Asymmetrically Permeable Membrane Channels in Cell Junction

Abstract. *Asymmetric membrane junctions were formed in culture by pairing two cell types which, in their respective homologous junctions, have cell-cell channels of different permselectivities. The channels in the asymmetric junction, presumably made of unequal channel precursors, displayed directional permselectivity; fluorescent labeled glutamic acid (700 daltons), but not smaller and less polar permeant molecules, traversed the junction more readily in one direction than in the other. The favored direction was the one where the permeant passed first through the cell membrane that would have the less restrictive channels in a homologous junction. This directional selectivity requires no electric field across the junction and is thus distinct from a rectifying junction. The physiological potential of such directional molecular sieving for partitioning communication between tissue cells of different function and developmental fate are discussed.*

Cells of tissues and cultures commonly form junctions that provide a path for direct diffusion from cell to cell (*1-4*). The junctional elements in this kind of cellular communication are large, specialized membrane channels linking one cell interior to the other (*1, 5*). The channels are detectable as quantal steps of cell-cell conductance in the nascent junction (*6*). They form between cells of the same kind (homologous junction) and frequently also between cells of different kinds (*4, 7*). In mammalian cell junction, they show polar permselective properties against negatively charged, 14-Å-wide molecules (*8*). The channels in a homologous junction seem to pass small inorganic electrolytes in one direction as well as in the other, and there are no grounds for suspecting that they are less symmetric with regard to larger permeant molecules (*4*). For this reason and others concerning junction formation and disjunction (*9*), the channel is envisioned as made of two identical parts, the protochannels, one contributed by each membrane (*1, 6*).

But is permeability symmetry also preserved in junctions between cells of different types? Asymmetry in the form of junctional rectification—that is, asymmetry of junctional current with respect to the direction of the electric field—is well known to occur in certain electrical nerve synapses. The asymmetry there is manifested in response to an electric gradient (*10*), and one would not expect to see it in ion fluxes driven by chemical gradients alone. There are, however, no significant electric gradients in cell junctions of many electrically inexcitable tissues.

The question remains whether heterologous junctions can display asymmetric permeability under chemical gradients alone. Cell heterology, even when it entails structural asymmetry of cell-cell channels, need not give rise to permeability asymmetry for all permeants; deviation from permeability symmetry requires interaction between permeant and channel. Thus, with a polar channel, one has a good chance to detect asymmetry by probing with charged molecules of a size close to that of the channel bore. This is the approach we take here. We induce junction between two cell types with (homologous junctional) channels of different permselectivities, and probe this hybrid membrane pair with a series of charged tracers of different sizes, the largest one approaching the permeation limit of the (homologous junctional) membrane that has the more restrictive channels. In terms of the model mentioned above, this would

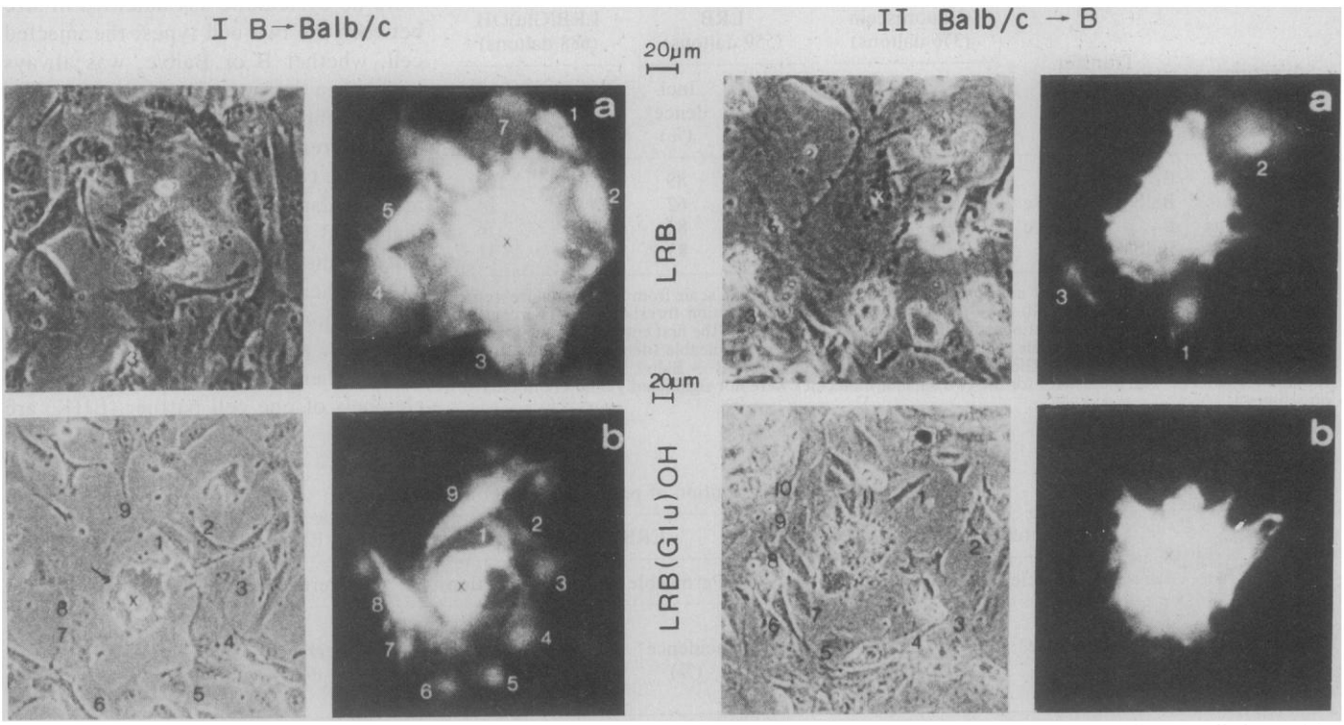

Fig. 1. Asymmetrically permeable B-Balb/c junctions. (I) Transfer of tracers from B to Balb/c cells. The B cell (*x*, in two different cell clusters) is injected with (a) LRB (559 daltons) and (b) LRB(Glu)OH (688 daltons). The tracers are seen in the dark-field photomicrographs to have passed to all first-order Balb/c neighbors [1 to 7 in (a) and 1 to 9 in (b)]. The B cells are labeled with latex particles, which the cells concentrate in the perinuclear region (arrows in the phase-contrast micrographs). All first-order neighbors here are Balb/c cells. (II) Transfer of tracers from Balb/c to B cells. The injected cell (x) is again the largest cell in the cluster. In (a) the LRB has passed to all three first-order neighbor B cells (1 to 3, latex-labeled), but note their relatively low fluorescence. In (b) the larger LRB(Glu)OH failed to pass detectably to any neighbor B cells (1 to 11). (The fluorescent spots at 1 and 6 o'clock are autofluorescent debris, visible as dark spots in the phase-contrast micrograph.) The Balb/c cells here are latex-labeled. All first-order neighbors are B cells (the nearest Balb/c neighbor is in contact with B cells 5 and 4, not with cell x). Calibration bars, 20 μm (different for top and bottom rows).

0036-8075/80/0215-0771$00.50/0

mean building a channel with dissimilar protochannels, namely, a channel more restrictive on one side than on the other. We find that the large-molecule permeability is, indeed, asymmetric in these conditions.

We used mammalian cell types Balb/c-3T3 and B in culture. The homologous junctional channels of the former are narrower or more polar than those of the latter (*8*). The two cell types were mixed and allowed to establish junction (*11*). The junctions were probed with 6-carboxyfluorescein (C-fluorescein, 376 daltons), lissamine rhodamine B (LRB, 559 daltons), and LRB glutamate [LRB-(Glu)OH, 688 daltons]. These fluorescent tracers are known to permeate the homologous junctional channels of both cell types, but while LRB(Glu)OH, the largest and most charged tracer molecule (with two negative amino acid charges) passes readily through the B-cell channels, it passes only marginally through the more restrictive Balb/c channels (the other two molecules pass readily through both) (*8*). The tracers were microinjected into the cells, and their spread inside the cells and from cell to cell was observed in a microscope dark field and photographed. To maximize sensitivity, we chose small cell groupings where the injected cell was the largest one and made the fluorescence in the injected cell (roughly matched in the various trials) much greater than the detection threshold. Cell-cell transfer was indexed by the peak fluorescence intensity of the first-order neighbors on a scale, from + (fluorescence of first-order neighbors just above detection threshold) to ++++ (first-order neighbor fluorescence approaching that of the injected cell) (*12*). We also scored the incidence of permeable first-order junctions—that is, the proportion of fluorescent cells among cells contiguous to the injected one. Higher order junctions were not considered (*13*).

The basic experiment was to inject a cell with a tracer and to determine its passage through the junctions (Fig. 1). The results are summarized in Table 1. Within the resolution of our method, C-fluorescein or LRB passed in either direction equally well across heterologous junction; the fluorescence intensity of the first-order neighbors and the incidence of permeable junctions were similar both ways. The cells were also symmetrically coupled electrically (*14*). However, the passage of the larger LRB(Glu)OH was clearly asymmetric; by the two indices, the transfer was greater from B to Balb/c than in the opposite direction. Invariably, the favored direction across heterologous junction was the one where the tracer first went through the cell membrane that would have had the less restrictive channels in homologous junction (Tables 1 and 2) (*15*).

We considered the possibility of an asymmetric artifact of microelectrode insertion. For example, if the Balb/c cell membrane sealed less well around the microelectrode, a moderate elevation of the cytoplasmic Ca^{2+} concentration due to heightened Ca influx might conceivably have caused a junctional permeability reduction for the larger LRB(Glu)OH molecule (*16*). This possibility was rendered unlikely by control experiments in which this red fluorescent tracer was injected into a B cell while a Balb/c neighbor was injected with green fluorescent FITC(Glu)OH, or vice versa (FITC = fluorescein isothiocyanate). The B-to-Balb/c direction continued to be the favored one (*17*). There were no systematic asymmetries in size between the two cell types; the injected cell, whether B or Balb/c, was always larger than the heterologous and homologous neighbors. Nor were there systematic differences in resting potential between the two cell types (*18*) or in rate of intracellular diffusion (*19*). We conclude that the B-Balb/c junction is asymmetrically permeable.

A further series of experiments was done on junctions of B cells with BHK cells (*20*), another heterologous combination where the homologous junctional channels of one cell partner (BHK) are

Table 1. Junctional transfer: summary.

Junction	Transfer direction	C-fluorescein (376 daltons)		LRB (559 daltons)		LRB(Glu)OH (688 daltons)	
		Intensity*	Incidence† (%)	Intensity*	Incidence† (%)	Intensity*	Incidence† (%)
Homologous	B → B	++++	93	++++	89	++++	100
	Balb/c → Balb/c	++++	82	++++	62	++	24
Heterologous	B → Balb/c	++++	73	++++	82	++++	76
	Balb/c → B	++++	88	+++	83	+	31

*Fluorescence intensity in first-order neighbors (transferring junctions) on a scale from ++++ (fluorescence approaching that of the injected cell) to + (fluorescence just above detection threshold). †Percent incidence of first-order permeable junctions; cumulative scores. For example, the first entry, 93 percent, represents the aggregate result of 24 trials where 55 of 59 junctions were permeable (details in Table 2). The statistical confidence level for LRB(Glu)OH permeable junction incidence B → Balb/c > Balb/c → B is better than 0.1 percent. The incidences for the two smaller molecules were not significantly different in the two directions.

Table 2. Frequency distribution of permeable junction incidence.

Transfer direction	C-fluorescein (376 daltons)					LRB (559 daltons)					LRB(Glu)OH (688 daltons)				
	Permeable first-order junctions					Permeable first-order junctions					Permeable first-order junctions				
	Incidence* (%)	Frequency†				Incidence* (%)	Frequency†				Incidence* (%)	Frequency†			
		0	20–49	50–89	90–100		0	20–49	50–89	90–100		0	20–49	50–89	90–100
B → B	93 (55: 59;24,11)	1	0	1	22	89 (31:35;15, 8)	0	0	2	13	100 (37: 37;12, 6)	0	0	0	12
Balb/c → Balb/c	82 (27: 33;13, 8)	0	1	3	9	62 (60:97;22,12)	1	2	16	3	24 (25:105;39,13)	24	5	6	4
B → Balb/c	73 (100:137;37, 7)	3	2	18	14	82 (18:22; 4, 2)	0	0	2	2	76 (73: 96;15, 7)	0	1	10	4
Balb/c → B	88 (7: 8; 4, 4)	1	0	0	3	83 (50:60;14, 4)	1	0	5	8	31 (66:213;48,14)	27	6	8	7

*Percent incidence of permeable junction of cells microinjected with tracer. In parentheses, in the following order are the number of permeable first-order junctions (when more than two per test), the total number of first-order junctions, the number of microinjections (trials), and the number of culture dishes examined. All permeability tests were made on subconfluent cultures. †Frequency distribution of scores of permeable first-order junctions from individual trials, arranged in intervals of 20 to 49, 50 to 89, 90 to 100, and 0 percent. There are no scores in the 1 to 19 percent interval; the minimum score criterion was 2 and, at the cell densities of the experiments, the maximum number of first-order junctions was 10.

more restrictive than those of the other (*8*). Here, too, the favored direction of junctional transmission [LRB(Glu)OH] was from the B cell to the partner.

A permeability asymmetry has two requisites. There must be a spatial asymmetry of channel structure, and this must give rise to an asymmetry of interaction between permeant and channel (*21*). The asymmetry condition here presumably is given by the pairing of unequal protochannels. We note in this connection that this condition is not met by merely pairing two different cell membranes; directional permselectivities were not apparent when we built heterologous junctions with mammalian cell membranes of equal homologous junctional permeability [tested on cell types with channels of permeability group II (*8*)]. As to the nature of the interaction, our earlier results with the homologous junctional Balb/c channel, probed with molecules of a wider range of sizes and charges (*8*), lead us to believe that it is either electrostatic, namely, an interaction with a fixed or induced charge guarding the channel, or a hydrogen bonding with the channel—and here one thinks first of the channel mouth as the locus of interaction.

An asymmetrically permeable cell-cell channel of the sort we have described has interesting physiological potential. If it occurs in heterologous junction of organs or tissues—and heterologous junctions are abundant—it could set up subtle internal boundaries where the traffic of certain molecules is effectively one-way. Selective boundaries of this sort could be important for partitioning tissues into cell domains of different physiology or different developmental fate. There is evidence that some domains of this kind are coupled by permeable junction (*2, 4, 22*). Little is known about the permeabilities of the channels at such boundaries, but it would be surprising if they were not somehow more limited than within the domains.

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11. The Balb/c cells (from confluent stock) were seeded in plastic dishes together with the B cells or 18 to 24 hours after seeding the latter. Junctional permeability was tested on subconfluent cocultures 18 to 24 hours after the last seeding. One of the cell types (alternated) was labeled, before coculture, with 1-μm latex particles, which the cells took up from the medium (Fig. 1). Besides, there was a natural marker; the Balb/c nucleus is granular. The growth medium was Eagle-Dulbecco's with 10 percent calf serum (Balb/c) or fetal calf serum (B). For coculture we used one serum or the other, with similar results.
12. The half-times of tracer fluorescence loss, determined by means of a photodiode system that images the cells through the microscope, were $>$ 30 minutes (continuous illumination); junctional transit times were $<$ 20 seconds; where junctional transit was scored negative, observation times were at least 10 minutes. The photodiode system was designed by R. Rick, B. Rose, and J. Gray. For a description, see J. L. Flagg-Newton and W. R. Loewenstein [*J. Membr. Biol.* **50**, 65 (1979)]. The fluorescences of the first-order neighbors (invariably greater than those of second-order ones) were averaged in the various trials. Deviations from the mean, in terms of our scale, were $\leq 1+$.
13. A junction here means two cells in contact as seen in phase contrast. Injected cells were multijunctional, each trial giving information on several junctions. The acceptance criterion for a positive score was a minimum of two 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 ^{3}C-fluorescein together with the larger LRB(Leu)$_3$(Glu)$_5$OH (1158 daltons; Leu = leucine), which is mammalian-channel impermeant (*8*); there was no instance of transfer of the larger molecule through junctions scored positive for C-fluorescein transfer. For properties and purification of the tracers, see I. Simpson, B. Rose, and W. R. Loewenstein [*Science* **199**, 294 (1977)] and S. J. Socolar and W. R. Loewenstein [*Methods Membr. Biol.* **10**, 121 (1979)].
14. Electrical measurements were taken simultaneously with tracer injection on 22 cell pairs. Electrical coupling was symmetric within the resolution of the method even in conditions, as in Fig. 1, IIb, where LRB(Glu)OH failed to pass altogether from Balb/c to B.
15. The transfer asymmetry is apparent by both cumulative indices. In particular, the frequency distribution in Table 2 reveals that although not a single junction exhibited LRB(Glu)OH transfer from Balb/c to B in 27 of 48 trials, all trials scored $>$ 48 percent permeable junctions in the opposite direction.
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17. Of ten cell pairs thus examined, nine transmitted LRB(Glu)OH or FITC(Glu)OH from B to Balb/c with strong first-order neighbor fluorescence (++++); and of these same nine pairs, only one transmitted in the reverse direction and just detectably (+). In another control series, the Balb/c neighbor was simply impaled on a microelectrode (without injection) while the B cell was injected with LRB(Glu)OH; of the 11 pairs examined, all transmitted the tracer from B to Balb/c (++++).
18. The resting potentials of contiguous cells were not sensibly different. The permeability asymmetry was present when the tracers were iontophoresed with trains of (hyperpolarizing) pulses (2.5 to 10 nA, 10^{-2} second, five per second) of durations three orders of magnitude shorter than the tracers' junctional transit times as well as with pulses of longer durations, and in conditions of voltage symmetry, when the tracers were pulsed simultaneously into two heterotypic cells (*17*).
19. Intracellular diffusion velocities were determined by imaging two regions of a given (large) cell, separated by a known distance, on an array of two photodiodes. The diffusion constants in a given cell type varied ≤ 8 percent and did not differ significantly in Balb/c and B cells, thus excluding the possibility of asymmetries arising from differences in tracer binding to cytoplasm or intracellular sequestering.
20. No double-impalement controls (*17*) were made in this series.
21. It follows from first principles (thermodynamics) that directional selectivity in passive diffusion can result only through an interaction of the permeant with the channel, or with another permeant species if the independence principle is violated. (An analysis of this point by J. N. Barrett is in preparation.)
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23. We thank I. Simpson for tracer synthesis, J. N. Barrett and S. J. Socolar for valuable discussion, J. Gray and C. Freites for construction of apparatus. J.F.-N. held postdoctoral fellowship 5F32 GM05802 from the National Institutes of Health. The work was supported by research grant CA14464 from the National Cancer Institute.

26 September 1979

Camouflage by Integumentary Wetting in Bark Bugs

Abstract. *Unlike most insect integuments, the body surfaces of certain bark-inhabiting bugs are wettable. A thin film of water reduces the reflectivity of the insect, resulting in a close match with the wettable bark upon which it rests. Wettability probably aids in concealing the insects from predators.*

Insects that spend much of their lives exposed on tree trunks provide some of the best examples of camouflage in the animal kingdom. Pattern and resting behavior together serve to hide many nocturnal Lepidoptera from diurnal predators (*1*). The protective adaptations of nonlepidopterous bark-resting insects are less well known. We now report a method of background matching in certain true bugs (Heteroptera) which compensates automatically for changes in background reflectance.

When wet with rain, the bark of trees

0036-8075/80/0215-0773$00.50/0