP (0.1 and 1 mM) and Na butyrate (1 mM) had no noticeable junctional effects. The incidence of $LRB(Glu)_3OH$-transferring B cell junctions and of $LRB(Glu)_2OH$-transferring 3T3-BalbC junctions were not significantly changed (Table 6),

Table 6. Control treatment: 5′ AMP and Na butyrate. Incidence of transferring junctions

Time after start of treatment (hr)	B cells [LRB(Glu)$_3$OH]	3T3-BalbC [LRB(Glu)$_2$OH]
0	**12** (12:100; 13, 2)	**15** (11:72; 12, 3)
4		[a] **12** (8:68; 11, 2) [b] **14** (13:94; 15, 3)
24	[a] **13** (9: 67; 8, 2) [b] **10** (8: 79; 9, 2)	

[a] 5′AMP treatment, 0.1 and 1 mM.
[b] Na butyrate treatment, 1 mM. μm^2

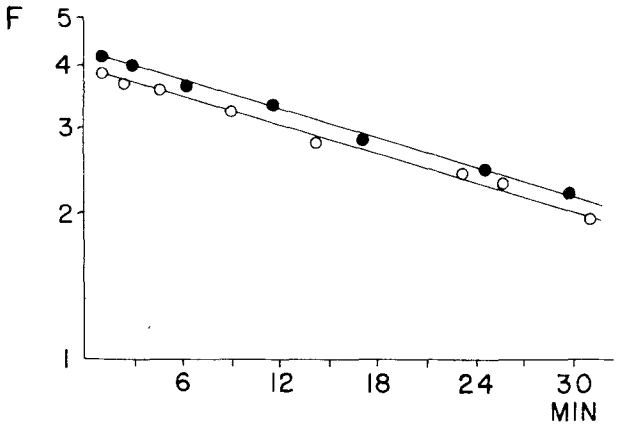


Fig. 10. Fluorescence loss. The LRB(Glu)$_3$OH fluorescence intensity (F) in the source cell as a function of time before (○) and after (●) a 4-hr treatment with db-cAMP. A two-cell system in a B cell culture. The tracer is injected at time 0; the F record starts after the tracer had passed to the sink cell (junctional transit time, 18 sec). Fluorescence is excited intermittently with pulses of 6-sec duration

nor was the fluorescence intensity in first-order neighbors different from those of controls.

Controls against Effects on Permeabilities Not Mediated by Cell-to-Cell Channels

The tracer molecules used in the present work are rather specific probes of junctional membrane; they normally do not significantly permeate nonjunctional membrane (Simpson et al., 1977; Flagg-Newton et al., 1979). However, two possible nonjunctional cyclic nucleotide effects needed to be considered: (i) a change in nonjunctional membrane permeability to the probes, in particular, a reduction; and (ii) an increased frequency of (coarse) cytoplasmic bridges between cells (such bridging does not seem to occur in cells of organized tissues, with cytokinesis complete, but it seems to occur with low frequency in culture, presumably as the result of incomplete cell division or cell membrane fusion).

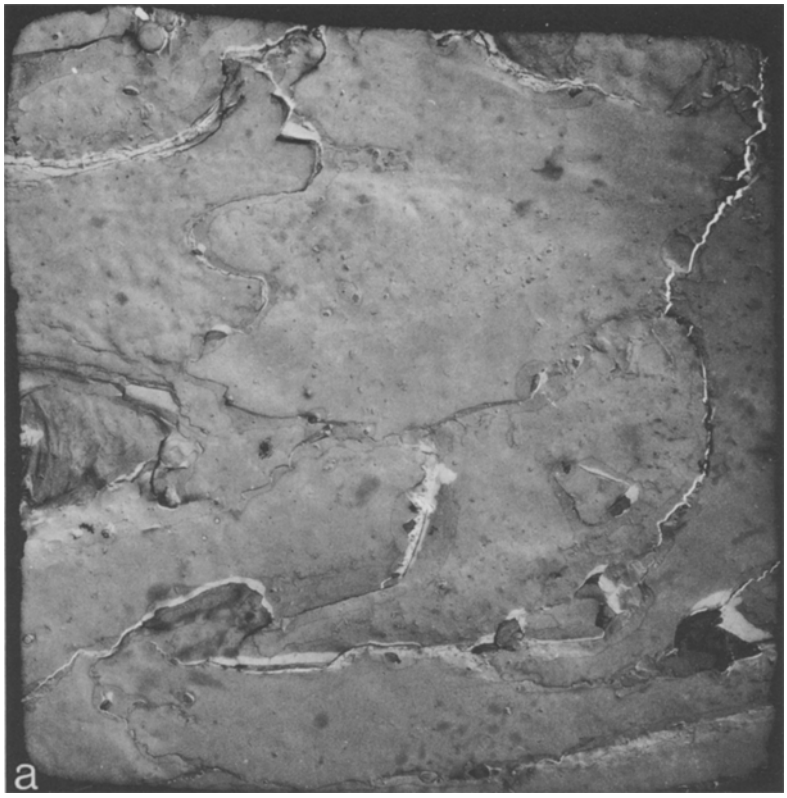
The first possibility was examined by measuring the rates of the probes' fluorescence loss from two- and three-celled systems in B cultures. We injected the sink cells with LRB(Glu)$_2$OH or LRB(Glu)$_3$OH to a standard fluorescence intensity, and determined the rate of fluorescence loss – which includes all losses, losses through nonjunctional membrane leakage, photodecomposition, etc. – at 4 hr and 24 hr after db-cAMP-caffeine treatment, with the aid of a photodiode system. The rates of loss were not sensibly affected by the treatment. Figure 10 exemplifies this for a measurement taken on a two-cell system at time 0 and at 4 hr, when the incidence of transferring junctions, as determined on other 26 junctions in this dish, had risen by 62%.

The second possibility could also be ruled out. We evaluated the frequency of cytoplasmic-bridge transfer using the cell-to-cell passage of FITC-fibrinopeptide as an index. This peptide molecule, unlike the linear peptides of the glutamic series, has a tertiary structure stabilized by H bonds, that exceeds the size limit for cell-to-cell channel permeation; it is junctional membrane-impermeant and nonjunctional membrane-impermeant (Simpson et al., 1977; Flagg-Newton et al., 1979). The incidence of randomly chosen cell pairs with positive FITC-fibrinopeptide transfer in (subconfluent) B cultures was 0.3%, and this incidence did not rise upon cyclic nucleotide treatment.

Gap-Junctional Membrane Particles

The junctions of the 3T3-BalbC cultures, in various experimental conditions, were examined electron microscopically. The junctions exhibited a single kind of membrane differentiation, the typical particle clusters of gap junction (Figs. 11 and 12). The particle spacing in the clusters was 9.5 ± 2.5 nm (SE; $n = 200$ spacings in different, randomly selected clusters) and this particle packing and the scatter of spacings was about the same in the db-cAMP-caffeine treated cells. There were no tight junctions. The cells in these one-layer cultures freeze-fractured mainly in the plane of their surface membranes (fractures across cytoplasm were rare), thus providing a favorable preparation for a quantitative study of gap junction.

The frequency of gap junctions in different cell surface samples and the size of the individual gap junctions varied greatly. Therefore, for quantitative comparisons, it was necessary to sample large surface membrane areas. We scanned 47,000–150,000 μm^2 for a given experimental condition (except for the treat-



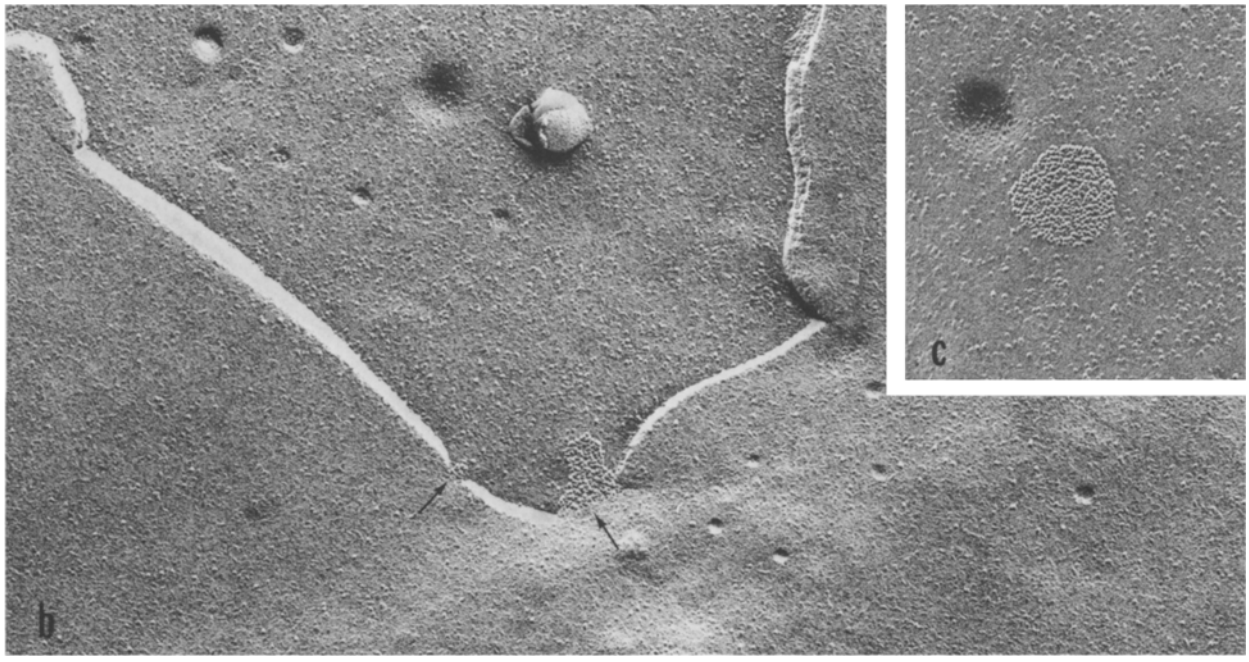


Fig. 11. Freeze-fracture electron micrographs of 3T3-BalbC cells. (*a*): A representative low-magnification view of a unit square, as used for the present morphometry; the field is occupied by cell membrane fracture faces. Such squares were scanned at high magnification for identification of gap junctions and measurements of their areas. (*b*): Typical gap junctional particle clusters (arrows) in cell treated with db-cAMP-caffeine (24 hr); (*c*): in cells treated with puromycin (24 hr) gap junctions of considerable size are found; gap junctions as large as these are typical after protein synthesis inhibition, although their frequency is lower than in controls. Magnifications: (*a*) 1,600×, (*b*, *c*) 48,000

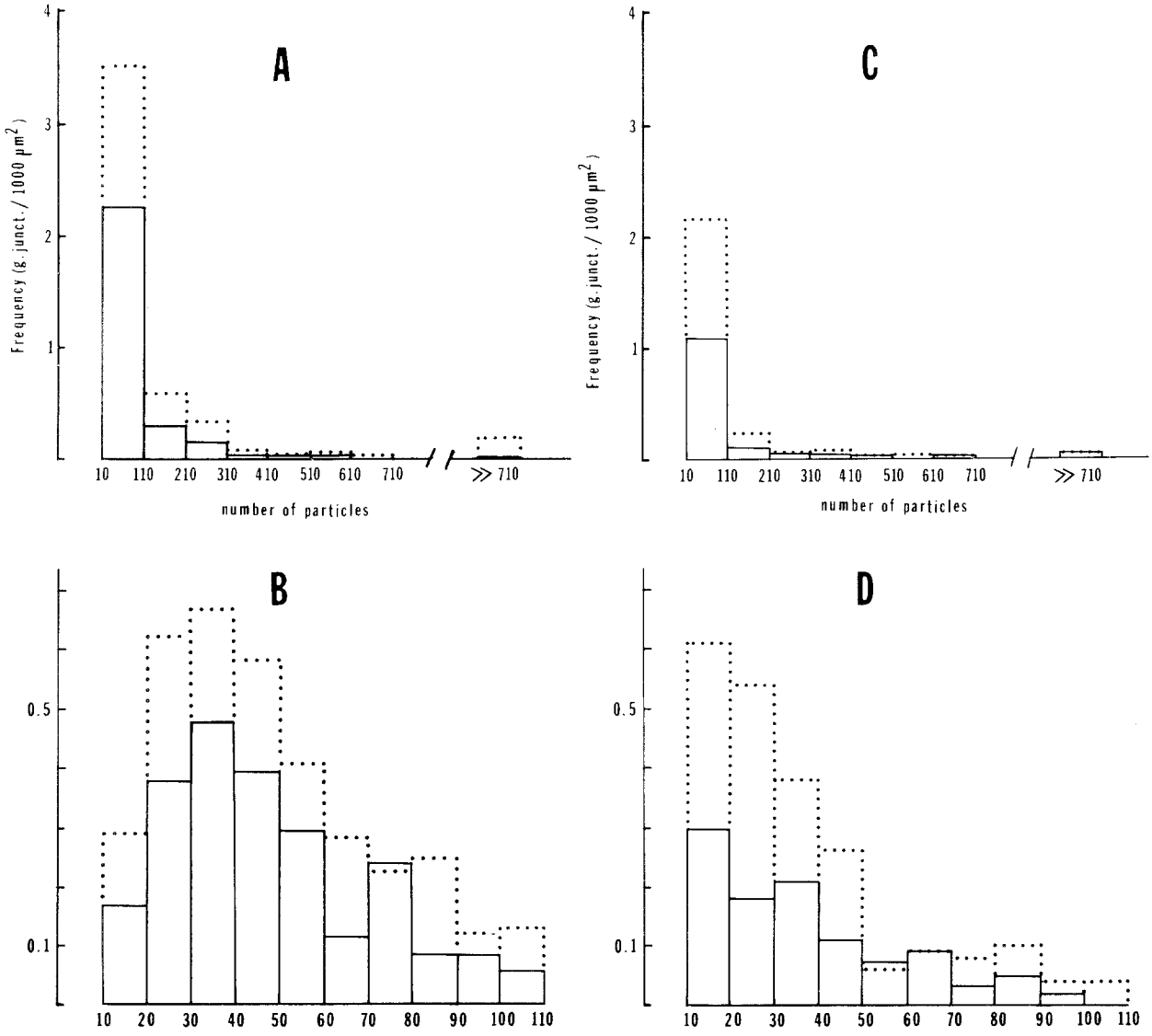


Fig. 12. *Top:* frequency distribution of gap junction size in control (solid lines) and after a 24-hr (*A*) or a 4-hr (*C*) db-cAMP-caffeine treatment (dotted lines). Gap junction size is given in number of particles; and frequency, in gap junctions per 1000 μm² of cell membrane. *Bottom:* the corresponding data from the 10–110 particle size interval expanded, (*B*) 24 hr, (*D*) 4 hr. Data are based on the following number of gap junctions: (*A*) control, 204; db-cAMP-caffeine, 540; (*B*) control, 137; db-cAMP-caffeine, 267

ments with protein synthesis inhibitors alone) and determined the frequency and area of gap junctions (the primary data) and the number of gap junctional particles (Methods).

The db-cAMP-caffeine treatment produced an increase in the number of gap junctional particles on the cells: the gap junction frequency increased and the gap junction size, i.e., the number of particles in the gap junctions, increased significantly ($P < 0.001$) over a 24-hr period (Table 7). During that time, the frequency increased over a broad spectrum of gap junction sizes; the distribution of frequency roughly paralleled that of the controls, but extending far beyond in the large-size range (Fig. 12*A*). At an early phase, at 4 hr, the frequency peaked at a gap junction size smaller than at 24 hr (or in controls) (Fig. 12*B*). The time course of the increase of the total number of particles is shown in Fig. 13.

Exposure of the cells to the protein synthesis inhibitors cycloheximide (10 μg/ml) or puromycin (20 μg/ml) prevented the db-cAMP-caffeine effect. The treatment schedule was to expose the cells first for 1 hr to the protein synthesis inhibitor and then to both, the inhibitor and db-cAMP-caffeine for the

Table 7. Gap junction frequency and size. 3T3-BalbC cells

Condition	Mean gap junction frequency		Mean gap junction size		Membrane area scanned
	Per 1,000 μm^2	Per equivalent cell	nm^2	Number of particles	μm^2
Control	2.84 ± 0.59 (35)	19.88 ± 4.13	8,609.2 ± 986.1 (204)	95 ± 11	71,750
db-cAMP-caffeine	4.88 ± 0.83 (54)	34.16 ± 5.81	13,861.9 ± 1,382.4 (540)	154 ± 15	110,700
db-cAMP-caffeine + cycloheximide	0.60 ± 0.16 (23)	4.20 ± 1.12	34,293.9 ± 9,777.9 (62)	380 ± 108	47,150
db-cAMP-caffeine + puromycin	0.34 ± 0.12 (30)	2.38 ± 0.84	13,847.9 ± 3,985.9 (21)	153 ± 44	61,500
Cycloheximide	0.73 ± 0.30 (10)	5.11 ± 2.10	24,301.0 ± 5,272.8 (15)	269 ± 58	20,500
Puromycin	0.44 ± 0.20 (10)	3.08 ± 1.40	7,515.2 ± 3,696.5 (9)	83 ± 41	20,500

All test conditions after 24-hr treatment.

Means ± SE; in parenthesis, *n*, the number of unit squares (2,050 μm^2 each) or the number of gap junctions.

Confidence levels of differences (*p*) for frequency: control/db-cAMP-caffeine, $<0.1>0.05$; control/cycloheximide db-cAMP-caffeine, <0.005; control/puromycin db-cAMP-caffeine, <0.001; control/cycloheximide, $<0.1>0.05$; control/puromycin, <0.05; for size: control/db-cAMP-caffeine, <0.025; control/cycloheximide db-cAMP-caffeine, <0.001; control/puromycin db-cAMP-caffeine, NS; control/cycloheximide, <0.001; control/puromycin, NS.

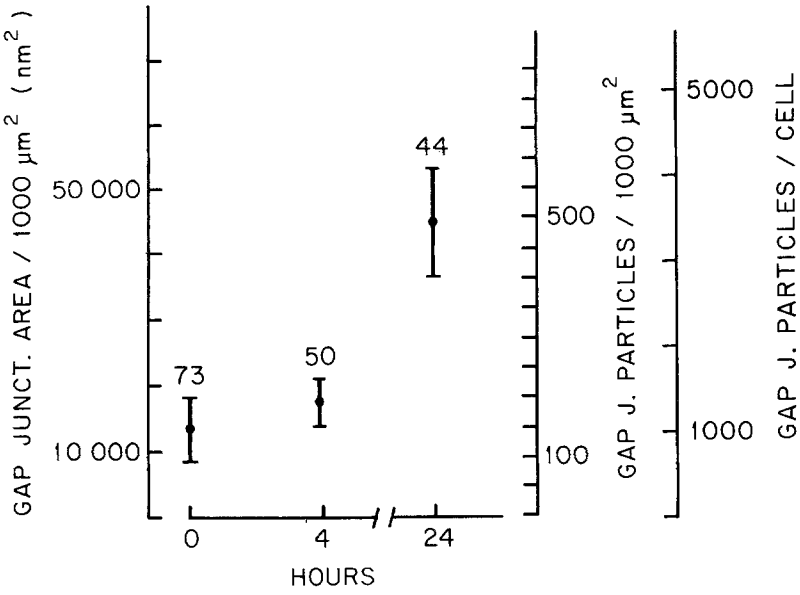


Fig. 13. The time course of the increase of gap junction area. 3T3-BalbC cells treated with db-cAMP. Each point represents the mean of the data pooled from 3 runs each on several parallel culture dishes. Bars subtend the standard error; on top, *n*, the number of unit squares scanned. The area data are given as: (i) the total gap junction area (nm^2) on 1,000 μm^2 of cell membrane (left ordinate); (ii) the number of gap junctional particles for that same area; and (iii) the total number of particles per equivalent cell membrane area (3,500 μm^2) (*see* Methods). The total areas of cell membrane scanned were 149,650 μm^2 (0 hr), 102,500 μm^2 (4 hr) and 90,200 μm^2 (24 hr). Statistical confidence levels of the difference: control *vs.* 4 hr, $P>0.05$; control *vs.* 24 hr, $P<0.001$

next 24 hr. There was no gap junctional particle proliferation in these conditions. In fact, there were then fewer particles than in the controls ($P<0.005$) (Fig. 14).

It would appear that the inhibitors themselves produced particle diminution. At least, with treatments of puromycine alone, the diminution was significant ($P<0.05$). There were, however, always gap junctions left over after the inhibitor treatments. (*Compare* with work on other cell cultures: Epstein, Sheridan & Johnson, 1977; Williams & DeHaan, 1981.) The leftovers were all of relatively large size; the sizes with 10–20 particles, abundant before, were missing (Table 7).

Discussion

Junctional Permeability Modulation

The present results show that the rate of transfer of molecules across junction is increased by action

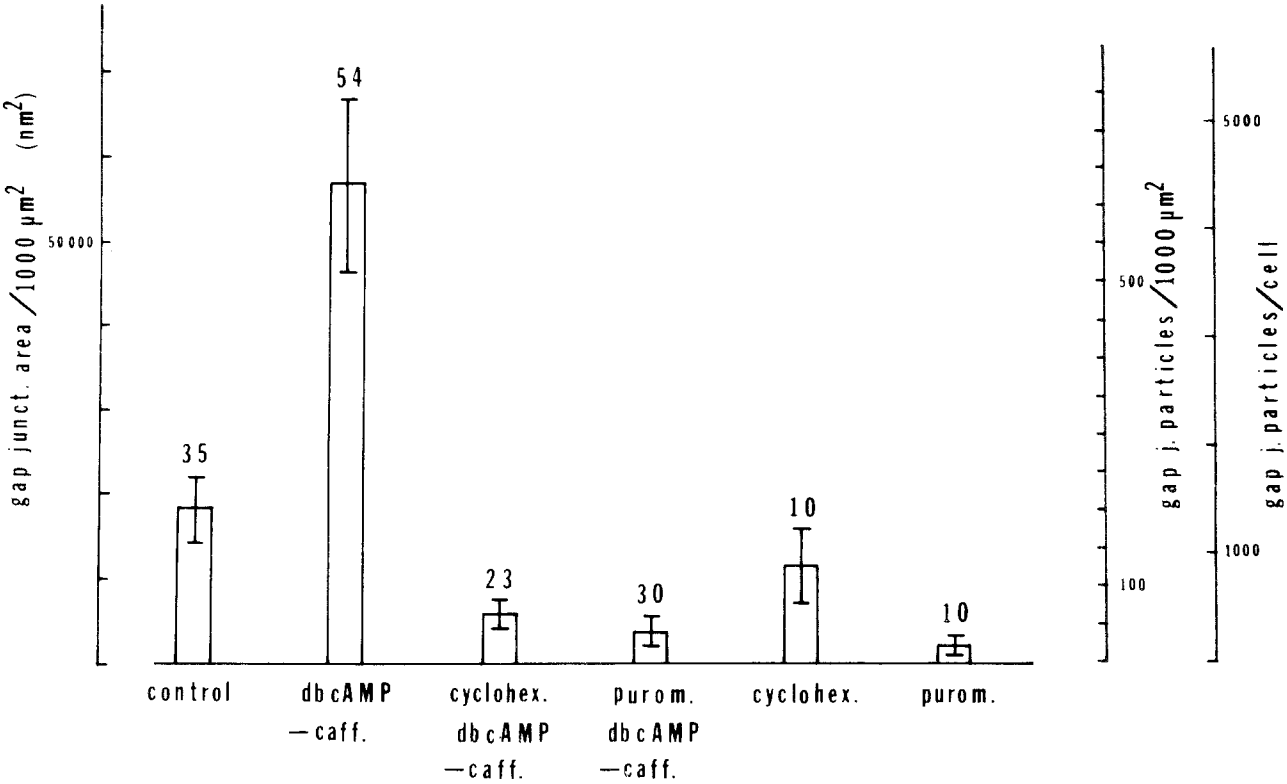


Fig. 14. The effect of inhibitors of protein synthesis. 3T3-BalbC cells. Ordinates and data presentation as in Fig. 13. Data were pooled from three runs of several parallel culture dishes, except for cycloheximide and puromycin (1 run). Significance levels of control *vs.*: db-cAMP-caffeine, $P < 0.001$; cycloheximide + db-cAMP-caffeine, $P < 0.025$; puromycin + db-cAMP-caffeine, $P < 0.005$; cycloheximide, and puromycin, NS; db-cAMP-caffeine *vs.* cycloheximide + db-cAMP-caffeine, $P < 0.001$; puromycin + db-cAMP-caffeine, $P < 0.001$

of cyclic nucleotides and that this increase is not due to a change in nonjunctional membrane permeability. The effect thus is an increase in junctional membrane permeability.[1] Such a permeability change could be brought about by an increase in the number of cell-to-cell channels and/or an increase in the unit channel permeability, i.e., an increase in the effective channel diameter. At least, the first seems a plausible mechanism in view of the finding of an increased number of gap junctional membrane particles, if we may assume that this larger number of particles indeed reflects a larger number of open channels.

This affords not only an explanation for the increased junctional transfer rates, but it may even account for the rise in the permeation cutoff, as observed with the present probe series. With this series of linear molecules of rather constant width (14–16 Å) – a width smaller than the channel diameter – the probability for channel permeation is determined by molecular length and negative charge (Flagg-Newton et al., 1979); and, since, in the glutamic series, negative charge increases inherently with molecular length, the permeability falls steeply from Glu to Glu-Glu-Glu. Thus, if the junctional permeability for LRBGlu-Glu-GluOH is ordinarily below the detection threshold (as it is, for example, in RL cells), the permeability may conceivably be raised to threshold by a channel proliferation – a proliferation by opening of preexisting channels or by new channel formation.

The finding of an increased number of gap junctional membrane particle points to a proliferative mechanism of the last kind. The effects at both, the levels of junctional structure and transmission seem slow enough for new channel formation, even one from scratch including channel precursor synthesis; the entire synthesis and assemblage of gap junction components (Revel, Yancey, Meyer & Nicholson, 1980) or of cell-to-cell channels (Dahl, Azarnia & Werner, 1981) seems to take on the order of 3–4 hr. We have no experimental clues on how cAMP may affect the channel formation process, but, as in many cAMP-regulated processes (Kuo & Greengard, 1969; Krebs, 1972; Nimmo & Cohen, 1977), it could con-

[1] The only alternative, an increase in junctional transfer due to an increased driving force is unlikely. There were no systematic differences in the transjunctional chemical gradients of the probe molecules, and there is no evidence for junctional transfer being anything other than dissipative diffusion (Loewenstein, 1981).

ceivably promote a critical phosphorylation step. The possibilities here are manyfold in a process that presumably involves accretion of channel precursor elements on the two membranes, as well as their interlinkage between the membranes (Loewenstein, Kanno & Socolar, 1978); and that, as preludes, must involve steps of precursor synthesis and of close membrane apposition, if not adhesion. cAMP might act at any of these stages, and the finding that its effect is blocked by puromycin and cycloheximide sheds no light in this regard; a protein synthesis requirement is consistent with an action at any of these stages, even with a proliferative mechanism involving the opening of pre-existing channels.

Does the particle proliferation reflect new gap junction formation or addition of particles to old gap junction? This question is not simply answered by the result of an increased gap junction frequency, because our criterion for gap junction was a minimum of 10 particles (Methods). But some information is gleaned from the frequency distribution of gap junction sizes. Earlier work had indicated that gap junction grows by a progressive increase in the number of membrane particles (Johnson et al., 1974), in agreement with the notion that permeable junction formation proceeds by an accretion of cell-to-cell channels (Ito, Sato & Loewenstein, 1974). In the present *control* conditions, we are presumably dealing mostly with old junction. This is what one would expect as the cell cultures were dense enough for extensive cell contact and had ample time for gap junction formation (they were left unpassaged for more than 24 hr before the experiments); and the fact that there were more large-size than small-size gap junctions in the 10-to-100 particle range in the controls (and that the size distribution shifted to larger sizes on protein synthesis inhibition (Table 7)) would seem to attest to this (Fig. 12). After the db-cAMP-caffeine treatment, the peak of the frequency distribution over that range shifted to the smaller gap junction sizes (at 4 hr) before the distribution eventually paralleled that of the controls (24 hr). This suggests that part of the particle proliferation, at least, represented new gap junction. This provides no decisive argument in the question of where in the junction formation process cAMP is acting, but it leads one to weigh the possibility of an effect at the stage of membrane apposition at least as heavily as that of an effect on the other three stages, particularly in view of its known cytoskeletic actions (*cf.* Dedman, Brinkley & Means, 1979). For instance, cAMP might promote critical membrane appositions and, as in one model of permeable junction genesis (Loewenstein, 1981), cell-to-cell channel formation would follow suit. The present cell types showed, at the light microscope level, none of the conspicuous shape changes after cyclic nucleotide treatment, common in transformed cell types; but there may have been changes in fine-scale cell approximation. Regions of membrane apposition $\leqq 10$ nm were, in fact, more frequent after the treatment, but since they were also invariably occupied by gap junction, this does not answer the question.[2]

The aforegoing interpretation of the results as a proliferation of cell-to-cell channels – and we include here a possible recruitment from a pre-existing pool of non-patent channels – was guided by the findings of a proliferation of gap junctional particles in the present cell types and the cAMP-stimulated emergence of such particles in the channel-deficient cell type described in the third paper of this series. However, a permeability change by an increase in the effective channel diameter is, of course, not excluded by the results and, indeed, could be envisioned as an additional or even an alternative mode of cAMP action (for example, by way of a cytoplasmic Ca^{2+}-mediated channel regulation, Loewenstein, 1981).

Physiological Roles

We do not know whether the cAMP junctional effect has a counterpart in real organs and tissues. This is a common problem, of course, in studies with cell cultures, but perhaps a more acute one here because of the possibility of a cAMP action on membrane apposition. Such an action would not be less interesting from the point of view of junctional genesis, but its plausibility raises the question whether the cAMP junctional effect is at all present in organs and tissues where cell-mechanical conditions may be more static than in culture. The fact that it was seen at any of the cell densities, even at relatively high ones (Table 3), and that it is not prevented by cytokinesis inhibitors (*see* paper III), and the electron microscopic findings in organized tissues mentioned below encourage us to think that the effect does occur in real life, and we discuss some general physiological possibilities.

In cell systems capable of making cell-to-cell channels, one may expect an increased junctional permeability to go hand in hand with conditions elevating the intracellular cAMP concentration, and the reverse. Consider, for instance, the action of hormones that activate adenylcyclase, elevating the cAMP concentration in their respective epithelial target cells.

[2] Regions of membrane apposition particle-free or particle-poor, such as described for certain other cell types, with forming gap junctions ("formation plaques," Johnson et al., 1974), if present at all, must have been rare. The present situation in this regard is similar to that in the gap junction proliferations of myometrium (Dahl & Berger, 1978) and regenerating liver (Yancey, Easter & Revel, 1979).

One would predict, as part of the cellular response, an augmentation of the junctional traffic of molecules – molecules involved in basic tissue homeostasis and perhaps of molecules specialized in intercellular signalling. Moreover, since cAMP itself, to judge by its molecular size and charge, should fit through the cell-to-cell channel (Schwartzmann et al., 1981) and may possibly spread from cell to cell (Tsien & Weingart, 1976; Lawrence, Beers & Gilula, 1978; Blalock & Stanton, 1980), there is potential for an interesting, subtle form of cellular response amplification where the gain is regulated by the magnitude or duration of the primary hormone signal (Loewenstein, 1981). This is merely one example of the several categories of cAMP-mediated cellular regulations; but given the ubiquity of cAMP and cell-to-cell channels in organs and tissues, it would be small wonder if the junctional cAMP effect had adapted to many regulatory functions.

There seems no point in speculating beyond these generalities at this time. But it is fitting to mention some morphological findings that hint at such a concomitant hormone action in vertebrate tissues. Gap junctions were shown to proliferate in thyroid hormone-stimulated ependymal epithelium (Decker, 1976). Although this electron-microscopic work did not tell about intracellular cAMP or junctional transfer, and it is not possible to infer that the gap junctional particles represented viable channels, a cAMP-mediated hormone action on junctional transmission seems a reasonable possibility in the light of the present results and of the knowledge that thyroxin causes elevation of cAMP concentration in other cell types. Moreover, after the announcement of the present results (Flagg-Newton, 1979), cAMP treatment was reported to produce increase of gap junction area in myometrium (Garfield, Merrett & Grover, 1980).

There are also indications of a junctional hormone action in tissues of arthropods, where the cell-to-cell channels have different size and permselectivity (Flagg-Newton & Loewenstein 1979; Schwartzmann et al., 1981): treatments of *Drosophila* salivary gland with ecdysterone and of *Tenebrio* epidermis with β-ecdysone produced increase of junctional electric conductance, effects that developed over several hours (Hax, van Venrooij & Vossenberg, 1974; Caveney & Blennerhassett, 1980). However, it is not known whether these junctional effects were associated with an elevation of the cellular cAMP concentration. Treatments with cAMP were reported to cause increase in the gland junctional conductance (simultaneous with a decrease in nonjunctional conductance) (Hax et al., 1974). But, the time course for this change (it set in within 5 min of db-cAMP application) would suggest mechanisms distinct from those in the vertebrate cells. In *Tenebrio* epidermis, the junctional effect of cAMP ran counter to that of β-ecdysone (Caveney, 1978).

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Appendix

Appendix-Table F3. Data detail for Fig. 3

Expt. #	Incidence (%) of LRB(Glu)$_2$OH-permeable junctions[a]	
	Control	4 hr
1	**13** (4:32; 5, 1)	**79** (41:52; 7, 1)
2	**12** (4:34; 5, 1)	**58** (18:31; 5, 1)
3	**12** (3:25; 4, 1)	**71** (29:41; 6, 1)
4	**20** (5:25; 4, 1)	**75** (27:36; 5, 1)
5	**27** (11:41; 6, 1)	**84** (38:45; 6, 1)
6	**35** (12:34; 5, 1)	**78** (32:41; 6, 1)
7	**35** (11:31; 4, 1)	**80** (35:44; 7, 1)

[a] In parentheses in the following order: the number of permeable first-order junctions, the number of first-order junctions, the number of microinjection trials, the number of culture dishes.

Appendix-Table F4. Data detail for Fig. 4

Time (hr)	Incidence of LRB(Glu)$_3$OH-permeable junctions (%)
0	**13** (12: 94; 10, 2)
1	**23** (18:78; 10, 2)
2	**44** (46:105; 15, 3)
3	**59** (61:104; 15, 3)
4	**72** (79:109; 15, 3)
24	**79** (103:130; 15, 4)
48	**74** (62: 84; 9, 2)

Appendix-Table F5. Data detail for Fig. 5

Fig.	Time (hr)	Incidence of LRB(Glu)$_2$OH-permeable junctions (%)
5I	0	**12** (3:25; 4, 1)
	1	**24** (16:67; 11, 2)
	2	**49** (36:74; 11, 2)
	3	**59** (20:34; 7, 1)
	4	**76** (55:72; 11, 2)
5II	0	**13** (4:32; 5, 1)
	3	**55** (28:51; 7, 1)
	4	**79** (41:52; 5, 1)
	5	**70** (31:44; 5, 1)
	6	**16** (6:37; 3, 1)
5III	0	**13** (6:46; 5, 1)
	3	**55** (27:49; 6, 2)
	4	**41** (18:44; 5, 1)

Experiments *5II* and *5III* are consecutive on parallel subcultures.

Appendix-Table F7. Data detail for Fig. 7

Time (hr)	Incidence of LRB(Glu)$_2$OH-permeable junctions (%)
0	**27** (11: 41; 10, 2)
3	**82** (53: 65; 14, 2)
4	**80** (65: 81; 14, 2)
5	**82** (60: 73; 14, 2)
6	**78** (29: 37; 8, 2)
24	**31** (51:162; 21, 4)
26	**63** (34: 54; 6, 1)
27	**63** (49: 78; 12, 2)
28	**48** (52:108; 15, 3)
29	**44** (46:104; 14, 2)
30	**30** (20: 67; 11, 2)

Cell density during the 0–6 hr period, 0.703 (10^4 cells/cm^2); during 24–30 hr period, 1.35–1.67 (10^4 cells/cm^2).

Appendix-Table F6. Data for Fig. 6

Time after start of the db-cAMP-caffeine treatment (hr)	LRB(Glu)$_2$OH-permeable first-order junctions									
	Single db-cAMP-caffeine application					Repeated db-cAMP-caffeine application				
	Incidence (%)[a]	Frequency[b]				Incidence (%)	Frequency			
		0	10–49	50–89	90–100		0	10–49	50–89	90–100
0	**40** (65:162; 23, 5)	4	6	13	0					
4	**77** (106:137; 21, 4)	0	1	12	8	**70** (143:205; 30, 6)	1	3	20	6
6	**67** (77:115; 17, 4)	0	3	13	1	**64** (95:149; 24, 5)	4	3	12	5

[a] [b] *See* footnotes to Table 2.