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Experimental Depression of Junctional Membrane Permeability in Mammalian Cell Culture. A Study with Tracer Molecules in the 300 to 800 Dalton Range

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Summary. Cell-to-cell junctional permeability in mammalian cell cultures was probed with a series of fluorescent tracers ranging 300 to 800 in molecular weight, during treatment with metabolic inhibitors, Ca-transporting ionophore, and carbon dioxide. Treatment with the combination of cyanide and iodoacetic acid (1–2 mM each), but not with either one alone, caused reversible junctional blockade to all tracer molecular species, large and small. (Electrical coupling, however, persisted in a proportion of the junctions tested.) Treatment with the ionophore A23187 (2–10 μM) or with CO_2 (an atmosphere of 100% CO_2 equilibrated with the medium) produced selective junctional blockade: transmission of a 688 and an 817-dalton tracer was generally blocked, while that of a 376-dalton tracer and, in certain conditions, that of a 559-dalton one, persisted. The junctional effect of the ionophore required the presence of Ca in the external medium; and effective junctional blockade by CO_2 required pretreatment in medium with high Ca concentration or, interchangeably, pretreatment in medium with high CO_2 concentration. In one cell type, prolonged exposure to medium with high Ca concentration alone sufficed to block transmission of the 688-dalton tracer. These effects are discussed in terms of the Ca hypothesis of junctional permeability regulation. In comparison with mammalian (or other vertebrate and invertebrate) organized tissues or with insect cell cultures, the mammalian cell cultures are more resistant to junctional blockade. This difference in transmission stability is discussed in terms of intracellular Ca-buffering capacities of the junctional locales; in particular, in terms of the electron-microscopic finding in the mammalian cultures of fine, bilateral cell processes connected by gap junctions.

The cell junctions in tissues and cultures are permeable to a range of molecules in the cell-to-cell direction (Loewenstein, 1966; 1975; Furshpan & Potter, 1968; Simpson, Rose & Loewenstein, 1977; Pitts, 1977; Flagg-Newton, Simpson & Loewenstein, 1979). This permeability can be experimentally depressed by intracellular injection of Ca or by treatments with Ca-transporting ionophore, metabolic inhibitors, or carbon

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dioxide (Loewenstein, Nakas & Socolar, 1967; Politoff, Socolar & Loewenstein, 1969; Rose & Loewenstein, 1971; 1975*b*; 1976; Ito, Sato & Loewenstein, 1974; De Mello, 1975; Délèze & Loewenstein, 1976; Gilula & Epstein, 1976; Peracchia & Dulhunty, 1976; Rose, Simpson & Loewenstein, 1977; Turin & Warner, 1977; Loewenstein, Kanno & Socolar, 1978*b*; Baux *et al.*, 1978; Rose & Rick, 1978). In all of these conditions, the cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) is elevated (Baker, Hodgkin & Ridgway, 1971; Rose & Loewenstein, 1976; Rose & Rick, 1978; Lea & Ashley, 1978), and in *Chironomus* salivary gland it has been shown that the permeability depression is associated with $[Ca^{2+}]_i$ elevation in the junctional region (Rose & Loewenstein, 1976; Rose & Rick, 1978).

The junctional permeability depression has been observed in invertebrate and vertebrate organized tissues and in insect cell culture: upon sufficient $[Ca^{2+}]_i$ elevation, the junctions seem to be blocked to all molecular species normally transmitted, including the small electrolytes. The junctions of cultured mammalian cells proved to be more resistant, at least to such complete blockade: 3T3 cells and Novikoff tumor cells treated with cyanide, dinitrophenol, or iodoacetic acid stayed electrically coupled, and the junctional effects of Ca-transporting ionophore were not separable from those of general cell deterioration (Gilula & Epstein, 1976; also D. Garrison & W. Loewenstein, *unpublished work*; M. Epstein & J. Sheridan, *personal communication of unpublished work*). Effects on the permeability of larger molecules were not studied in these mammalian cell cultures.

We look for such effects in the present work. We are prompted by the finding that the permeability depression in *Chironomus* salivary gland is selective for large molecules at moderate $[Ca^{2+}]_i$ elevations: with elevations in the range of $10^{-7} - \sim 5 \times 10^{-5}$ M, cell-cell transmission of large molecules is blocked while that of small ones continues; only with higher elevations is the transmission blocked to all molecules, large and small (Rose, Simpson & Loewenstein, 1977). Thus, it seemed possible that in the aforementioned mammalian cell experiments, the junctions underwent selective permeability changes. For example, the $[Ca^{2+}]_i$ elevation in the junctional locale might have been prevented from reaching the level for full blockade because of residual Ca-sequestering mechanisms in that locale.

We probe the junctions of a mammalian epithelioid and a mammalian fibroblastic cell line in culture with linear fluorescent-labelled molecules in the 300–800 dalton range during treatment with metabolic inhibitors, Ca-transporting ionophore, and CO_2. We find that the junctional per-

meability of the larger molecules in this range is, indeed, depressed and, in certain conditions, even that of the small ones.

Materials and Methods

Cell Types and Media

We used the rat epithelioid liver cell line RL (*see* Fentiman, Taylor-Papadimitriou & Stoker, 1976) and the rat fibroblast cell line B (Azarnia & Loewenstein, 1971). Both have normal growth patterns and their junctions have similar permeability properties (Flagg-Newton, Simpson & Loewenstein, 1979). For a comparison with insect cell cultures we used the fibroblast line AC-20 from the homopteran leafhopper *Agallia constricta* (Chiu & Black, 1967).

The mammalian cells were grown in Eagle-Dulbecco (high-glucose) medium (Vogt & Dulbecco, 1960) complemented with 10% fetal calf serum (*growth medium*), at 37 °C in an atmosphere of 5–10% CO_2 and air. The insect cells were grown in medium 199 plus Melnick's medium supplemented with 2 mM glutamine and 10% fetal calf serum, at room temperature in an air atmosphere. All cells were grown in plastic dishes (Falcon, 60 mm diameter) and were passaged in a Ca, Mg-free saline containing 0.5 g/liter trypsin and 0.2 g/liter EDTA.

The experiments with junctional permeability depressants were carried out in the growth medium or, over relatively short terms, in media of simpler composition. The composition of the media for the mammalian cultures is given in Table 1. Some comments follow.

Growth medium serum-free: To avoid foaming, we used it serum-free in the experiments in which CO_2 was bubbled through the medium reservoir.

Growth medium Ca-enriched: $CaCl_2$, variously ranging 6.8–30.0 mM, was added to the medium (Gibco, which was in powder form). There was some precipitation in the medium upon $CaCl_2$ addition. The Ca concentration in the supernatants, determined by atomic absorption spectrophotometry, were 6.5–25 mM. After solution in distilled water, the medium was ultrafiltered for sterilization. This medium was used for pretreatment in the CO_2 experiments before the permeability tests. The permeability tests themselves were done in regular growth medium (1.8 mM Ca), because the microelectrodes (*see* below) tended to clog in the Ca-enriched medium; besides, the incidence of permeable junctions was noticeably smaller in Ca-enriched medium. Perhaps the junctional permeability in some cases was then affected due to excessive Ca^{2+} influx at leaks around the microelectrodes.

Cyanide (CN) and *iodoacetic acid* (IAA) were dissolved in Ca,Mg-free Krebs-Ringer medium.

Ionophore A23187 was dissolved in dimethyl sulfoxide (DMSO), and the solution added to the Krebs-Ringer medium. The concentrations of the ionophore in the medium ranged 2–10 μM. The concentration of DMSO was 0.005–0.025%. DMSO alone had no discernible effect on junctional permeability at these concentrations.

CO_2 pretreatment and treatment. For pretreatment, the cells were exposed to an atmosphere of 50% CO_2 and air in the incubator. For treatment (during the permeability tests), 100% CO_2 was bubbled through the medium reservoir (*see* below).

Junctional Permeability Determinations

Junctional permeability was probed with the fluorescent molecules listed in Table 2. Their preparation and purification are described elsewhere (Simpson *et al.*, 1977; Socolar

Table 1. Salt composition of media (mM)

Medium	Used in experiments on	NaCl	KCl	$CaCl_2$	$MgCl_2$	$MgSO_4$	$NAHCO_3$	NA_2HPO_4	KH_2PO_4	(Glucose)
Saline, phosphate-buffered	pH_e	137.9	2.68	0.9	0.49			8.05	1.47	-
Krebs-Ringer	Ionophore A23187	121.5	4.9	2.6	-	1.21	21.2	-	1.21	-
Krebs-Ringer, Ca,Mg-free	Ionophore A23187, CN, IAA, CN-IAA	121.5	4.9	-	-	1.21	21.2	-	1.21	-
Krebs-Ringer, Ca-enriched	Ionophore	114.5	4.9	7.6	-	1.21	21.2	-	1.21	-
Growth medium Dulbecco-Eagle	CO_2	110	5.03	1.80	-	0.81	44	0.91	-	25
Growth medium Dulbecco-Eagle, Ca-enriched	CO_2	100	5.03	6.5–25.3[a]	-	0.81	44	0.91	-	25

The compositions listed are complete for the saline and Krebs-Ringer medium. The growth medium contains 10% fetal calf serum (except for certain conditions stated in Materials and Methods), $Fe(NO_3)_3$ 0.0024 mM, and other organic components; *see* Vogt & Dulbecco, 1960.

[a] Actual Ca concentrations in solution (*see* Materials and Methods).

Table 2. Permeability probes

Probe	Mol wt
6-Carboxy fluorescein	376
LRB	559
LRB (Glu) OH	688
LRB $(Glu)_2$ OH	817

LRB = Lissamine rhodamine B (or rhodamine B sulfonate). In previous publications from this laboratory, this molecule was denoted as LRB SO_3H. (Glu)OH = glutamic acid.

& Loewenstein, 1979). The molecules were iontophoresed into the cells with the aid of a microelectrode; the iontophoretic currents ranged 2.5–10 nA (Flagg-Newton *et al.*, 1979). To minimize Ca influx, the microelectrode was withdrawn as soon as enough of the fluorescent tracer was seen in the cell.

Usually 3 to 5 cells were microinjected in a given culture dish, each injection providing information about several junctions. A "junction" here simply signifies two cells in contact as seen in the phase-contrast microscope. We scored the proportion of permeable first-order junctions, by which we mean the percentage of fluorescent cells among cells contiguous to the injected one[1]. Higher-order junctions were not considered. The acceptance criterion for a positive score was a minimum of 2 fluorescent first-order neighbors, to make sure that the tracer had not passed between incompletely divided daughter cells. The soundness of this criterion was checked by injecting C-fluorescein together with the larger LRB$(Leu)_3$ $(Glu)_2OH$ (1,158 daltons) which is mammalian-junction impermeant (Flagg-Newton *et al.*, 1979); there was no instance of transfer of the large molecule through junctions scored C-fluorescein-transfer positive. The scores tabulated are cumulative, from several microinjections and often from several culture dishes in the same experimental conditions. For example, the score of 67% in Table 3, expt. *1*, represents the aggregate of several individual tests, such as the one shown in Fig. 2, in which 37 first-order junctions were permeable out of a total of 55. We also tabulated the frequency distributions of the scores from the individual microinjections. These are arranged in the intervals 10–49, 50–89, 90–100, and 0%. There were no scores in the 1–9% interval; at the present cell densities, the maximum number of first-order junctions was 12 for RL and 20 for B cells, and, as already mentioned, our minimum score criterion was 2.

The permeability determinations were performed on subconfluent cultures of densities of $1.7–2.5 \times 10^4$ cells/cm^2. The determinations were carried out in the culture dishes (outside the incubator) at 35–36 °C. While they were outside the incubator, the cells were superfused, at a rate of about 2 ml/min, with medium from a heated 100-ml reservoir. For the CO_2 treatments, the reservoir medium was bubbled with 100% CO_2. The flow of the medium was stopped from the moment of microelectrode insertion to its withdrawal. The pH in the culture dish, monitored periodically, was 7.4 in all experiments, unless stated otherwise.

Fluorescence quantitation. For determination of the rates of tracer loss from individual cells and tracer transjunctional transit times, we used a photodiode system devised by

[1] This scoring procedure presupposes that the tracer transfer between the contiguous cells is primarily via the direct junctional route rather than via a more circuitous one mediated by additional junction. Our justification for this assumption is the fact that the fluorescein intensity of the first-order neighbors was invariably greater than that of the second-order ones.

R. Rick, B. Rose and J. Gray in this laboratory. The system consists essentially of an array of sensitive, low-noise silicone photodiodes (10^{-7} ft-candle, threshold) and an amplifier stage (*see* Socolar & Loewenstein, 1979, for a description). The photodiode array was positioned selectively over a microscope-projected image of a single cell for fluorescence loss determination, or over the image of a cell pair for determination of junctional transit time. Except for a short initial phase, presumably dominated by photodecomposition, the fluorescence decay curves from individual cells could be fitted by a single exponential.

Freeze-fracture electronmicroscopy (by G. Dahl). The cells were freeze fractured on the dishes according to the technique of Pauli *et al.* (1977). Besides RL cultures, the human skin fibroblast strain LN, derived from a Lesch-Nyhan patient (Azarnia, Larsen & Loewenstein, 1974), was examined.

Results

Metabolic Inhibitors

The junctions of the RL and B cultures in normal growth medium have previously been found permeable to 6-carboxy-fluorescein (C-fluorescein), Lissamine rhodamine B (LRB) and Lissamine rhodamine B glutamate (LRB(Glu)OH) (Flagg-Newton *et al.*, 1979) (Table 2). In tests preliminary to the following experiments, we found them to stay permeable also in the simpler Krebs-Ringer medium or in Ca,Mg-free Krebs-Ringer medium for at least 50 min after substitution of the growth medium. We performed the experiments with metabolic inhibitors in the Ca,Mg-free Krebs-Ringer medium.

We treated the cells with Na-CN (CN; 2 or 3 mM) and with iodoacetic acid (IAA; 1 or 2 mM). The former blocks electron transfer in the respiratory chain and the latter inhibits glycolysis: either poison alone would be expected to leave a remnant of ATP synthesis. With either one, in treatments over periods ranging 7–50 min, a large proportion of the junctions tested continued to transfer the LRB(Glu)OH (and presumably also the smaller probes) (Fig. 1). While the overall incidence of LRB(Glu)OH-permeable junctions was little changed with CN, the frequency distribution of the incidence for the various trials suggests a permeability depression: the incidence of LRB(Glu)OH-transferring RL junctions was 0% in one-third of the trials, as against an incidence $> 50\%$ in all control trials (Table 3, frequency distribution). However, many junctions evidently escaped blockade in this condition.

When treated with the combination of CN and IAA (1 or 2 mM each), all RL cell junctions failed to transmit the LRB(Glu)OH and C-fluorescein, and the B cell junctions failed to transmit at least the LRB(Glu)OH (C-fluorescein-permeability was not tested). Figure 1 sum-

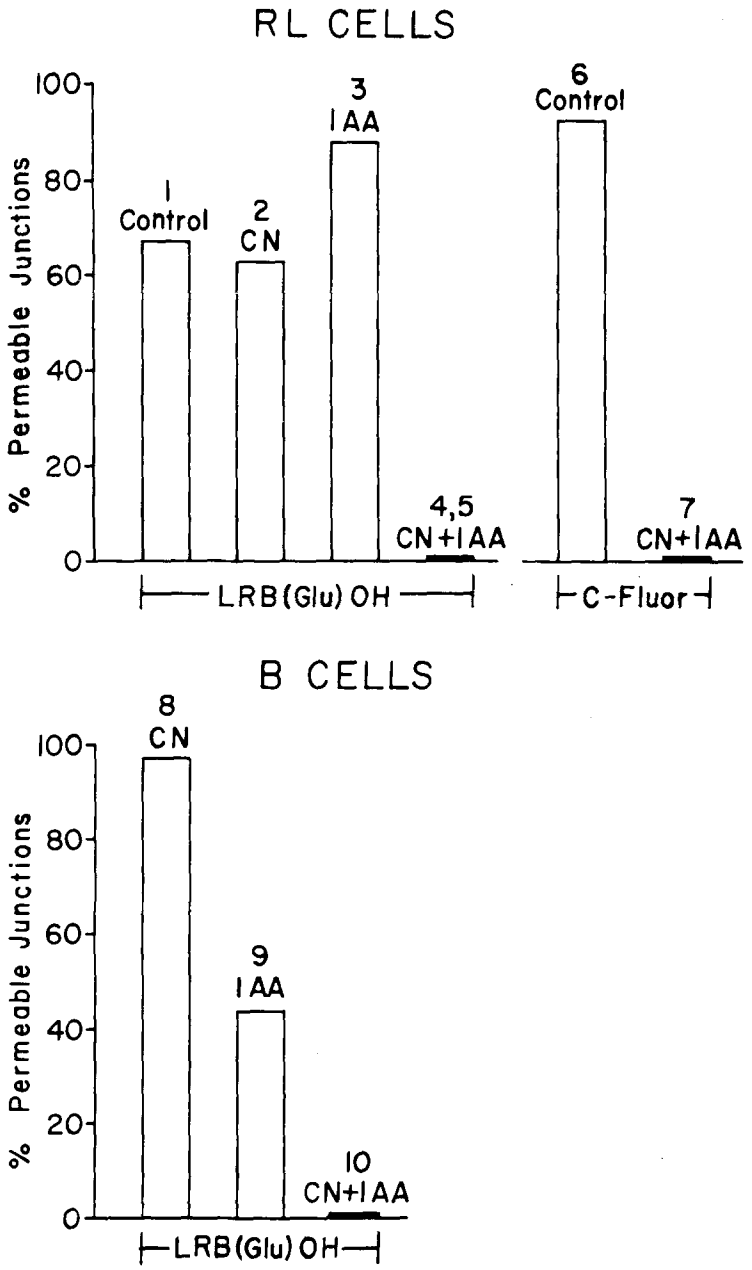


Fig. 1. Effect of metabolic inhibitors on junctional permeability. RL cell junctions are probed with LRB(Glu)OH (688 mol wt) (Expt. series 1–5) and 6-carboxy-fluorescein (376 mol wt) (series 6–7); B cell junctions are probed with LRB(Glu)OH. The graphs represent the proportion of permeable first-order junctions (cumulative scores from all tests) after treatment with 2–3 mM cyanide (CN), 1–2 mM iodoacetic acid (IAA), and both poisons combined (1–2 mM each). All experiments were in Ca,Mg-free Krebs-Ringer medium. The total treatment periods were 38–50 min; permeability tests (microinjections) were begun 6, 7 or 15 min after the start of this period and continued throughout it. Data detail in Table 3; the numbers on each bar graph identify the experiment series of the table

Table 3. Effect of metabolic inhibitors

Inhibitor			Permeable first-order junctions											
			RL cells							B cells				
			LRB(Glu)OH					C-Fluorescein		LRB(Glu)OH				
	Concentration (mM)	Exposure[a] (min)	Expt.		Frequency[c]			Expt.		Expt.		Frequency[c]		
			#	%[b]	0	10–49	50–89	90–100%	#	%[b]	#	%[b]	0	10–49	50–89	90–100%
–	0	–	1[e]	67 (37: 55; 5, 1)	0	0	4	1	6	93 (15: 16; 3, 1)						
CN	3	15–50	2	63 (67: 107; 12, 3)	4	0	3	5								
		7–40									8	96 (27: 28; 4, 1)	0	0	0	4
IAA	2	6–38	3	88 (42: 48; 6, 3)	0	0	1	5								
		7–40									9	43 (9: 21; 3, 1)	1	0	2	0
CN+IAA	1[d]	7–40	4	0 (0: 64; 6, 3)	6	0	0	0	7	0 (0: 21; 3, 1)	10	0 (0: 18; 3, 1)	3	0	0	0
	2[d]	7–40	5	0 (0: 34; 4, 4)	4	0	0	0								

marizes the results, Table 3 gives their detail, and Fig. 2 shows samples of tests of LRB(Glu)OH- and C-fluorescein-permeability.

The transmission block by CN-IAA was reversible. The recovery of junctional permeability was followed by testing the LRB(Glu)OH permeability, in parallel cultures, at 30, 60 and 80 min after washout of the poisons. By 30 min some junctions were permeable, and by 60 min the number of permeable junctions was comparable to that of the controls (Fig. 3).

Dr. R. Azarnia determined the effects of CN-IAA treatment (2 and 4 mM) on the electrical coupling in RL cultures, and kindly provided us with the results: within 30 min, the electrical coupling fell below detectable level in about half of the junctions tested, of cells that had retained high input resistances; but at least some degree of coupling persisted in the remainder of the junctions. With CN alone, all junctions tested stayed electrically coupled.

In these as well as all other experiments of this paper, the medium flowed over the cells (*see* Materials and Methods). This flow was important. In stagnant medium containing CN-IAA, fewer junctions showed permeability depression over the 30-min observation period. Presumably the hydrodynamic boundary layer, the thin unstirred liquid layer above the cells (*cf.* Stoker, 1973; Maroudas, 1974), limits the entry of the CN-IAA into the cells. (However, with CN alone, the junctions stayed electrically coupled regardless of medium flow.)

All permeability tests, including those showing full blockade, were performed on cells well attached to the dishes and to each other (*see*

[a] Range of exposure time to metabolic inhibitors. The first number is the time at which the first microinjection was made in a given culture dish; second number, that of the last microinjection.

[b] Incidence (%) of permeable first-order junctions, cumulative score of tests. In parenthesis, in the following order: the number of permeable first-order junctions (when >2 per test); the total number of first-order junctions; the number of test microinjections (trials); the number of culture dishes examined. All experiments in Ca,Mg-free Krebs-Ringer medium.

[c] Frequency distribution of the permeable-junction incidence. Tabulated are the number of individual trials for 0 incidence and for three incidence intervals. The interval 1–9% is unoccupied; the minimum scoring criterion was 2 permeable first-order junctions (*see* Materials and Methods) and the cell densities were such that the maximum number of first-order junctions was 12 for RL and 20 for B cells.

[d] CN and IAA each at that concentration.

[e] The general experimental conditions (superfusion of the cultures outside the incubator, temperature, etc.) were the same as those in experiments 2–5. Experiment 1 controls also for the time outside the incubator; the first of the 5 trials was done at 1 min and the last at 50 min, outside the incubator.

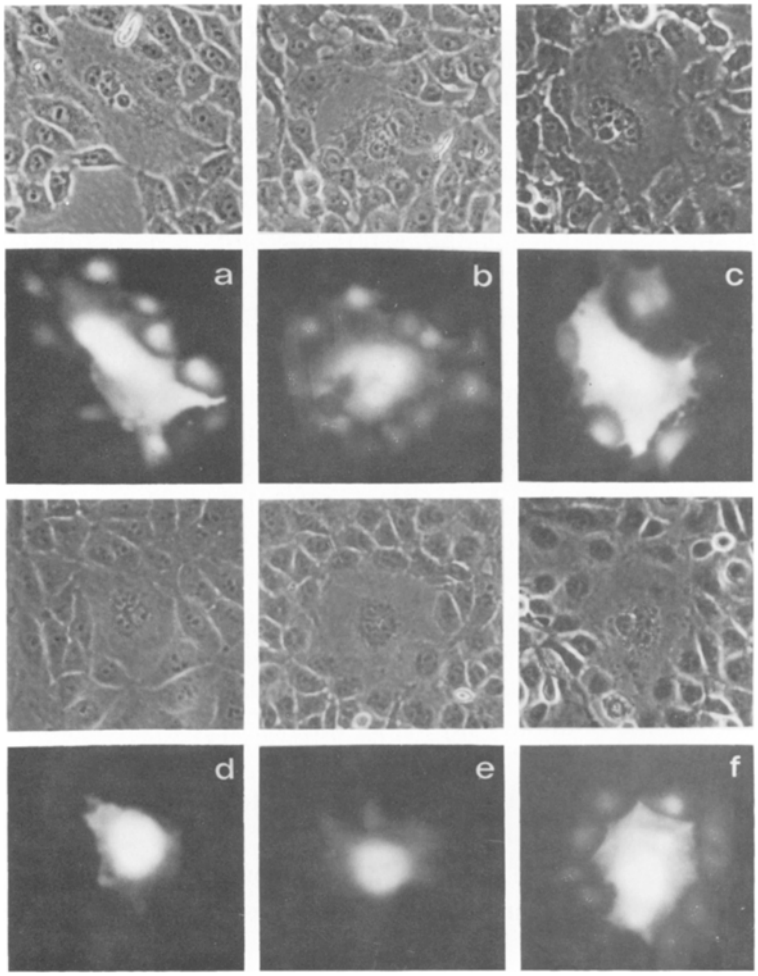


Fig. 2. Metabolic inhibition and cell-cell transfer. The darkfield photomicrographs were taken after microinjection of the fluorescent probe into an RL cell (the largest one in each RL cell cluster): (*a*) in Ca, Mg-free Krebs-Ringer (control) and in this medium plus (*b*) 3 mM CN, (*c*) 2 mM IAA, (*d* and *e*) 1 mM CN+IAA, (*f*) 60 min after washout of 1 mM CN-1 mM IAA in control medium. The cultures had been exposed to the poisons for: (*b*) 24 min, (*c*) 20 min, (*d*) 9 min, (*e* and *f*) 20 min. The probe is LRB(Glu)OH in *a, b, c, d, f*; and C-fluorescein in *e*. Figs. *b* and *c* show examples of cell junctions which remained permeable to LRB(Glu)OH in the presence of CN and IAA, respectively (*see* Fig. 1); *d, e* exemplify the junction behavior of CN+IAA poisoning. On top of the darkfield photographs are the same cell fields in phase contrast. Photographic exposure of darkfields, 10 min

Fig. 2). Cultures showing signs of detachment during the treatment were discarded. The controls and tests were done on parallel cultures (the same passages) of a stock, of the same density – and this applies also

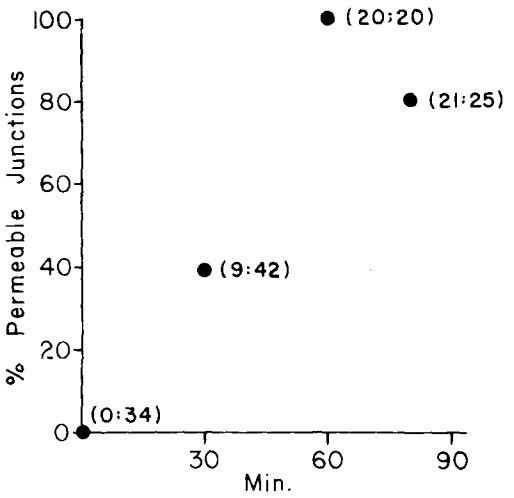


Fig. 3. Recovery of junctional permeability after CN-IAA poisoning. RL cultures were treated for 12–15 min with 2 mM CN + 2 mM IAA in Ca,Mg-free Krebs-Ringer medium, and their LRB(Glu)OH-junctional permeability was tested at the end of this period (time 0). The cultures were then washed and returned to the incubator for 30, 60 or 80 min, and the junctions were then tested. Permeability was tested on 4 parallel culture dishes (1 microinjection in each) at time 0 and on three of these cultures at the other times (1 culture for each time; several microinjections in each culture). The data points are cumulative scores; in parentheses, the number of permeable first-order junctions followed by the number of first-order junctions tested

to any given experiment series in the following sections. This was necessary; as we report elsewhere, the state and density of the cultures influence junctional permeability (Flagg-Newton & Loewenstein, 1979).

Ionophore A23187

The experiments with this Ca-transporting ionophore were performed on RL cells. The ionophore (2–10 μM) was applied in Krebs-Ringer medium, which contains 2.6 mM Ca, or in Krebs-Ringer medium, Ca,Mg-free. In a few experiments, the cells had been in Ca-enriched Krebs-Ringer medium (7.6 mM Ca), before the ionophore application.

In the Ca-containing media, most of the ionophore-treated cells failed, within 4–20 min, to transmit LRB(Glu)OH detectably to their neighbors (Figs. 4 and 5). Only 9% of the first-order junctions tested transmitted this molecule as against 94% in the controls.

In Ca,Mg-free medium, the ionophore had little effect. Most junctions then passed the LRB(Glu)OH (Fig. 4; Table 4). The present cells differ

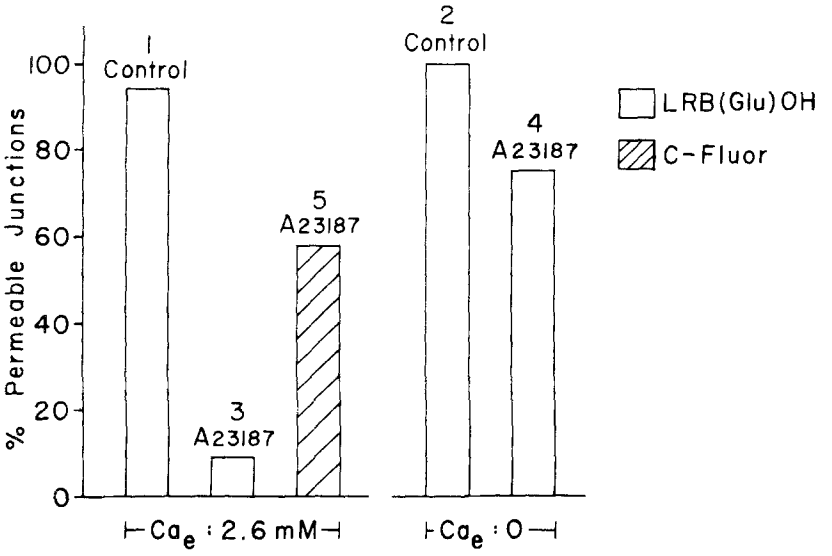


Fig. 4. Effect of ionophore A23187 on junctional permeability. Junctions of RL cell cultures are probed with LRB(Glu)OH (white bar graphs) and C-fluorescein (hatched). The graphs represent the proportion of permeable first-order junctions in Ca-containing Krebs-Ringer medium (2.6 mM, *control 1*) and after addition of 2–10 μM A23187 to that medium (*3*, *5*); in Ca,Mg-free Krebs-Ringer medium (*control 2*); and after addition of A23187 to this medium (*4*). Periods of exposure to A23187 ranged 4–20 min at the time of junctional testing. Most junctions are blocked to LRB(Glu)OH (*3*) but not to C-fluorescein (*5*) by the A23187 treatment in Ca-containing medium. As in all other experiments in this paper, all tests and control experiments were done on parallel cultures from the same passages. No control in this strict sense was done for experiment 5; in other experiment series with RL cells in Krebs-Ringer medium, the incidence of C-fluorescein-permeable first-order junctions ranged 75–94%. Data detail in Table 4

in this regard from *Chironomus* salivary gland cells where the ionophore produces junctional permeability depression, even complete transmission block, in the absence of Ca in the medium bathing the gland exterior (Rose & Loewenstein, 1976). However, in the latter case the gland lumen may still have contained Ca.

Our permeability tests covered a period of 4 to 20 min following ionophore application. During this period, the effect on junctional transmission seemed selective; the smaller C-fluorescein continued to be transmitted by a large proportion of the junctions (Fig. 4, *#5*).

During the test period (4–20 min), the cells looked normal in phase contrast, flat, well-attached to the dishes and to each other. Functional contact was evidently also retained since the junctions continued to pass C-fluorescein. Eventually and inexorably the cells deteriorated: they be-

Table 4. Effect of ionophore A23187. RL cells

Ionophore (μM)	$[Ca]_e$ (mM)	Permeable first-order junctions											
		LRB(Glu)OH							C-Fluorescein				
		Expt.		Frequency					Expt.		Frequency			
		#	%	0	10–49	50–89	90–100%		#	%	0	10–49	50–89	90–100%
0	2.1	1	94 (51: 54; 6, 2)	0	0	0	6							
0	0	2	100 (17: 17; 2, 1)	0	0	0	2							
2–10	2.1	3	9 (15:171;19,10)	16	1	2	0		5	58 (57:98;10,2)	2	1	5	2
2–10	0	4	75 (132:176;19, 9)	1	2	9	7							

Exposure times were 4–20 min; first microinjection at 4 min, last at 20 min.
Notation of permeable junction-incidence and related primary data were as in Table 3; *see* footnotes *b* and *c* to that table.

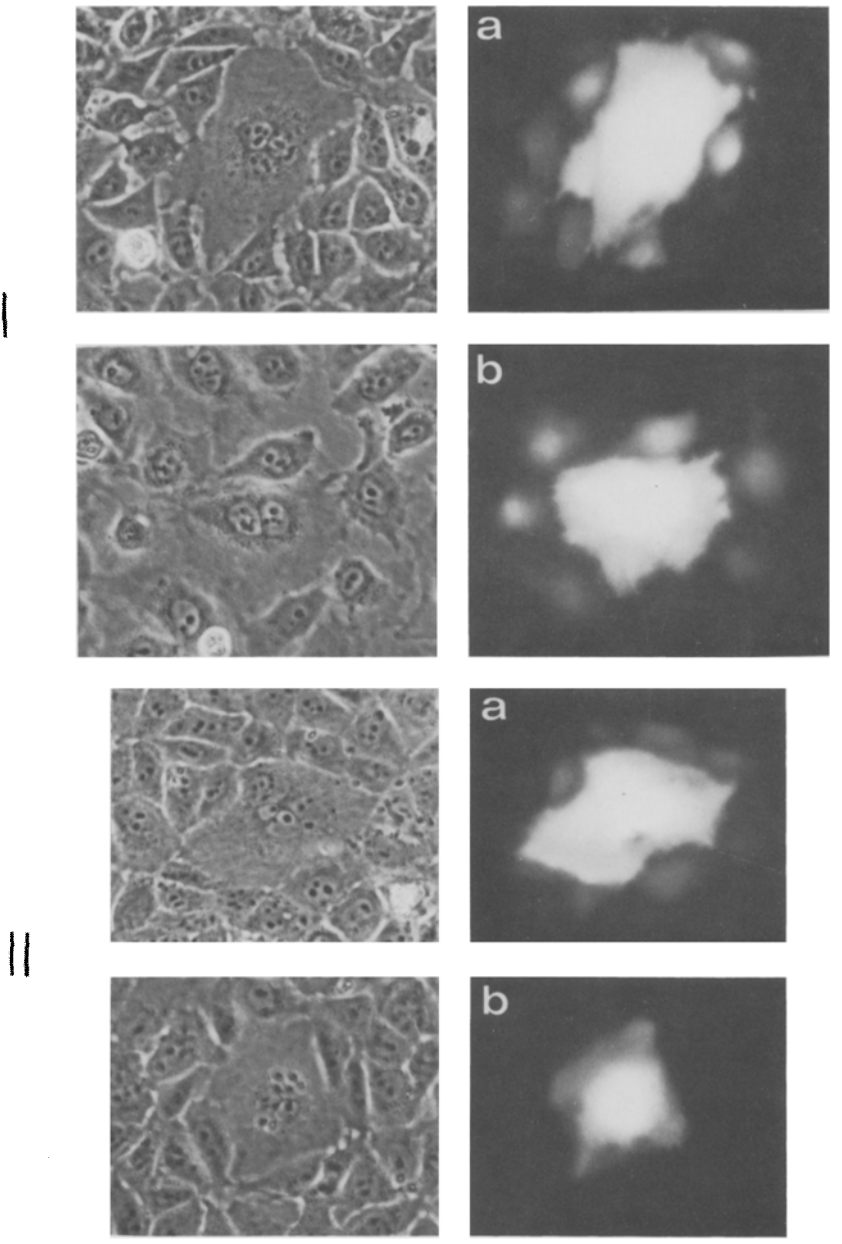


Fig. 5. Ionophore A23187 and cell-cell transfer. Darkfield photomicrographs after microinjection of LRB(Glu)OH into a cell (the largest) of an RL cluster. (*I*): Experiment in Ca,Mg-free Krebs-Ringer. (*II*): Experiment in Krebs-Ringer containing 2.6 mM Ca. (*a*): Control; the medium contains 0.025% DMSO. (*b*): After addition of 2 μM ionophore (and 0.025% of the solvent DMSO). At the time of the microinjection, the cells had been in the control or test medium for 22 min in *Ia* and *IIa*, 13 min in *Ib*, and 12 min in *IIb*. Phase contrast photomicrographs are at left

came granular, "rounded up," and detached from the dishes. The higher the ionophore concentration, the earlier the deterioration tended to set in. At the concentrations used (2–10 μM), the earliest signs of deterioration were seen 25 min after ionophore applications in Ca medium. This inevitably clouds our view of the mechanism of the junctional change. It may be noted, however, that the first permeability tests, which were completed by 4 min, gave essentially the same results as those completed by 20 min.

As in other cell cultures and tissues, the effects of the ionophore were not reversible by washout. However, the permselectivity shown here was analogous to that produced by the junctional effect of CO_2 which was reversible (*see* below). It was also analogous to the reversible junctional effects of direct Ca injection into *Chironomus* salivary gland cells (Rose *et al.*, 1977).

CO_2

Two kinds of experiments were done to test the effects of CO_2 on junctional permeability. In one kind, the cells were exposed, for periods ranging 1–70 min, to growth medium containing a high concentration of CO_2 (treatment). In another, the treatment was preceded by exposure, for 18–24 hr, to growth medium containing a high concentration of Ca (6.5–25 mM) and/or a high concentration of CO_2 (50%) (pretreatment)[2].

RL cells. The treatment by itself had no extreme effect on junctional permeability in RL cells: the frequency of LRB(Glu)OH-transferring junctions was reduced, but more than half of the junctions tested still transferred this tracer (Fig. 6; Table 5, expt. *3*). More pronounced uncoupling ensued when the cells were pretreated. Upon pretreatment with either a high [Ca] or a high [CO_2], followed by treatment with 100% CO_2, the incidence of LRB(Glu)OH-permeable junctions fell to a small fraction of that in the controls (expts. *4* and *5*), and all junctions were blocked when the Ca- and CO_2 elevations in the pretreatment were combined (expt. *6*; *see also* Fig. 7).[3]

The junctional block was reversible. After lowering of the [CO_2] in the medium to 5%, the incidence of LRB(Glu)OH-permeable junctions rose over the first 4 hr and reached a level comparable to control by

[2] The normal growth medium contained 1.8 mM Ca and was equilibrated with 5% CO_2.

[3] Shorter treatments (1–30 min) were less effective even when combined with pretreatment (expts. *7* and *8* of Fig. 6 and Table 5).

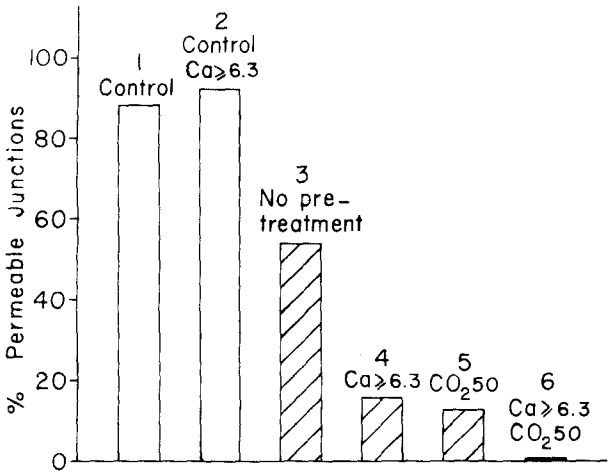


Fig. 6. Effect of CO_2 treatment on junctional permeability. RL cell junctions are probed with LRB(Glu)OH. The bar graphs represent the proportion of permeable first-order junctions in untreated control cultures (white) and in cultures treated with 100% CO_2 (hatched). The pretreatments with high [Ca] and/or [CO_2] are indicated on top. The treatments lasted for a total of 60–70 min; the permeability tests were begun 25 or 35 min after the start of the treatment and were then continued throughout the period of treatment. The pretreatment periods were 18–24 hr. Pretreatments were in growth medium; treatments (including the controls for equivalent period), in serum-free growth medium (*see* Materials and Methods). Data detail in Table 5

24 hr (Fig. 8). Nevertheless, the LRB(Glu)OH permeability seemed not yet quite normal at that time; the (first-order) transjunctional fluorescence intensity was, in many cases, weaker than in the controls.

The permeability depression seemed selective. In RL cultures, LRB and C-fluorescein continued to be passed by a high proportion of the junctions; in particular, C-fluorescein was passed by all junctions tested in conditions where the larger LRB(Glu)OH and LRB$(Glu)_2$OH failed to be passed by most (Fig. 9; Table 6). Even in conditions maximizing the depression, where all junctions failed to pass LRB(Glu)OH, still 75% of the junctions transferred C-fluorescein (expts. *6* and *7* of Fig. 9 and Table 6).

The 18–24 hr pretreatment by itself had variable effects on RL cultures. In some trials, we saw no major change in junctional permeability, even with the combined high [Ca] and 50% CO_2 pretreatment (not followed by treatment). In others, such pretreatment reduced the incidence of LRB(Glu)OH-permeable junctions to as low as 30%.

Table 5. RL cell junctional permeability to LRB(Glu)OH after CO_2 treatment at various exterior [Ca]

Expt. #	Pretreatment[e]	Treatment % CO_2	CO_2 duration[a] (min)	pH_e[d]	Permeable first-order junctions				
						Frequency[c]			
					%[b]	0	10–49	50–89	90–100%
1	–	–	–	7.4	88 (46: 52; 10, 3)	0	0	4	6
2	Ca 6.5–25.3 mM + CO_2 5%	–	–	7.4	92 (94: 102; 11, 5)	0	0	2	9
3	–	100	25–60 } 35–70 }	6.0	54 (50: 93; 11, 4)	3	1	5	2
4	Ca 6.5–14.6 mM + CO_2 5%	100	25–60 } 35–70 }	6.1	16 (15: 92; 13, 4)	9	2	1	1
5	Ca 1.8 mM + CO_2 50%	100	25–60	6.1	13 (16: 120; 14, 3)	10	2	2	0
6	Ca 6.5–25.3 mM + CO_2 50%	100	25–60 } 35–70 }	6.1	0 (0: 144; 15, 4)	15	0	0	0
7	Ca 14.6–18.8 mM + CO_2 50%	50	1–30	6.5	30 (82: 274; 29, 6)	14	6	7	2
8	Ca 6.5–25.3 mM + CO_2 50%	100	1–40	6.1	45 (68: 152; 20, 5)	9	2	5	2

[a b c] *See* footnotes to Table 3. [d] pH of medium. [e] Pretreatment durations: 18–24 hr.

No pretreatments in experiments 1 and 3, and no treatments in 1 and 2; the cells were in control medium containing 1.80 mM Ca, equilibrated with 5% CO_2.

The first of 3–4 microinjections in each culture dish were made at time 25 or 35 min (expt. #3–6) and 1 min (#7, 8) after treatment, and the last microinjections at 60 or 70 min (#3–6) and 30 and 40 min (#7, 8); in the controls (#1), the microinjections extended over the period of 25–70 min outside the incubator. Cultures in experiments 1 and 3 were passaged 24 hr before treatment; in experiment 5, immediately before pretreatment; in all other experiments, 48 hr before treatment.

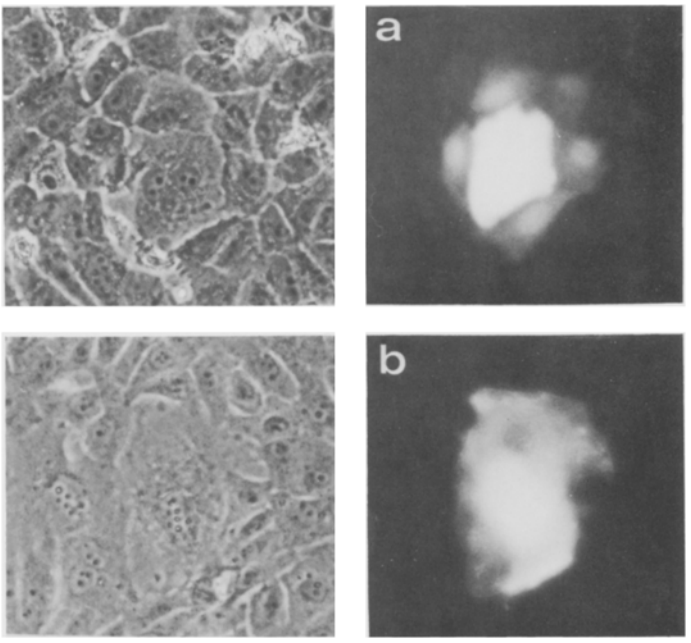


Fig. 7. (*b*): An example of blockade of cell-cell transfer from experiment series 6 of Fig. 6. The RL culture was pretreated, for 18 hr, in medium with [Ca] raised to 18 mM and [CO_2] raised to 50%, and then treated, for 30 min, in medium (1.8 mM Ca) equilibrated with 100% CO_2. (*a*): Same pretreatment, but no treatment (following the pretreatment the cells were for 25 min in medium containing 1.8 mM Ca, equilibrated with 5% CO_2). LRB(Glu)OH was injected into the largest cell of each cluster. The darkfield pictures in *a* and *b* show the LRB(Glu)OH fluorescence after injection of this probe

B cells. B cultures were more susceptible to junctional uncoupling. Here, interestingly, pretreatment with 6.5 mM Ca alone blocked LRB(Glu)OH permeability in all junctions. Furthermore, this pretreatment combined with a 100% CO_2 treatment caused marked depression of junctional permeability to LRB, in addition to depression of permeability to LRB(Glu)OH; with 15–60 min treatments, the incidence of LRB-permeable junctions fell to 0 (Fig. 10; Table 7).

Effects of external pH. Upon exposure to 100% CO_2 the medium pH fell from 7.4 to 6.1–6.0. Such acidification of the external medium by itself had little effect on junctional permeability: the incidence of permeable junction did not change significantly when the pH of the growth medium (exposed to 5% CO_2) was lowered to 6.0 by adjusting its bicarbonate buffer; and the change was relatively small upon similar

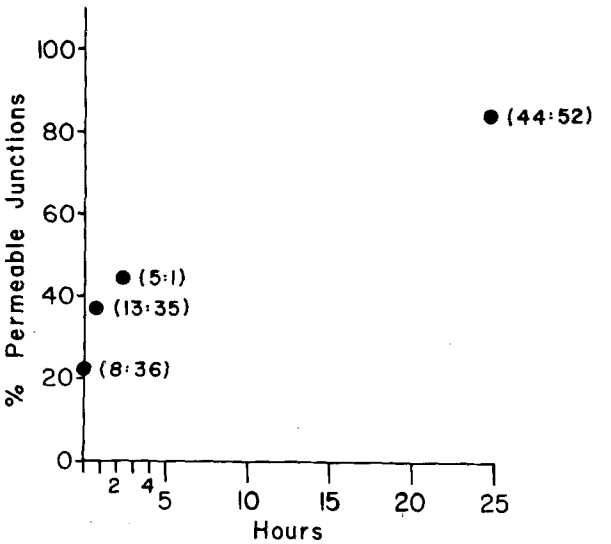


Fig. 8. Recovery of junctional permeability after CO_2-treatment. Four parallel RL cultures were pretreated, for 18 hr, in medium with raised [Ca] (15 mM) and raised [CO_2] (50%) and then treated, for 13–15 min, with medium (1.8 mM Ca) with 100% CO_2. At the end of the treatment (time=0), the LRB(Glu)OH-junctional permeability was tested in each culture (1 microinjection in each). The medium was then equilibrated with 5% CO_2 (in the incubator) and the junctional permeability tested at various times (in 1 culture for each time; several microinjections). In parentheses, the number of permeable first-order junctions followed by the number of first-order junctions tested

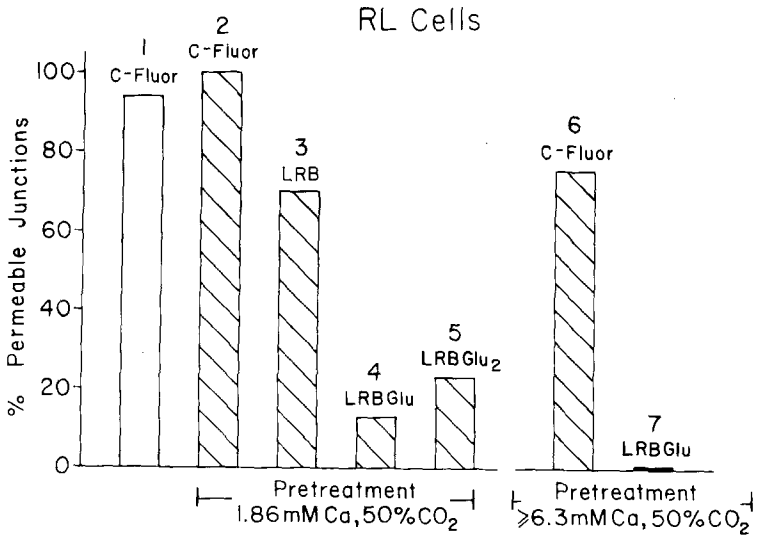


Fig. 9. Selective changes of junctional permeability by CO_2 treatment. RL cultures. Junctions are probed with C-fluorescein, LRB, LRB(Glu)OH, and LRB$(Glu)_2$OH. The cultures are treated, for 25–60 min, with 100% CO_2. They are also pretreated for 18–24 hr: one group of experiments in medium with 50% CO_2 and the other group in medium with [Ca] raised to $\geqq$6.3 mM, in addition. Bar graphs represent the percentage of first-order junctions permeable to the various probes. Control, white; treatments, hatched. Data detail in Table 6

Table 6. RL cell junctional permeability to various tracers after CO_2 treatment[a]

#	Probe	Mol wt	Pretreatment	Treatment	Permeable first-order junctions				
						Frequencies[c]			
					%[b]	0	10–49	50–89	90–100%
1	C-fluorescein	376	–	–	94 (15: 16; 2, 1)	0	0	0	5
2	C-fluorescein	376	Ca 1.8 mM, CO_2 50%	Ca 1.86 mM, CO_2 100%	100 (14: 14; 2, 1)	0	0	0	2
3	LRB	559	*id.*	*id.*	70 (21: 30; 7, 2)	3	1	2	1
4	LRB(Glu)OH	688	*id.*	*id.*	13 (16:120;14, 3)	10	2	2	0
5	LRB$(Glu)_2$OH	817	*id.*	*id.*	23 (38:167;17, 5)	8	5	3	1
6	C-fluorescein	376	Ca 6.5–25.3 mM, CO_2 50%	*id.*	75 (27: 36; 5, 2)	0	0	3	2
7	LRB(Glu)OH[a]	688	*id.*	*id.*	0 (0:144;15, 4)	15	0	0	0

Durations of pretreatment, 18–24 hr; of treatment 25–60 min.

[a] Experiments #4 and 6 of Table 5. [b] [c] *See* footnotes to Table 3.

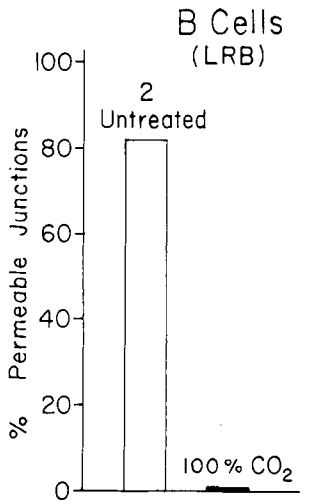


Fig. 10. Effects of CO_2 treatment on B cell junctions. The junctions are probed with LRB in untreated cultures (white bar graph) and in cultures treated, for 15–60 min, with 100% CO_2. In both series of experiments the cultures were pretreated, for 18–24 hr, with medium with [Ca] raised to 6.5 mM. Table 7 gives the data detail and also the results of an unpretreated control and those of shorter treatment periods

Table 7. B-cell junctional permeability to LRB after CO_2 treatment at high [Ca] in medium

Expt. #	Treatment[e]		pH_e	Permeable first-order junctions				
	CO_2 %	Duration[a] (min)		%[b]	Frequency[c]			
					0	10–49	50–89	90–100%
1	–	–[d]	7.4	93 (40:43; 4,1)	0	0	0	4
2	–	–[d]	7.4	82 (36:44; 6,2)	1	0	3	2
3	100	5–40 7–50	6.0	28 (24:87;14,3)	7	4	3	0
4	100	15–40 20–60	6.0	0 (0:25; 5,2)	5	0	0	0

[a b c] *See* footnotes to Table 3.

[d] This experiment also controls for the time outside the incubator; the first of the microinjections were started at 1 min, the last at 35 min.

[e] Pretreatment for 18–24 hr in medium with 6.5 mM, 5% CO_2 in experiments #2, 3, and 4. No pretreatment in experiment #1. Compare experiment #4 with #6 of Table 5.

lowering of the pH, by adjustment of the phosphate buffer in saline solution (Fig. 11). In this regard, the junctions of the mammalian cultures behave like those of organized tissues (Loewenstein *et al.*, 1967; Turin & Warner, 1977; Rose & Rick, 1978).

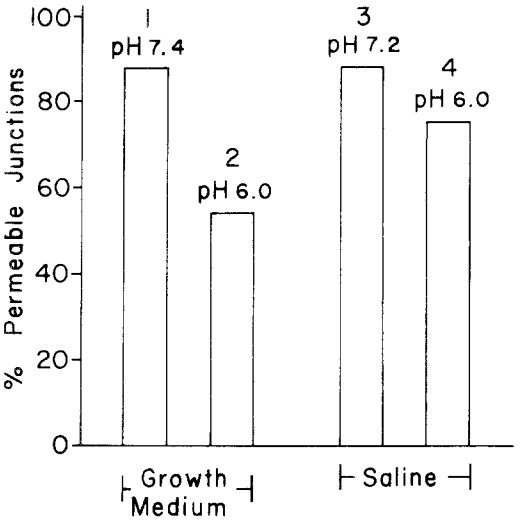


Fig. 11. Junctional permeability at low external pH. LRB(Glu)OH-permeability of RL cultures in growth medium buffered with $NaHCO_3$ to pH 7.4 (*1*) (the normal medium) and 6.0 (*2*); and in saline buffered with phosphate to 7.2 (*3*) and 6.0 (*4*). The numbers of permeable first-order junctions and of first-order junctions tested were: (*1*) 46:52 (same experiment series as in Fig. 6, *1*); (*2*) 50:93; (*3*) 23:26; (*4*) 46:61

Discussion

On the Junctional Uncoupling Mechanism

The present results show that treatments with metabolic inhibitors, Ca-transporting ionophore, or CO_2 produce depression of junctional permeability in mammalian cell cultures. Predominantly the permeabilities of the larger junction-permeant molecules are affected here, and for two of the treatments the permeability depressions depend on external Ca. It seems possible to account for these results in terms of the Ca hypothesis of junctional permeability regulation (Loewenstein, 1966; 1967). Thus, we will attempt to interpret them in the light of known mechanisms of cellular Ca metabolism, particularly in the light of the correlation between $[Ca^{2+}]_i$ and junctional permeability established earlier in this laboratory in cells of *Chironomus* salivary gland. In these large cells, the changes in $[Ca^{2+}]_i$ were directly monitored with the aid of aequorin during junctional permeability depression – a monitoring not feasible in the small, mammalian cells.

It is known that treatment with metabolic inhibitors, ionophore A23187, or CO_2 causes elevation of $[Ca^{2+}]_i$ in various cell types (*cf.* Baker, 1972; Loewenstein & Rose, 1978; Lea & Ashley, 1978; Rose & Rick, 1978). In *Chironomus* salivary gland cells, such $[Ca^{2+}]_i$ elevation is associated with depression of junctional permeability when the elevation occurs in the junctional locale (Rose & Loewenstein, 1976; Rose & Rick, 1978). The molecular size limit for junctional permeation decreases gradually with rising $[Ca^{2+}]_i$ in the range of 10^{-7} to $\sim 5 \times 10^{-5}$ M. At higher $[Ca^{2+}]_i$ the junctional pathway is blocked to all molecular species (*cf.* Loewenstein & Rose, 1978). The CO_2 treatment and, in certain conditions, $[Ca^{2+}]_i$ elevation on its own, causes also a decrease in cytoplasmic pH (pH_i) (Jacobs, 1920; Thomas, 1976; Boron & De Weer, 1976; Meech & Thomas, 1977). But a decrease in pH_i is not necessary for the depression of junctional permeability: $[Ca^{2+}]_i$ elevation in *Chironomus* salivary gland cells causes junctional permeability depression when pH_i is kept constant (buffered) and even when pH_i is substantially increased. Moreover, treatment with cyanide causes $[Ca^{2+}]_i$ elevation and junctional permeability depression without a significant pH_i decrease, and the ionophore A23187 can produce these effects associated actually with a slight pH_i increase (Rose & Rick, 1978). In sum, $[Ca^{2+}]_i$ elevation by itself seems sufficient for junctional permeability depression.

In this light, a simple interpretation of the present results is in terms of $[Ca^{2+}]_i$ elevation. The permeability depressions produced by the various treatments and the depression or facilitations of the depression by the pretreatments would all be due to $[Ca^{2+}]_i$ elevation in the junctional locale: elevations just sufficient to block junctional transmission of the larger probe molecules (Table 2) – in the case of the ionophore or CO_2 treatments –, higher elevations blocking transmission to all probe molecules – in the case of the cyanide-iodoacetate poisoning –, and subthreshold elevations (threshold in B cells) – in the case of the pretreatments in high-[Ca] medium. The heights of the $[Ca^{2+}]_i$ elevations will depend on how much the Ca influx, the efflux, and the Ca_i sequestering are affected by the various treatments and pretreatments. All of the treatments are, in fact, known to alter one or more of these Ca fluxes in such a way as to cause $[Ca^{2+}]_i$ elevation. The ionophore treatment increases the rate of Ca influx (*cf.* Baker, 1972); the CO_2 treatment reverses the vectors of Ca sequestering and causes Ca release (Rose & Rick, 1978), but it also decreases the Ca influx (Baker & Honerjäger, 1978); metabolic inhibition increases the Ca influx, decreases the efflux and the Ca_i sequestering (Baker, 1972; Brinley *et al.*, 1977); and a rise in external [Ca]

(pretreatment), within certain limits, causes an increase in the rate of Ca influx (*cf.* Baker, 1972) and thus perhaps increases the Ca load of the sequestering sites.

Alternatively, one may consider a less sweeping explanation singling out the CO_2-induced junctional effect. This treatment causes substantial internal acidification in cells in general, and so one may think of a direct effect of H^+ on junctional permeability, not mediated by Ca^{2+}. This is a possibility to be considered *a priori* in view of the long-known fact that H^+ competes with Ca^{2+} for binding on cell membranes (e.g., Carvalho, Sanui & Pace, 1963; Katchalsky, 1964; *see also* Politoff *et al.*, 1969, for a discussion on this point). There is also experimental evidence showing CO_2-induced intracellular acidification accompanied by junctional uncoupling (Turin & Warner, 1977; Rose & Rick, 1978; Spray, Harris & Bennett, 1979). But such acidification is also accompanied by $[Ca^{2+}]_i$ elevation (Rose & Rick, 1978). Moreover, it has been demonstrated that intracellular acidification actually causes $[Ca^{2+}]_i$ elevation; injection of H^+ into cells was shown, by use of the aequorin technique, to release Ca from intracellular stores (Rose & Rick, 1978). Thus, so long as there is no evidence that H^+ alone is sufficient for junctional uncoupling – evidence that is available for Ca^{2+} – the broader Ca hypothesis seems more attractive.

On Resistance to Junctional Uncoupling

The problem of the mechanism aside, the question needs to be answered, why are the mammalian cultures more resistant to junctional uncoupling than the organized tissues and insect cultures heretofore studied? The last two are uncoupled readily for all permeant molecular species by any one of the treatments used here, including the metabolic poisons applied singly (*see* Introduction). The mammalian cultures are for the most part uncoupled solely for the larger molecular species. The uncoupling is most complete with the cyanide-iodoacetate combination, effecting junctional blockade to molecules of our probe series as small as C-fluorescein (376 daltons). As far as the available evidence goes, this resistance to uncoupling seems *peculiar to mammalian cell cultures*, not to mammalian (or vertebrate) cells in general and not to cell culture in general – and, with respect to the mechanisms discussed in the preceding paragraph, it is worth emphasizing that the resistance applies to uncoupling induced by CO_2 as well as to uncoupling induced by metabolic inhibitors.

Is this peculiarity inherent in the junctional channels? The junctional channels of mammalian cultures are different from those of insect cells: they are narrower or more polar (Flagg-Newton *et al.*, 1979). But this channel property seems to be shared by mammalian organized tissues; tests with some of the present peptide probes on the junctions of rat heart (auricle) indicate a permeation limit as low as that in the mammalian cultures (J. Délèze, *personal communication*). There is also no evidence thus far leading us to believe that the permeability regulation mechanisms of vertebrate junctional channels in general are basically different from those of the insect channels which have been studied most extensively and directly in this respect. Both seem sensitive to Ca: the cells of insect tissue as well as vertebrate (and mollusk) tissues lose electrical coupling upon microinjection of Ca: *Chironomus* salivary gland (Loewenstein *et al.*, 1967; Rose & Loewenstein, 1975*b*; 1976), dog heart (DeMello, 1975), *Xenopus* embryo cell aggregates (Loewenstein, Kanno & Socolar, 1978*a*), *Navanax* neurons (Baux *et al.*, 1978). It seems therefore more likely that the resistance to uncoupling resides in an extrajunctional mechanism.

We consider the possibility that it resides in the buffering mechanisms of intracellular Ca. Here the mitochondria, endoplasmic reticulum, and certain cytoplasmic proteins are the best-known elements. The Ca sequestering by the first two is energized by ATP (Lehninger, Carafoli & Rossi, 1967; Poisner & Hava, 1970; Moore *et al.*, 1974; Bruns, McDonald & Jarrett, 1976; Hales *et al.*, 1974; Moore & Pastan, 1978; *see also* Hasselbach, 1974; Blaustein, Ratzlaff & Kendrick, 1978) and that by the last is high-capacity chemical binding (Wasserman & Corradino, 1973; *see also* Baker & Schlaepfer, 1975; Requena *et al.*, 1977). Such elements are present in organized tissues as well as in cell culture, but they are not necessarily evenly distributed or of equal power. Thus, in principle, it is possible to account for the greater resistance to uncoupling, if the mammalian cultures possess Ca sequestering mechanisms severely restricting the access of this ion to the junctional channels. In fact, there is direct evidence for such shielding in *Chironomus* salivary gland, where energized sequestering mechanisms can protect the junctional channels from substantial $[Ca^{2+}]_i$ elevations occurring only short distances away from the junctions (Rose & Loewenstein, 1975*a*; 1976). One would have to assume that the shielding is better in the mammalian cultures.

A shielding of this sort may conceivably result from (i) special, powerful Ca sequestering processes in the vicinity of the junctional channels;

(ii) special conditions of cell geometry where the channels are far enough away from sites of $[Ca^{2+}]_i$ elevation, with ordinary sequestering processes intervening; or (iii) a combination of the two. As far as the present evidence is concerned, we need to explain the resistance to uncoupling primarily for conditions where *intracellular* Ca stores are the sources of $[Ca^{2+}]_i$ elevation: The experiments with metabolic inhibitors were done in Ca-free media; and the CO_2 treatments, as discussed in the preceding section, may be expected to produce $[Ca^{2+}]_i$ elevation by a release of intracellular Ca. Thus, a shielding of type *ii* may perhaps suffice.

In this connection, electronmicrographs of the RL cultures, kindly provided to us by Dr. G. Dahl, reveal a suggestive feature. The cells commonly make junctions, namely gap junctions, at fine processes, cellular protrusions 0.05–0.1 μm in diameter and up to 10 μm long (Figs. 12–14). These kinds of gap-junctional protrusions have also been seen in other mammalian cell cultures (Dahl, Schudt & Gratzl, 1978; Lawrence *et al.*, 1979). They seem to be specializations of culture. They have not been seen in organized tissues and are not present in the insect AC-20 cultures, where the gap junctions are on "flat" contact surfaces (in mammalian cultures, they are on the flat surfaces in addition). The protrusions of the mammalian cultures are devoid of mitochondria (they are too thin) and hence devoid of major cyanide- and H^+-sensitive Ca release sites[4]. Dahl finds two types of gap-junctional protrusions, which are diagrammed in Fig. 15. Type *A* is of immediate interest to the point of discussion. Here the gap junction, on both cell sides, lies a good distance away from the main cell body, i.e., away from the nearest mitochondrion[5]. Thus, without our assuming anything beyond the ordi-

[4] Mitochondria release Ca in the presence of cyanide (Chance, 1965; *cf.* Lehninger, 1970) or low pH (Åkerman, 1978; Åkerman & Saris, 1978). On the other hand, the ATP-dependent Ca sequestering by endoplasmic reticulum of mammalian cell cultures is quite insensitive to blocking agents of mitochondrial Ca uptake; the endoplasmic reticulum loses its Ca-holding capacity only upon ATP depletion which requires block of glycolysis in addition to block of electron transfer (Moore & Pastan, 1977). We have not determined the ATP levels in our cells. M. Epstein and J. Sheridan (*personal communication*) found an ATP remnant of 20% in Novikoff hepatoma cell cultures with established junctions after a 1-hr exposure to 1 mM iodoacetate, with persistent electrical coupling (*see also* Epstein, Sheridan & Johnson, 1977, for action of iodoacetate on forming junction).

[5] A gap junctional arrangement on a one-sided protrusion, the other type of protrusive gap-junctional interaction present in mammalian culture (type B, Fig. 12), would not offer adequate shielding, since Ca^{2+} access to one side of the junction suffices for uncoupling (Oliveira-Castro & Loewenstein, 1971; Rose & Loewenstein, 1976). A type-B interaction would be as prone to uncoupling as a gap-junctional interaction on "flat" membrane presumably the common situation in organized tissues.

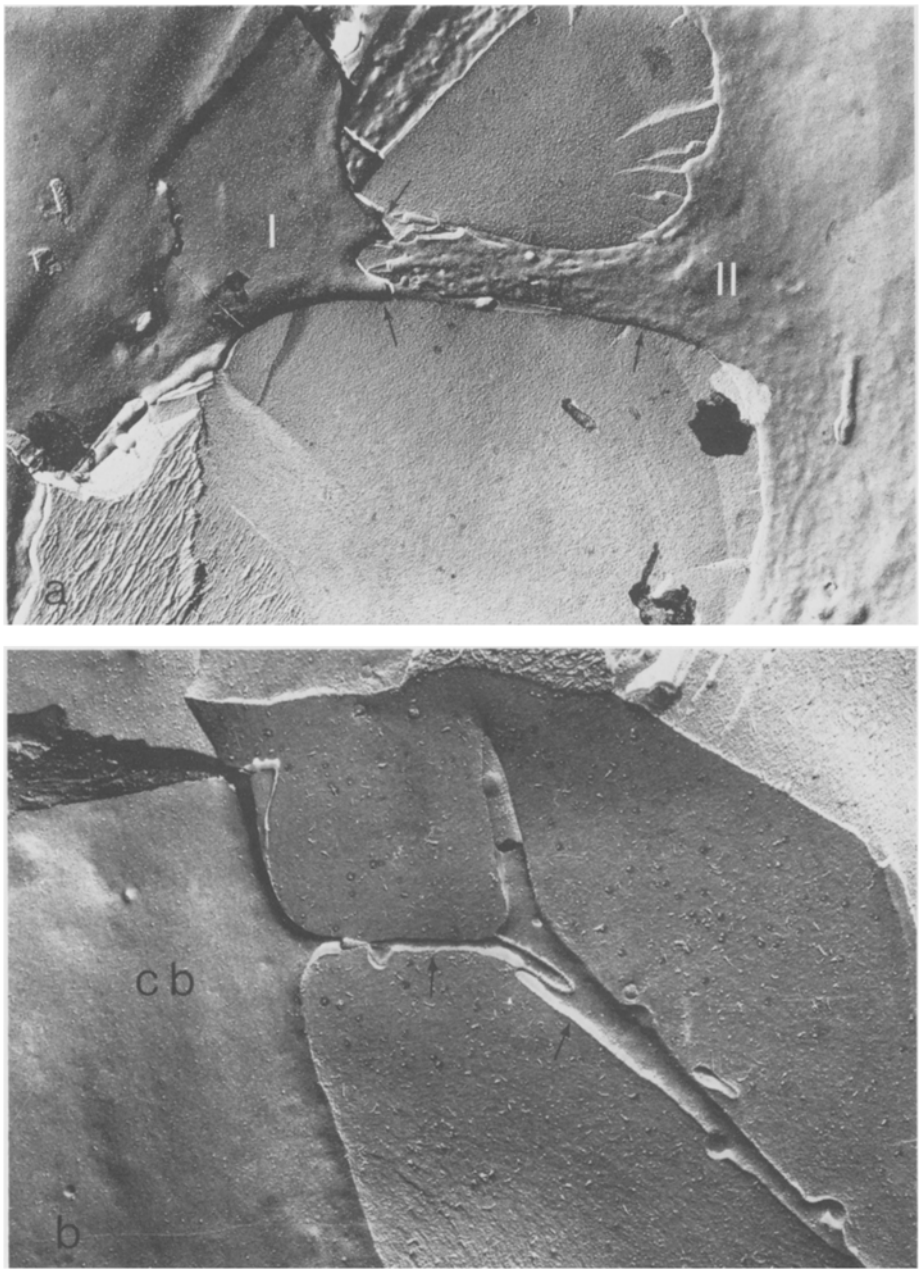
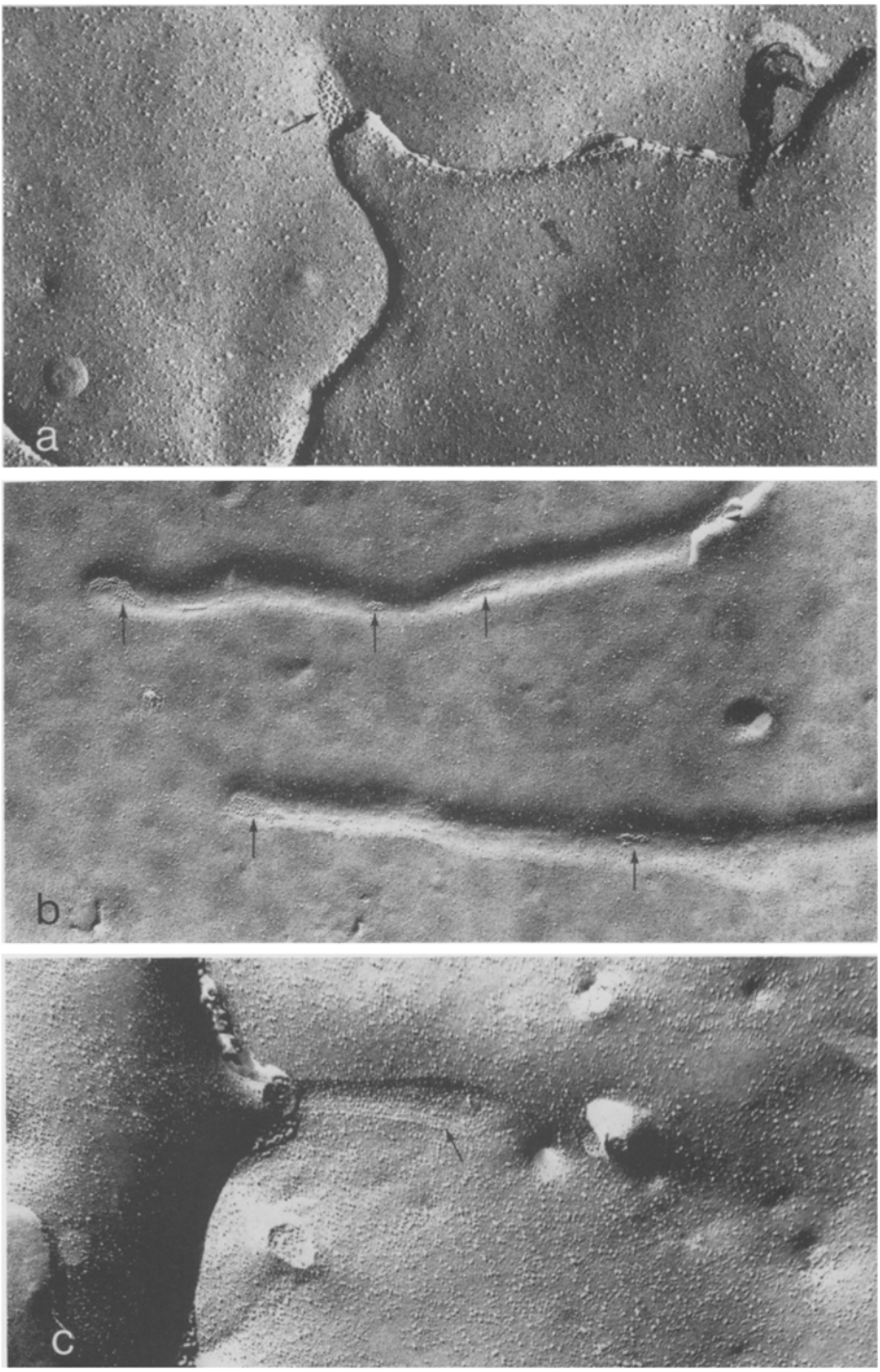


Fig. 12. Electromicrographs by G. Dahl of freeze-fractured mammalian cell cultures: *a*, human fibroblastic LN cells; *b*, rat epithelioid RL cells. Cell-cell contacts in these cultures occur between cell bodies, between cell body and cell processes, and between two processes of neighboring cells. Illustrated here are contacts of the latter kind. In *a*, the processes (arrows) of cell *I* originate from narrow bases, and the process of cell *II*, from a broad base. In *b*, interaction of two thin processes (arrows), one is seen in continuity with the cell body (*cb*). Magnification: (*a*) 12,300×; (*b*) 30,000×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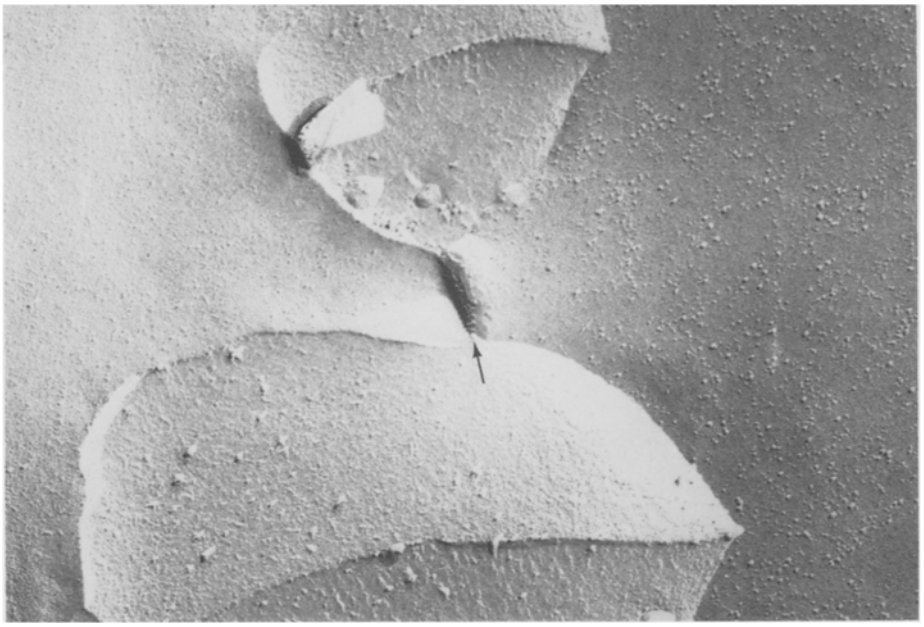

Fig. 14. A process-process contact between two RL cells. A gap junction (arrow) links the tips of the two cell processes. Magnification: 60,000×. Electronmicrographs from G. Dahl

nary cellular properties, the protrusions would seem to offer the means for junctional shielding: the Ca sequestering by their endoplasmic reticulum and the Ca outward transport through their plasma membrane could keep the $[Ca^{2+}]_i$ level low relative to that in the main cell body so long as there is ATP to energize them. In addition, there would be shielding contributed by nonenergized Ca sequestering by protein and Ca outflux by ion exchange with the medium. Neither a blocker of electron transfer alone, such as cyanide, nor a blocker of glycolysis alone, such as iodoacetate, would thus suffice for cutting off junctional transmission of all molecular species.

Fig. 13. Cell body-cell process contacts in mammalian RL (*a*), mammalian LN (*b*) and insect AC-20 cell cultures (*c*). The mammalian cell contacts are studded with gap junctions (arrows) (*a*, *b*); the insect cell contacts have septate junctions (arrow) instead (*c*). (In the insect cells, gap junctions, however, are present in body-body contacts; not shown.) The areas of the body-process contacts can be very extensive, as in *b*, where the fingerprint of a process exhibits several groups of gap junctional particles. Magnification: (*a*) 49,500×; (*b*) 30,000×; (*c*) 40,500×. Electronmicrograph from G. Dahl

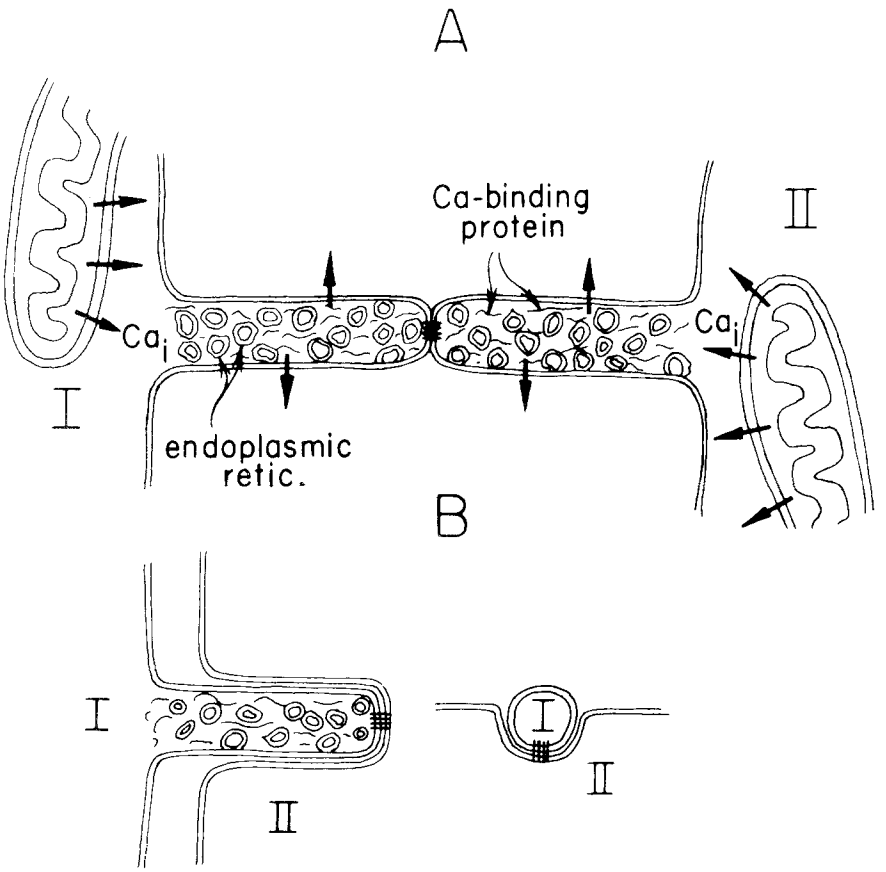

Fig. 15. Schematic representation of a hypothetical mechanism for shielding of cell-cell channels from $[Ca^{2+}]_i$ elevations. Diagrammed are the two types of protrusive gap-junctional interactions found by G. Dahl in the mammalian cell cultures. In the type-*A* interaction, Ca sequestering by endoplasmic reticulum (energized) and by cytoplasmic protein (non-energized), and Ca extrusion (energized and ion exchange; thick arrows) would maintain a low $[Ca^{2+}]_i$ in the gap-junction locale relative to that in the main cell body where the mitochondria release Ca (arrows). In the type-*B* interaction, the channels would be, on one side, more accessible to the cell-body Ca^{2+}, a situation conferring no more protection than a nonprotrusive gap-junctional interaction where channels are accessible on both sides

This tentative picture is predicated on the assumption that the gap junction contains the cell-cell channels. It would be invalid, at least in the simple form presented, if the septate junction contained such (Ca-sensitive) channels, too; for similar protrusions studded with septate junctions are found in insect AC-20 cultures (Fig. 12), and these are the only kind of protrusive junctional arrangement in these cultures (the gap junctions are on the "flat" cell surfaces here). The junctional

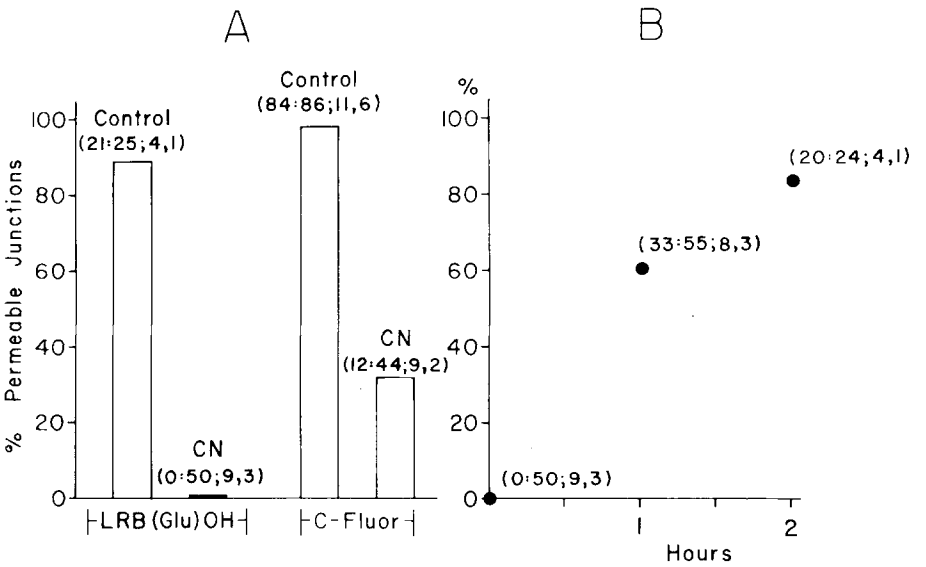

Fig. 16. Effect of cyanide on insect AC-20 cell cultures. (*A*): Graphs represent the proportion of first-order junctions permeable to LRB(Glu)OH and C-fluorescein before and after 25–60 min exposure to 1 mM CN in the cells' growth medium. (*B*): Time course of recovery of LRB(Glu)OH-permeability after CN washout (time=0). In parentheses in the following order: the number of permeable first-order junctions, the number of first-order junctions, the number of microinjections, and the number of culture dishes tested

permeability to LRB(Glu)OH and C-fluorescein of these cultures are readily and reversibly depressed by cyanide alone, as shown by the experiment in Fig. 16, and they can become uncoupled electrically (Gilula & Epstein, 1976).

The specially obdurate resistance to uncoupling of the small electrolyte transmission in mammalian cells may be accommodated by this general explanation: blockade of this transmission requires the highest $[Ca^{2+}]_i$ elevation (Rose *et al.*, 1977). But one cannot help being impressed by the high proportion of the junctions that continue to be electrically coupled after 30 min of metabolic poisoning. In conjunction with the result that the large-molecule transmission is uncoupled, this makes one wonder whether there might not be in the mammalian cells a second class of junctional channels, narrower and less Ca-sensitive (or better protected from Ca), in addition to the channels transmitting the larger molecules. The possibility of a dual junctional pathway for the small electrolytes is raised also by the finding of certain clones of mammalian cell hybrids between communication-competent and communication-incompetent cells: the junctions of the competent parent cell transmit the small electrolytes and larger molecules; the junctions of the incompe-

tent parent cell transmit neither class of molecules; and the junctions of the hybrid clones in question transmit only the small electrolytes (Azarnia *et al.*, 1974; Azarnia & Loewenstein, 1977). This intermediate expression of transmission phenotype may reflect the presence of aberrant channels that are products of an incomplete genetic dominance of the competent genome in the hybrids. But it might also reflect segregation of two channel classes. Interestingly, these hybrid clones express also a phenotype intermediate in junctional structure. Their predominant differentiation is a junction of the *occludens* type, namely intramembranous strands; the competent parent cell expresses typical gap-junctional particle aggregates plus the strands, whereas the incompetent parent cell expresses neither (Larsen, Azarnia & Loewenstein, 1977; Loewenstein, 1979*b*).

On the Selectivity of Uncoupling

In the experiments with the ionophore (Table 4) and, particularly, with CO_2 (Table 6) the cell-to-cell transfer of LRB(Glu)OH was depressed more than that of the smaller C-fluorescein. The treatments did not seem to have major effects on the (outward) nonjunctional membrane permeability of these tracer molecules; the rates of fluorescence loss were not significantly changed. This point was ascertained by injecting single cells (not in contact with other cells) with the tracer molecules and determining the rates of fluorescence loss with the aid of a photodiode system onto which the cells were imaged through the microscope (*see* Materials and Methods). As in the normal, untreated conditions, the half times of fluorescence loss were for all tracers >30 min, while the transjunctional transit times of the tracers were <20 sec. Thus, the effects on junctional transmission seem permselective according to size or charge of the probe molecules – resembling the selective permeability depression produced by experimental elevation of $[Ca^{2+}]_i$ in *Chironomus* salivary gland cells. In the latter, several molecular size-dependent permeability gradations were noticeable during $[Ca^{2+}]_i$ elevation, showing that the effective bore size of the cell-cell channels is gradually reduced or their fixed charge is gradually changed. In the mammalian cells, which have normally more restrictive channels to start with (Flagg-Newton *et al.*, 1979), only one gradation was distinguished with the more limited series of probes here[6]; this leads us to surmise that these channels can undergo such graded changes, too.

[6] The present probe series is a subset of the series used in the *Chironomus* cell experiments.

The fact that the transmission of the small electrolytes (electrical coupling) is the last to go (if at all) in the various treatments, is consistent with this notion; a gradual reduction in the bore of the channels or a gradual increase in their polarity would be less limiting for the inorganic electrolytes than for the much larger probe molecules. However, on its own this fact provides no basis for inferring selectivity; electrical coupling is a nonlinear function of junctional permeability, and the sensitivities of the methods for probing changes in electrical coupling and molecular permeability are unequal (*see* Délèze & Loewenstein (1976) and Socolar (1977) for further discussion of this point).

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