

Diffusion Rates of Cell Surface Antigens of Mouse-Human Heterokaryons

III. Regulation of Lateral Diffusion Rates by Calcium Ions

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ABSTRACT In mouse-human heterokaryons, the lateral diffusion of major histocompatibility (MHC) antigens in the plasma membrane is enhanced by treatment of parent cells with ouabain. Ouabain treatment is ineffective if the medium lacks calcium ion, or if Verapamil, a blocker of calcium channels, is present. The divalent ionophore A23187 also enhances lateral diffusion of MHC antigens, to the same extent as ouabain. Like ouabain, A23187 is effective only if calcium is present in the medium. Thus it appears that increased levels of cell calcium release constraints to lateral diffusion of MHC antigens.

Lateral diffusion of membrane proteins is demonstrable by several independent methods in both organelle and cell surface membranes (reviews, 2, 5, 19). In most cells, the diffusion rates measured for a number of different surface membrane proteins are 5–100-fold slower than expected from a consideration of protein size and of viscosity of membrane lipids. Indeed, in epithelial cells lateral diffusion of components is so restricted that surfaces remain organized in functional domains for days to weeks (4, 29).

The locus of restrictions to lateral diffusion is probably in the cytoplasm immediately adjacent to the cell surface. The observation that lateral diffusion of band 3 protein is 100-fold faster in erythrocyte ghosts lacking spectrin (10, 23) than it is in normal ghosts makes a strong case for the effect of the erythrocyte cytoskeleton on diffusion. Recent work comparing lateral diffusion of membrane proteins in blebs, free of underlying cytoplasm, with diffusion of the same protein species in unperturbed membranes of muscle cells and myoblasts also indicates that rates of diffusion in the plasma membrane are affected by interactions with the underlying cytoplasm (1, 26). Such interactions are also implied by the effect of cytochalasin B on lateral diffusion (8, 21), by association of plasma membrane integral proteins with actin in cell extracts (14) and by similar associations in detergent-extracted ghosts (18).

For a number of years we have studied the lateral diffusion of antigens of the major histocompatibility complex (MHC antigens), H-2 of mouse and HLA of humans in terms of the rate of intermixing of these antigens in mouse-human heterokaryons (8). MHC antigens diffuse much more slowly in some cells than in others; a range of 20-fold or more is found for MHC antigen diffusion in the population of heterokaryons. Though it contrasts with the range of values for diffusion of

most other membrane proteins (2, 5), this range is not peculiar to the cells that we use, or to virus-treated heterokaryons. A similarly large range has recently been reported for lateral diffusion of MHC antigens, measured by photobleaching techniques (17, 24) (9a).

Human and mouse MHC antigens appear intermixed in ~10% of the cells in the earliest sample taken of the heterokaryon population, 3 min after initiating fusion. MHC antigens in these cells must diffuse with $D > 2-3 \times 10^{-9} \text{ cm}^2 \text{ sec}^{-1}$. This approximates the fastest rate measured for lateral diffusion for integral membrane proteins (26, 28), a rate predicted by theory from membrane viscosity and the size of the diffusing species (13). We found that we could increase the proportion of heterokaryons in which MHC antigens intermixed within 3 min of initiating fusion by treating the parent cell populations, with agents expected to reduce their membrane potential (9). Hanks' balanced salt solution 56 mM in K^+ , or containing ouabain, produced heterokaryon populations in which up to 60% of cells allowed maximal lateral diffusion of MHC antigens. Measurements of ^{35}SCN partition in treated and control cells showed ouabain and high $[\text{K}^+]$ did in fact lower the transmembrane potential of c1 1d and VA-2 (6).

We now show that, in addition to depolarization, Ca^{++} is required to increase rates of lateral diffusion of MHC antigens. Release of constraints to diffusion may also be effected by the divalent ionophore A23187, but only if Ca^{++} is present in the medium.

MATERIALS AND METHODS

Hanks' BSS buffered with HEPES to pH 7.35 (HH) was the basis for most drug solutions, as noted in the text. Isotonic phosphate-buffered saline 0.1% in glucose (PBSG) was used in some experiments. Ouabain was obtained from Sigma

Chemical Co. (St. Louis, MO). Verapamil (20) was the generous gift of Dr. Douglas Fambrough, Department of Embryology, Carnegie Institution. The calcium ionophore A23187 (19) was the gift of Dr. Robert Hamill, Lilly Research Laboratories (Indianapolis, IN). It was made up as needed from a 1 mg/ml stock in dimethylsulfoxide.

Culture of mouse c1 1d and human VA-2 cells and details of the antibodies used for visualizing the H-2 and HLA antigens of these cells by indirect immunofluorescence have been described in a previous publication (8). In later experiments in this series H-2 antigens were directly visualized with tetramethylrhodamine-labeled Fab fragments of a mouse monoclonal antibody and the human surface antigens were labeled with a fluorescein-labeled Fab of rabbit anti-WI-38 human cells. Details of the preparation and labeling of the Fab fragment of mouse IgG2a will be published elsewhere (9a).

Mouse-human heterokaryons were formed by fusing parent cells, removed from plates with 2.5% chicken serum:0.2% trypsin:0.002% collagenase (CTC) with 70–100 HAU of Sendai virus per 3×10^6 cells. We have previously shown that varying conditions of cell removal from plates using trypsin or EDTA, or varying amounts of Sendai virus used for fusion does not affect the quantitative result of the experiment (8). Formation of heterokaryons was stopped 3 min after initiating fusion at 37°C by 10-fold dilution of the cell suspension in Hanks' BSS containing 10% fetal calf serum (Reheis, Phoenix, Arizona). From 3 to 15% of cells formed heterokaryons under these conditions. The percentage of fused cells varied from experiment to experiment, but not as a function of drug treatment.

Cells treated with drugs other than A23187 were exposed to the drugs for 30 min at 37°C before fusion with Sendai virus. Cells were treated with A23187 at 0°C for 30 min before fusion, unless otherwise noted.

Lateral diffusion in the population of mouse-human heterokaryons formed by virus fusion is estimated in timed samples in terms of the proportion of doubly stained cells with separate membrane areas of red and green fluorescence, "segregates", at various times after fusion. Segregates predominant in samples taken within a few minutes after initiating fusion. They are a minority, compared with cells with completely overlapping rings of green and red, in samples taken 40–60 min after initiating fusion. The change in heterokaryons from segregate to intermixed is due to lateral diffusion of the antigen and the data may be analyzed to yield diffusion coefficients (8).

All stained cell samples were chilled to 0°C and fixed in 0.5% paraformaldehyde in PBS. Scoring of all experiments was done by one individual. Cells scored as segregate contained some detectable region of membrane that fluoresced in only one color. The size of these regions ranged from an entire hemisphere to a patch at one pole of a cell about $20 \mu\text{m}^2$.

Time of sampling, but not treatment, was known for each sample. From 20–400 cells were examined in each experiment.

Data from all experiments involving a given treatment were pooled and the regression of fraction of segregate cells, Y , versus time was calculated to correctly weight values of Y (25). Regression analysis was performed in the usual way. Confidence limits for the regression lines are presented as standard errors of the mean, SEM, for calculated Y values; these are larger than SEM for measured Y values. Details of number of cells and number of experiments are given in the legend for each figure. In some instances, the 95% or 99% confidence intervals for individual values were estimated from Pearson's graph of confidence intervals for proportions (in reference 3).

$^{45}\text{Ca}^{++}$ influx was measured on c1 1d plated at 3×10^5 cells/well in six-well plastic plates. After washing in HH, $2 \mu\text{Ci}$ of $^{45}\text{Ca}^{++}$ was added to each well. At intervals four replicate wells were quickly washed in cold PBS (less than 20 s for all three washes) and the cells were dissolved in 1.5 ml of 0.5 N NaOH. Cells for efflux measurements were labeled by incubation in $0.5 \mu\text{Ci}$ $^{45}\text{Ca}^{++}$ /ml growth medium for 20 h at 37°C. The cells were then washed three times with H-H and exposed to the agents under test for 0–120 min. Quadruplicate wells were sampled every 10 min. The wells were washed twice with ice-cold HH and then the cells were dissolved in 0.5 N NaOH.

RESULTS

Treatment of parent cells with 3 mM ouabain in Hanks' BSS (HH) (9) resulted in heterokaryon populations in which 40% of cells had intermixed their MHC antigens (Fig. 1) compared with 14% of control heterokaryons. Ouabain treatment and fusion of cells in HH lacking Ca^{++} yielded mostly segregates at 3 min after fusion (Fig. 1, \square). The 99% confidence limits for the measured fraction segregates are 0.13. Thus, significantly fewer heterokaryons incubated and formed in Ca^{++} -free medium intermix their MHC antigens during the first 13 min after fusion than do heterokaryons treated with ouabain in Ca^{++} -containing medium. The effect of ouabain treatment is also abrogated in 8 $\mu\text{g}/\text{ml}$ Verapamil, an agent blocking calcium

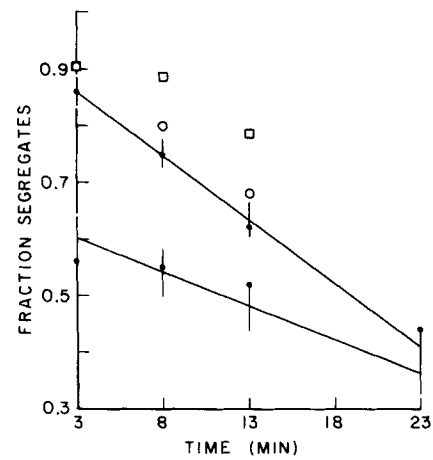


FIGURE 1 Effect of ouabain on lateral diffusion of MHC antigens in heterokaryons. Upper line, fraction of segregates as a function of time in heterokaryons formed from untreated cells, incubated in HH for 30 min before fusion. Data of 21 experiments, 2,576 cells. Lower line, fraction of segregates as a function of time in heterokaryons formed from cells incubated in 3 mM ouabain in HH for 30 min, 37°C. Data of 17 experiments, 2,115 cells. Bars given the standard error of the mean of the regression line through the weighted data points (\bullet). (\square) Cells treated with ouabain in Ca^{++} -free HH, one experiment, 55 cells. (\circ) Cells treated with ouabain in HH containing 8 $\mu\text{g}/\text{ml}$ Verapamil, two experiments, 516 cells.

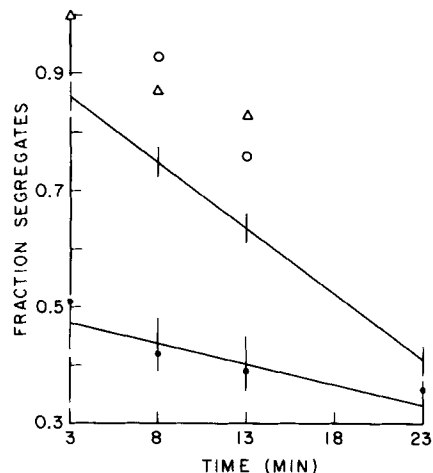


FIGURE 2 Effect of A23187 on lateral diffusion of MHC antigens in heterokaryons. Upper line, data on control heterokaryons, from Fig. 1. Lower line, fraction of segregates as a function of time in heterokaryons formed from cells incubated with 5–10 $\mu\text{g}/\text{ml}$ A23187 in HH at 0°C for 30 min before fusion. Data of 13 experiments, 1,390 cells. Bars give the standard error of the mean of the regression line through the weighted data points (\bullet). (\circ) Cells treated with A23187 in PBSG, one experiment, 89 cells. (Δ) Cells treated with A23187 in Ca^{++} -free HH 1 mM in EGTA, two experiments, 225 cells.

currents (20) (Fig. 1, \circ). 99% confidence limits for the fraction segregates in these experiments are 0.08.

~50% of heterokaryons formed from parent cells treated with the ionophore A23187 (20) had intermixed surface antigens within 3 min of initiating fusion (Fig. 2). Ionophore treatment of parent cells in Ca^{++} and Mg^{++} -free medium (PBSG) or in HH lacking Ca^{++} and 1 mM in EGTA had no effect on lateral diffusion (Fig. 2, Δ). Indeed, cells incubated and fused in medium lacking Ca^{++} appear, in both Fig. 1 and Fig. 2, to produce fewer heterokaryons that allow rapid diffu-

TABLE I
Effect of A23187 Added to Heterokaryons, Rather Than to Their Parent Cells

Group	Fraction segregates at		
	3 min	5 min	17 min§
Controls-no A23187	0.86 (0.03)*	0.80 (0.03)	0.59 (0.03)
A23187 before fusion	0.47 (0.04)	0.46 (0.04)	0.37 (0.04)
A23187 3 min after fusion	—	0.91 (0.77-0.96)‡	0.30 (0.18-0.44)‡

* () standard deviation.

‡ () 99% confidence interval.

§ Time after initiating fusion by warming the mixture of cells and virus to 37°C.

sion of MHC antigens than do controls. However, the 99% confidence limits for the limited number of experiments overlap with the limits for the controls.

Cells exposed to ouabain in HH and then washed and fused in Ca^{++} -free medium behave approximately like control cells not treated with ouabain, whereas cells treated with ouabain in the absence of Ca^{++} and then fused in HH behave like cells treated with ouabain in HH (data not shown). The time for changing medium by washing and for fusing the cells is about 15 min. Thus calcium ions need to be present for no longer than 15 min to affect lateral diffusion in ouabain-treated cells. We refined this time limit in another experiment in which A23187 (0.5–5 μ g in three experiments) was added to heterokaryons, instead of to parent cells. All three concentrations of A23187 used had an effect at the same time after fusion and so the pooled results are given in Table I. It appears that exposure of heterokaryons to A23187 for 2 min does not alter lateral diffusion rates, whereas exposure for 15 min does. Thus 15 min appears to be about the minimum time required for a Ca^{++} effect on lateral diffusion.

We next examined the effects of 56 mM K^+ (used in previous experiments (9) to depolarize cells) and of ouabain on Ca^{++} fluxes in one of the heterokaryon parents, c1 1d. Fig. 3 shows the time course of $^{45}Ca^{++}$ entry into control cells, cells in the presence of ouabain and cells in 56 mM K^+ . The initial rates of uptake are similar for all three treatments. However, ouabain treated cells take up the tracer to a higher steady-state level (~25% greater than in control) than do control or 56 mM K^+ -treated cells. (The standard errors for all values are between 0.01 and 0.001). Equilibrium is reached at around 12 min in control and 56 mM K^+ -treated cells and at around 16–20 min in ouabain treated cells.

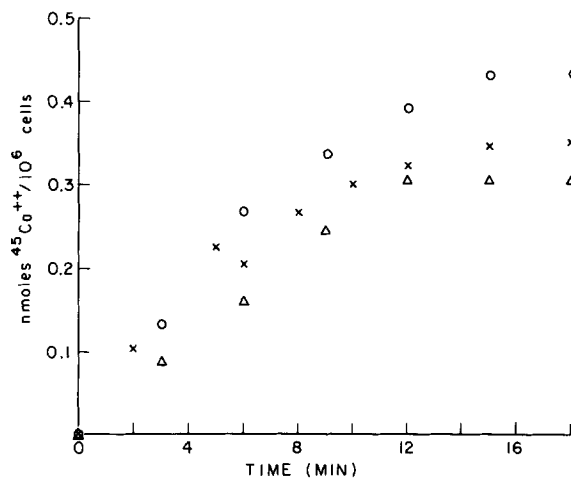


FIGURE 3 Influx of $^{45}Ca^{++}$ into c1 1d. (X) Cells in HH. (O) Cells in 3 mM ouabain in HH. (Δ) Cells in 56 mM K^+ in HH.

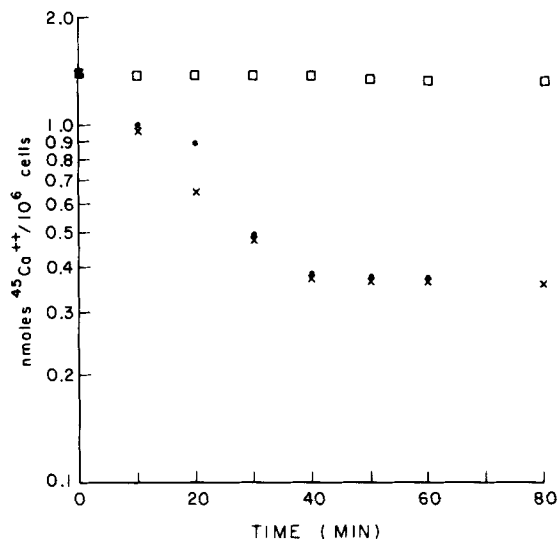


FIGURE 4 Efflux of $^{45}Ca^{++}$ from c1 1d. (X) Cells in HH. (●) Cells in Na^+ -free HH. (□) Cells in 5 mM NaCN plus 5 mM NaF in HH.

TABLE II
Rates of $^{45}Ca^{++}$ Efflux from c1 1d at 37°C

Treatment	Flux rate \pm SD <i>log nmol/10⁶ cells/min</i>
HH-control	-0.0145 \pm 0.00065
Na^+ -free*	-0.0136 \pm 0.0018
3 mM ouabain in HH	-0.0157 \pm 0.0016
HH 8-10°C	-0.0088 \pm 0.001
56 mM K^+ in isotonic HH	-0.0094 \pm 0.00018

* NaCl was replaced by Choline Cl.

The $^{45}Ca^{++}$ efflux rate in 56 mM K^+ HH was significantly less than the rate in HH $t = 6.87$, $n = 10$, $p < .01$.

Ca^{++} efflux from c1 1d is shown in Fig. 4. In agreement with Lamb and Lindsay (16) we found that Ca^{++} is extruded from L cells by an ATP-dependent mechanism, presumably a pump, and not by Na^+ -dependent exchange. The efflux curves are well approximated by single exponentials. Thus, it was possible to compare efflux rates with different cell treatments. These are tabulated in Table II. Ouabain has no effect on efflux, whereas 56 mM K^+ slows efflux as much as incubation at 8–10°C.

DISCUSSION

Ouabain affects lateral diffusion of MHC antigens in heterokaryons only when Ca^{++} is present in the medium and can enter the cells. The ouabain effect, appearance in the heterokaryon population of many cells in which MHC antigens mix within 3 min of formation, $\geq 2 \times 10^{-9} \text{ cm}^2 \text{ sec}^{-1}$, is abrogated

in Ca^{++} -free Hanks' solution, and by the calcium channel blocking drug Verapamil.

The divalent ionophore A23187 affects lateral diffusion of MHC antigens in the same way as ouabain. Like ouabain, A23187 is only effective if Ca^{++} is present in the medium. Though L cells are reported to contain calcium stores in a modified endoplasmic reticulum (12) these apparently are not mobilized by ionophore in our experiments. Because two different drugs, ouabain and A23187, both affect lateral diffusion to the same extent and apparently in the same subpopulation of heterokaryons (see next paragraph) we believe that the effects are Ca^{++} -specific and not due to nonspecific interactions of the drugs with cell membranes.

If intermixing of heterokaryon MHC antigen is followed for longer times than used here, up to 3 h after fusion, it is found that a portion of the population bear MHC antigens that are immobile on the time scale of the experiment, $D < 2 \times 10^{-11} \text{ cm}^2 \text{ sec}^{-1}$ (6). If all heterokaryons were affected by ouabain or A23187 and diffusion was increased in these cells by a constant factor we would expect the regression lines of Figs. 1 and 2 to be parallel. In fact, the slopes of the lines for ouabain or A23187-treated cells are significantly smaller than the slope of the control line. This is expected if ouabain or A23187 increases lateral diffusion mainly in cells which would in any case have intermixed MHC antigens during the first 40 min of the experiment. That is, ouabain or A23187 treatment results in a heterokaryon population that contains mainly cells in which $D > 2 \times 10^{-9} \text{ cm}^2 \text{ sec}^{-1}$ or $D < 2 \times 10^{-11} \text{ cm}^2 \text{ sec}^{-1}$. This interpretation is borne out by results on lateral diffusion of H-2 antigens in single cl 1d cells measured by fluorescence photobleaching and recovery (FPR) (9a). A23187 treatment reduces the proportion of cells in which D is of the order of $1-2 \times 10^{-10} \text{ cm}^2 \text{ sec}^{-1}$ while significantly increasing the proportion of cells in which MHC antigens are either immobile or diffusing in the range $2-5 \times 10^{-9} \text{ cm}^2 \text{ sec}^{-1}$.

Ouabain-treated cells must be exposed to external Ca^{++} for ~15 min before lateral diffusion is affected. This timing is similar to that required for the effect of depolarization by 56 mM K^+ on lateral diffusion (9). Our interpretation of the results assumes that the drug treatments of parent cells change restraints to diffusion rather than promoting selective fusion of some cells in which lateral diffusion of heterokaryons is intrinsically faster than in cells which do not fuse. Selective fusion of cells in which $D > 1 \times 10^{-9} \text{ cm}^2 \text{ sec}^{-1}$ could produce most of the results that we obtained. However, A23187 increases lateral diffusion rates of MHC antigens when added to heterokaryons, rather than to parent cells (Table I). This result, together with the observation that A23187 also increases the rate of lateral diffusion, measured by fluorescence photobleaching and recovery, of H-2 antigens in single cl 1d (9a) indicates that the drug treatments described effect changes in constraints to lateral diffusion in the parent cell population, rather than biasing the formation of heterokaryons.

We have directly examined the effects of ouabain and 56 mM K^+ level on Ca^{++} fluxes in cl 1d. Ouabain had no effect on Ca^{++} efflux, compared to controls, whereas 56 mM K^+ slowed Ca^{++} efflux considerably. The results suggest that, though 56 mM K^+ and ouabain work in different ways, they both take about 15 min to raise cell Ca^{++} content. The flux data are complicated by the presence of multiple Ca^{++} compartments in cells and by the multiplicity of effects on cell metabolism of both ouabain and high $[\text{K}^+]$.

Given the observations on interaction of cytoskeleton, es-

pecially of cytoplasmic filaments with integral membrane proteins, we must consider the ways in which increase in intracellular calcium content might affect these interactions. Actin and its associated proteins form a polymer system sensitive to calcium concentration (discussion in reference 11), and observations on the levels of free calcium during cell locomotion (27) and on the effects of A23187 on capping (22) indicate that the filament system is modified by calcium *in situ*. Hence, the effects that we described on lateral diffusion could be due to Ca^{++} -induced modification of actin filament systems. An alternative possibility is that pumping of excess free Ca^{++} by an ATP-dependent pump, depletes cell ATP and this in turn affects polymerization.

We are left then with the finding that entry of Ca^{++} into cells, resulting in an increase in cell Ca^{++} , leads to a release of constraints to lateral diffusion of MHC antigens. This result has also been found for single cells, by FPR. We expect that the single cell measurements together with work on cell ghosts or membranes, will further define the steps between Ca^{++} entry and changes in the diffusion rates of MHC antigens in the plane of the plasma membrane.

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