Relative roles of gap junction channels and cytoplasm in cell-to-cell diffusion of fluorescent tracers

(fluorescence photobleaching recovery/cytoplasmic diffusion coefficient/cell-to-cell membrane channels/junctional permeability)

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ABSTRACT Intercellular (tissue) diffusion of molecules requires cytoplasmic diffusion and diffusion through gap junctional (or cell-to-cell) channels. The rates of tissue and cytoplasmic diffusion of fluorescent tracers, expressed as an effective diffusion coefficient, D_e , and a cytoplasmic diffusion coefficient, D_{cyt} , have been measured among the developing epidermal cells of a larval beetle, *Tenebrio molitor* L., to determine the contribution of the junctional channels to intercellular diffusion. Tracer diffusion was measured by injecting fluorescent tracers into cells and quantitating the rate of subsequent spread into adjacent cells. Cytoplasmic diffusion was determined by fluorescence photobleaching. These experiments show that gap junctional channels constitute approximately 70–80% of the total cell-to-cell resistance to the diffusion of organic tracers at high concentrations in this tissue. At low concentrations, however, the binding of tracer to cytoplasm slows down the cytoplasmic diffusion, which may limit intercellular diffusion.

The pathway for the intercellular movement of molecules among the cells of a tissue consists of at least two components: a membrane component (mediated by gap junction channels) and a cytoplasmic component. While this movement is known to be influenced by the effective area of junctional channels, it is unclear whether diffusion through the cytoplasm can be rate limiting.

Diffusion of small molecules in a homogeneous solution such as water is a function of molecular size and shape and of viscosity alone. The measured diffusion of small molecules in cytoplasm, expressed as a cytoplasmic diffusion coefficient, D_{cyt} , is less than in water by a factor of 2–5 (1–9); presumably proteins, inorganic ions, sugars, and metabolites create a more viscous cytosol.

The rate at which larger molecules diffuse in cytoplasm is influenced not only by the viscosity of the cytosol but also by the cytoplasmic matrix. Although the pores within the meshwork of cytoplasmic architecture (ref. 10, cf. ref. 11) could be considered a factor limiting cytoplasmic diffusion rates, a reduction in cytoplasmic diffusion rate occurs well before the size limit of the pores within the microtrabecular lattice is reached. Jacobson and Wojcieszyn (3) and Gershon *et al.* (12) have suggested that proteins may bind reversibly to components of the cytoplasmic matrix, thereby reducing their mobility within a cell. This binding could be more important than steric interactions.

Cytoplasm accounts for approximately 15% of the total resistance to the intercellular movement (through cytoplasm and gap junctions) of inorganic ions in the insect epidermis (13). That is, gap junctional channels account for the greatest proportion of the resistance (85%). For other molecules, such as small fluorescent tracers, the relative role of cytoplasm in

cell-to-cell diffusion is unknown, although there is a suggestion that it may be quite large (14).

In this paper, the rates of diffusion of fluorescent tracers within the cytoplasm (diffusion coefficient D_{cyt}) and among the cells of an insect epithelium (diffusion coefficient D_e) have been measured independently. When compared, an estimate can be made of the relative contributions of the cytoplasmic and membrane components to tracer diffusion in the tissue.

MATERIALS AND METHODS

Squares of epidermis were excised from the ventral regions of abdominal segments II–VII of newly molted larvae (NML) and midinstar (MIL) larvae (6 days after ecdysis) of *Tenebrio molitor* L. and placed in tissue culture medium (15). Only last-instar larvae were used (0.10 g). Each preparation consisted of about 60,000 cells attached firmly to a transparent cuticle. The epidermal cells are uniform in height (16–20 μ m in NML; 8–10 μ m in MIL). Preparations were clamped into 35-mm dishes, and cells in the center of each preparation were viewed with either a Zeiss IM35 inverted microscope for measurement of D_e or a Zeiss Universal microscope for measurement of D_{cyt} .

Fluorescence Tracers. Two fluorescent dyes, 6-carboxyfluorescein (CF) and lissamine rhodamine B200 (LRB; also known as sulforhodamine B), were used as cell-to-cell tracers. Microelectrodes (resistances < 300 M Ω) were filled with either 10 mM CF or 25 mM LRB. A higher concentration of LRB was necessary to obtain approximately the same intensity of fluorescence emission. Tracers were iontophoresed into the epidermis by using 6-nA hyperpolarizing pulses (200-ms duration, one per second). Only those electrode penetrations in which the membrane potential remained stable were used in the analysis.

For the measurement of intercellular diffusion (D_e), preparations were placed on the stage of a Zeiss inverted microscope, and the cell-to-cell spread of tracers was recorded with a video system described previously (15). CF and LRB were excited by light from a mercury vapor lamp (HBO 50 W) using the following Zeiss filter sets: excitation filters, BP485 (CF) and BP546 (LRB); barrier filters, BP520 (CF) and LP580 (LRB); with dichroic prisms: FT510 (CF) and FT580 (LRB). A 1.5 neutral-density (ND) filter was placed into the excitation path to reduce bleaching of tracer (15).

For the measurement of cytoplasmic diffusion (15), CF and LRB were excited by 476.5-nm or 514-nm light, respectively, from a 2-W argon-ion laser (coherent light). A 1.03 ND filter was placed into the monitor beam path to enhance the contrast between monitor and bleach excitation. All other ND filters (0.36–2.50), when used, were placed into the

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Abbreviations: NML, newly molted larvae; MIL, midinstar larvae; CF, carboxyfluorescein; LRB, lissamine rhodamine B200; ND filter, neutral-density filter; FPR, fluorescence photobleaching recovery.

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barrier path. The following filters were used: for CF, 2 × 1 mm BG38 red-absorbing filters, a 500-nm dichroic mirror, 2 × 3 mm 515-nm yellow filters, and a BP520–540 filter; for LRB, 580-nm dichroic mirror, 2 × 3 mm 570 OG red-orange filters, and a BP580 filter.

Calculation of the Effective Diffusion Coefficient D_e . As described (15), the intercellular diffusion of fluorescent tracers (in two dimensions) is described by the equation:

$$C(r,t) = \frac{S}{4\pi D_e h} E_1\left(\frac{r^2}{4D_e t}\right), \quad [1]$$

where $C(r,t)$ is the concentration of fluorescent tracer at a distance r from the source of continuous tracer injection at a time t from the start of injection, S is the tracer delivery rate, h is the thickness of the epidermal cells, and π is a constant (16, 17). E_1 denotes the exponential integral. Light intensity data are fitted to this equation by using nonlinear regression to obtain D_e . Bleaching of tracer or loss of tracer from the cell are not problems over the time course of the measurement of D_e (<1 min). "Uncoupled" cells (see figure 9 in ref. 15) retain their fluorescence for periods of at least 5 min after injections have been stopped. In addition, the determination of D_e is partly based on the maximum extent of diffusion within the sheet for a given tracer delivery rate; loss of tracer results in a reduced delivery rate, which is independent of D_e (15).

Calculation of the Cytoplasmic Diffusion Coefficient D_{cyt} . Measurement was by fluorescence photobleaching recovery (FPR) in the standard way. From the fluorescence recovery after a bleaching pulse of light from a laser beam, a D_{cyt} is calculated from the formula:

$$D_{\text{cyt}} = \frac{w^2}{4\tau_D} \quad [2]$$

where τ_D is the characteristic time for recovery and w is the $1/e^2$ radius of the laser beam. In studies with cell membranes, the bleached region is an area, and fluorescence recovery is the result of two-dimensional diffusion of nonbleached molecules within the plane of the membrane. When bleaching fluorescent cells, a volume of cytoplasm is bleached. The combination of laser beam geometry and light-collecting apertures is such that the volume bleached is a thin cylinder into which diffusion is two- (and not three) dimensional (18). Typically, the following parameters were used: 0.25- to 5-ms sampling durations, 7- to 15-ms bleach pulses, and 15- to 20-ms postbleach pauses (to allow phosphorescence of filters to decay). Data were collected over a 3- to 5-min period (usually two to three bleaches). Recovery curves were analyzed by nonlinear least-squares regression to calculate τ_D .

The beam radius, w , defined as the laser beam width where light intensity falls to $1/e^2$ of its maximum value, was determined by using the methods in refs. 18 and 19. The average beam radius, w , was $1.4 \mu\text{m} \pm 0.13 \mu\text{m}$ ($n = 7$). D_{cyt} is then calculated using the above equation.

In a manner analogous to that described in ref. 15, the fluorescence from a series of CF solutions, 1 μM to 10 mM (expressed as fluorescence counts per millisecond), was measured by using the well of a microrefractometer slide (well depth of 8.32 μm ; similar to the height of the epidermal cells) as a defined path length. Only at concentrations greater than 1 mM did the relationship between concentration and fluorescence intensity become nonlinear.

RESULTS

Cytoplasmic and Junctional Diffusion of Fluorescent Tracers. To resolve a measured D_e for the movement of a molecule

in a tissue into its two components, one of the components—either diffusion through cytoplasm or diffusion across membrane junctions—also must be measured; the other is then calculated. As it was not feasible because of small cell size and tissue geometry to measure directly the junctional permeability of a tracer, its cytoplasmic diffusion was determined instead.

D_{cyt} was measured in the cytoplasm of a cell positioned 2 or 3 cells away from the cell injected with tracer (Fig. 1). CF and LRB were injected separately (because different excitation wavelengths were required of the laser) for about 15–20 s, and D_{cyt} was measured between 15 and 30 s after injection was stopped. Examples of FPR curves for the cytoplasmic

FIG. 1. Pattern of CF localization in NML and MIL epidermis. Injection of CF into NML (a) and MIL (b) epidermis results in distinctive patterns of localization. In NML cells highly fluorescent clumps are visible, while in MIL cells the nuclei stain brightly and no such clumps of fluorescence exist. The site of CF injection is shown by a "+," and the perimeter of the source cell, by the dashed lines in b. The diameter and typical position of the bleach beam, relative to the tracer source, are shown by the black dots. (Bar = 20 μm .)

diffusion of CF and LRB in MIL epidermis are given in Fig. 2.

Table 1 compares the values of D_{cyt} for CF and LRB in NML and MIL epidermis with those expected for cytoplasm with a viscosity 3 times that of water. Only the CF diffusion in MIL cytoplasm approaches the expected value. The reduced cytoplasmic diffusion of CF in NML epidermis may be caused by its strong cytoplasmic localization in the cells at this stage (Fig. 1). The diffusion of LRB is about half that expected in both NML and MIL cytoplasm.

The diffusion coefficients for the cell-to-cell movement of

FIG. 2. FPR curves for CF (Upper) and LRB (Lower) in MIL epidermis. Both FPR curves show characteristic recoveries. There is an initial fluorescence measurement, followed by a bleach and a pause period. The recovery of fluorescence follows this and ultimately is followed by assay of postrecovery fluorescence. The solid line shows the fitted curve derived from nonlinear regression analysis of the data in the recovery phase only. The gap in the data represents the duration of the bleach and the postbleach pause. The latter is the longer and is introduced to permit decay of phosphorescence in the dichroic and barrier filters. The data are fit with an absolute time reference centered in the bleach interval. With precise data, the fitting can be done correctly over any range of the recovery curve as long as the correct time after bleaching is used. The loss of information during the postbleach pause is not critical but does decrease the accuracy of the diffusion measurement. A more serious problem arises from the recovery occurring during the bleach interval. In the present cases, the minimum possible bleach times were used, but invariably the bleach and recovery times are comparable. There is no simple or quantitative way to account for the resultant effect on the measured recovery curves and fitted recovery times. Qualitatively, the effect is to increase the region (or volume) affected by the bleach, since a longer time is needed to give a specific fractional decrease in the fluorescence (a specific percentage bleached). Consequently, the recovery times will encompass a longer diffusion distance, and the recovery times will be slower, particularly in the "tail" region. Thus, the recovery times may be overestimates (or D_{cyt} values may be underestimates) of true values. For CF, $D_{\text{cyt}} = 1.53 \times 10^{-6} \text{ cm}^2/\text{s}$ ($\tau_D = 0.0032 \text{ s}$). For LRB, $D_{\text{cyt}} = 5.4 \times 10^{-7} \text{ cm}^2/\text{s}$ ($\tau_D = 0.0091 \text{ s}$).

Table 1. D_{cyt} values of two fluorescent tracers in the epidermis

Tracer	$D_{\text{cyt}} \times 10^7, \text{ cm}^2/\text{s}$		Predicted values [†]
	NML epidermis	MIL epidermis	
CF	4.8 ± 0.8 (5) [4.1–6.0]	11.7 ± 2.7 (4) [8.3–15.3]	12.0
LRB	5.7 ± 0.6 (3) [5.4–6.4]	6.2 ± 1.9 (3) [4.8–8.41]	10.1

*Means $\pm$ SD; the number in parentheses is the number of samples, and the numbers in brackets show the range.

[†]Predicted by Stokes-Einstein equation $D = kT/6\pi\eta r$, where $k = 1.38 \times 10^{-16} \text{ erg/deg K}$, $\eta = 0.03 \text{ erg s/cm}^2$, $r(\text{CF}) = 6.1 \times 10^{-8} \text{ cm}$, and $r(\text{LRB}) = 7.2 \times 10^{-8} \text{ cm}$; $1 \text{ erg} = 0.1 \mu\text{J}$.

CF and LRB were determined by coinjecting the two tracers into a cell in the epidermis; the spread of fluorescence over many cell diameters was videotaped and analyzed (refs. 15 and 20; R.G.A.S. and S.C., unpublished data). The effective tissue diffusion coefficients (D_e) obtained for the two tracers at both stages of epidermal development used are listed in Table 2. In all cases D_e is less than D_{cyt} .

In a simple linear system of cells, the resistance of the intercellular pathway is the sum of the cytoplasmic and junctional resistances in series (21, 22). In a two-dimensional system of hexagonally packed cells, the resistances of cytoplasm, R_{cyt} , and junctions, R_j (i.e., channels), are described by the equation $R_e = R_{\text{cyt}} + R_j/d^{3/2}$, where $d^{3/2}$ is the center-to-center distance between the cells (13) and d is the length of one side of a hexagonal cell.

Because diffusion is inversely proportional to resistance (i.e., a high resistance corresponds to a low diffusion coefficient and vice versa), this equation can be rewritten for diffusion as $1/D_e = 1/D_{\text{cyt}} + 1/D_j$, where $D_j = P_j \times d^{3/2}$, P_j being the permeability of the junctions in cm^2/s (20, 21) and $d^{3/2}$ being $1.08 \times 10^{-3} \text{ cm}$ (23). The junctional permeabilities calculated from measured values of D_e and D_{cyt} of CF and LRB in MIL epidermis are given in Table 2. These values are consistent with other calculated junctional permeabilities—usually $<10^{-3} \text{ cm}^2/\text{s}$ (24–29). The permeabilities of CF (or LRB) are not substantially different in NML and MIL epidermis.

Table 2 also lists the proportion of the resistance to diffusion due to cytoplasm ($D_e/D_{\text{cyt}} = R_{\text{cyt}}/R_e$). The higher value for CF in NML may well be the result of a cytoplasmic localization (Fig. 1a), thus decreasing D_{cyt} . In these experiments the cytoplasm plays an important but not a limiting role in tissue diffusion.

Concentration Dependence of D_{cyt} . The results of Table 2 are from measurements of D_{cyt} under conditions closely mimicking those of measurements of D_e —that is, at high dye concentration and within 1 min of injection. In early experiments, fluorescent tracers were injected into the epidermal cells using the apparatus for measuring D_e , and the preparation then was transported to another location for measurements of D_{cyt} . Approximately 15–20 min lapsed between tracer injection and the start of these measurements. The D_{cyt} values obtained were in the range of $0.1\text{--}2.4 \times 10^{-7} \text{ cm}^2/\text{s}$. In a few cases, D_e values were measured before and after FPR measurements; D_e values were consistently higher than this range of D_{cyt} . This is incompatible with the relationship between D_e and D_{cyt} unless other factors influence the diffusion in cytoplasm (i.e., the total resistance cannot be less than any one of the individual resistances). The largest D_{cyt} values were associated with high fluorescence counts, suggesting that D_{cyt} was concentration dependent. It was possible to test this by isolating squares of several hundred cells in single epidermal preparations (by killing intervening cells) and then injecting different concentrations of tracer (by injecting for different lengths of time) (Fig. 3). These exper-

Table 2. Comparison of junctional permeability and cytoplasmic diffusion

Tracer	Diffusion coefficients $\times 10^7$, cm^2/s		$P_j \times 10^4$, cm/s	$D_e/D_{\text{cyt}}^\dagger$
	D_e	D_{cyt}		
CF (NML)	2.4 ± 0.9 (15)	4.8 ± 0.8 (5)	4.6	0.51
CF (MIL)	3.4 ± 0.6 (7)	11.7 ± 2.7 (4)	4.5	0.30
LRB (NML)	1.4 ± 0.6 (15)	5.7 ± 0.6 (3)	1.7	0.24
LRB (MIL)	1.3 ± 0.4 (7)	6.2 ± 1.9 (3)	1.6	0.21

*Means $\pm$ SD. The number in parentheses is the number of samples.

† Fraction of intercellular resistance due to cytoplasm $(1/D_{\text{cyt}})/(1/D_e)$.

iments demonstrated a dependence of D_{cyt} on concentration, but the D_{cyt} values obtained were still lower than the D_e values. Reducing the time interval between completion of tracer injection and the measurement of D_{cyt} to 6–10 min resulted in higher D_{cyt} values ($1\text{--}5 \times 10^{-7} \text{ cm}^2/\text{s}$). The highest D_{cyt} values (those given in Table 1) were obtained by injecting immediately (15–20 s) before measuring D_{cyt} (done by assembling the apparatus used to inject tracer onto the FPR microscope). The results of all of these experiments are summarized in Fig. 4 where D_{cyt} is plotted against the prebleach fluorescence count. Higher D_{cyt} values are associated with higher initial fluorescence counts or concentration, with the highest D_{cyt} values occurring at concentrations of CF and LRB greater than 0.1 mM. Scatter in the data is likely due to uncertainty in the concentration estimate arising in part from cytoplasmic elements in the light path which either emit fluorescence or exclude the fluorescent dye.

DISCUSSION

D_{cyt} values for fluorescent tracers at high concentrations range from 6×10^{-7} to $12 \times 10^{-7} \text{ cm}^2/\text{s}$ in tissue in which the corresponding intercellular D_e values range from 1.4×10^{-7} to $3.7 \times 10^{-7} \text{ cm}^2/\text{s}$. These data show that at high tracer concentration, diffusion in cytoplasm approaches the maximum rate expected for a solution of viscosity about 3 times that of water. Under these conditions, cytoplasmic diffusion is more rapid than the permeation through gap junctions and, therefore, is not rate limiting. This conclusion also should be valid at lower tracer concentrations, provided neither D_{cyt} nor D_j is concentration dependent.

FIG. 3. Typical epidermal preparation used to measure the concentration dependence of D_{cyt} . Two different amounts of CF were injected into two isolated but adjacent areas of tissue. The upper region corresponds to a high concentration, where $D_{\text{cyt}} = 8.9 \times 10^{-8} \text{ cm}^2/\text{s}$, while the lower region corresponds to a low concentration, with $D_{\text{cyt}} = 4.1 \times 10^{-8} \text{ cm}^2/\text{s}$. (Bar = 100 μm .)

Our measurements of D_{cyt} for CF clearly show that D_{cyt} decreases with decreasing tracer concentration. Cytoplasmic diffusion would become the limiting factor in intercellular diffusion at low concentrations if D_j were not correspondingly concentration dependent. In any case, D_e must be concentration dependent to allow for changes in D_{cyt} and D_j , but D_e has not yet been measured well at low concentrations of tracers.

The D_{cyt} for LRB is similarly reduced at low concentrations and is always less than that for CF at comparable concentrations. A 2- to 3-fold difference in the mobility of these dyes has been observed in cytoplasm of fly salivary gland cells (31) and in the extracellular cuticular matrix to which the epidermis studied here is attached. Nevertheless, the resistance to intercellular diffusion of LRB is proportionally less than that for CF because the junctional permeability to LRB is one-third that for CF. Therefore, it appears that the molecular parameters that influence mobility in cytosol also affect permeability through the channel. Because of the similar molecular size and geometry of these tracers, it is likely that their distinct rates of intercellular movement result from different net charges (R.G.A.S. and S.C., unpublished data).

The origin of the concentration dependence of D_{cyt} is likely

FIG. 4. Concentration dependence of D_{cyt} . D_{cyt} values for CF from many experiments ($n = 46$) have been plotted against the prebleach fluorescence counts (per ms). The curve is described by $\log y = 0.6896 \log x + (-8.194)$; $r = 0.806$; $r^2 = 0.649$ [although there is no theoretical expectation that $\log D_{\text{cyt}}$ should vary linearly with $\log C$ in simple binding models (30)]. The most rapid D_{cyt} values are associated with the highest fluorescence counts (i.e., concentration). The absolute CF concentration is also shown on the x axis.

due to nonspecific binding to cytoplasmic constituents. If the fluorescent dye binds rapidly and reversibly, then the diffusion coefficient measured by FPR is the weighted average of diffusion coefficients of the free and bound molecules (30, 32). This implies that D_{cyt} should decrease at lower concentrations where the fraction of molecule bound is greater. The present data for CF are in accord with this model and support the notion that binding is as important as viscosity and steric effects in controlling movement of small molecules in cytoplasm.

Presuming that the difference between the observed and expected D_{cyt} values (Table 1) is due to binding, then the lower LRB diffusion can be understood if its less-negative charge results in a higher binding affinity. The lower mobility of CF in NML cytoplasm than in MIL cytoplasm is likely due to a greater binding in NML tissue to localized areas of the cytoplasm. Even though this binding must be rapid and reversible, it appears as a heterogeneous staining in Fig. 1a.

These findings with synthetic tracers may be relevant to models that assign a role to diffusible morphogens in development (21). An ideal morphogen should pass readily through membrane channels, diffuse rapidly in cytoplasm, and be degraded in, or lost from, the tissue at a low rate. At high morphogen concentration, the gap junctional channels may set an upper limit both to the molecular size and to the intercellular diffusion rate of a putative morphogen. In this case, modulation of junctional permeability, rather than cytoplasmic diffusivity, would be a more effective way to exert control over the rate of morphogen diffusion in a tissue. This is supported by the strong dependence of the cell-to-cell spread of high concentrations of injected tracer in the epidermis on junctional permeability (20). At low concentration, however, specific or nonspecific binding to cytoplasmic receptors may result in the cytoplasm rather than the junctions limiting the diffusion rate of the morphogen. Here the developmental regulation of cytoplasmic binding would determine the rate of morphogen diffusion.

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