J. Membrane Biol. 142, 223–228 (1994)

The Journal of
Membrane
Biology

Cheek Cell Membrane Fluidity Measured by Fluorescence Recovery after Photobleaching and Steady-State Fluorescence Anisotropy

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Received: 25 April 1994/Revised: 20 July 1994

Abstract. Membrane fluidity of human cheek cells was determined using fluorescence recovery after photobleaching (FRAP) and steady-state fluorescence anisotropy. The FRAP data showed that the lateral diffusion coefficient (D) and mobile fraction ($\%R$) of lipid in the plasma membrane of control cells were 2.01×10^{-9} cm^2/sec and 54.25%, respectively. Trypsin treatment increased D and $\%R$ to 6.4×10^{-9} cm^2/sec and 72.15%. In contrast, the anisotropy (r) for control cells was 0.270 which remained unchanged by trypsin treatment. The results show that diffusion of lipids in the plane of the membrane is restricted by trypsin-sensitive barriers.

Key words: Cheek cell — Membrane fluidity — FRAP — Anisotropy

Introduction

Human cheek (buccal epithelial) cells can be isolated by noninvasive methods and represent an accessible tissue on which to investigate the influence of diet on nutritional status (McMurchie et al., 1984). The isolation procedure relies on mechanical dislodgement of cells from the inner surface cheek and therefore isolates cells with associated extracellular protein (McMurchie et al., 1984). A number of investigators have isolated cheek cells using this method and have demonstrated that the cell membrane lipid composition can be influenced by the lipid composition of the diet (McMurchie et al., 1984; Sampugna et al., 1988). However, the biophysical properties of the cheek cell plasma membrane have not been investigated. Such studies are of importance since physical properties, such as membrane fluidity, may have a profound influence on cell function.

Membrane fluidity properties can be measured by ^{2}H-NMR, ESR, fluorescence recovery after photobleaching (FRAP), time-resolved and steady-state fluorescence anisotropy (Stubbs & Smith, 1984). However, these methods determine different aspects of membrane organization. For example, ^{2}H-NMR, ESR time-resolved and steady-state fluorescence anisotropy determine rotational motion of lipid in the membrane, whereas FRAP measures lateral motion in the plane of the membrane (Stubbs & Smith, 1984). The distinction between these measurements is important. Measurements of rotational diffusion provide information about molecular motion on a molecular length scale; in the case of FRAP, measurements of lateral diffusion provide information about molecular motion on a much larger scale, determined by the dimensions of the illuminated spot on the membrane surface (e.g., 1–5 μm). Thus, this latter measurement is sensitive to the presence of barriers to diffusion that may be present within the membrane resulting in domain formation. Physical barriers to diffusion in the membrane can originate from within the cell, where they are generally coupled to the cytoskeleton or can be located on the outer surface of the membrane, where they are referred to as the extracellular matrix (ECM).

The presence of such structures can cause the host cell to adopt a nonspherical appearance consistent with that observed with cheek cells. In this report, we describe measurements of the membrane fluidity of human cheek cells by steady-state fluorescence anisotropy (Kuhry et al., 1985) and FRAP (Axelrod et al., 1976). The possible presence of a membrane-bound, proteinaceous, ECM which compartmentalizes lipid in domains within the membrane and therefore restricts lateral diffusion has been investigated by combined mild trypsinolysis and FRAP.

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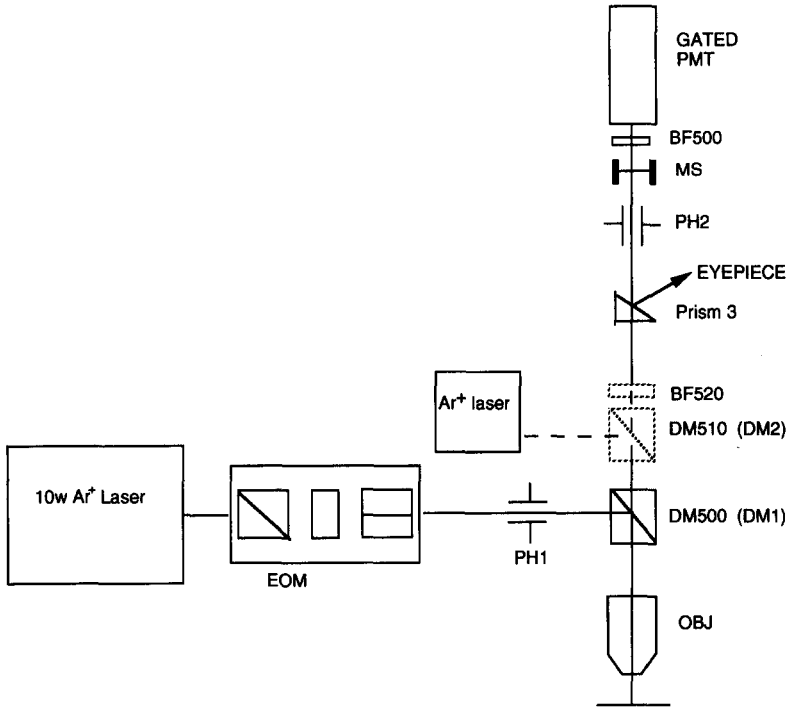

Fig. 1. Schematic representation of the photobleaching apparatus (abbreviations are discussed in the text).

Materials and Methods

Collection of Cheek Epithelial Cells

Cheek cells were collected by a modification of the method of McMurchie et al. (1984). Subjects initially cleaned their mouths with bottled mineral water (Malvern), then rinsed their mouth vigorously with 20 to 25 ml of mineral water. Cheek cells were collected by expectoration of the fluid into a container. Usually two of these collection cycles were performed after an initial cleansing rinse, with the final volume of washings collected amounting to approximately 40 ml. Penicillin/streptomycin was added to this suspension to a final concentration of 100 IU/ml and 100 mg/liter, respectively. The sample was then centrifuged at 15,000 × *g*, at 4°C for 30 min. After discarding the supernatant, the pellet of the cells was resuspended in 1 ml of Mg^{2+} and Ca^{2+} free phosphate-buffered saline (PBS buffer) at 4°C containing 100 IU/ml penicillin and 100 mg/liter streptomycin. The cells were then disaggregated by aspiration through a 1 ml syringe fitted with a 25 gauge needle. The cells were washed in PBS buffer by centrifugation at 3,500 × *g* for 5 min in a microcentrifuge. The cell pellet was finally resuspended in 1 ml of PBS-buffer at 4°C and stored on ice.

Trypsin Treatment

Isolated cheek cells (approx. 50,000/ml) were incubated in PBS containing 0.25% trypsin (EC 3.4.21.4, Imperial Laboratories) and 0.03% (w/v) EDTA at 37°C for 30 min. Control nontrypsin-treated samples were prepared in parallel. The progress of trypsinolysis was monitored by light microscopy since this treatment induced rounding of the cells and loss of their flattened polyhedral-like appearance. The cells were recovered after the incubation period by centrifugation at 400 × *g* for 5 min. The supernatant was discarded and the cells were resuspended in Hanks balanced salt solution pH 7.4 at 4°C, washed with buffer and harvested by centrifugation twice as above. The final cell pellet was resuspended in 0.5 ml of Hanks buffer. Cell viability was confirmed by their ability to hydrolyze fluorescein diacetate (Sigma).

Fluorescence Labeling of Cheek Cells

Cheek cells were incubated in Hanks buffer containing 2 μmol/liter 5-N-(octadecanoyl) aminofluorescein (ODAF, Molecular Probes) on ice for 15 min. The cells were then recovered by centrifugation at 400 × *g* for 5 min. The supernatant was discarded, and the cells were resuspended in Hanks buffer then washed twice as described above. The final cell pellet was resuspended in 0.5 ml of Hanks buffer. The cells were then drawn into 100 μm thick, 50 mm long rectangular cross-section microslides (Camlab Cambridge) by capillary action (Bloom & Webb, 1983) and the ends of the slides were sealed with Critoseal (Hawksley and Son). The slide was then placed on a temperature-controlled microscope stage ready for photobleaching experiments. All samples were labeled and ready for analysis within 3 hr. Photographs were taken with Nikon FX35DX camera on Kodak Ektachrome 400 ASA film.

Fluorescence Recovery after Photobleaching (FRAP)

Lateral diffusion measurements of the fluorophore, ODAF, in the plasma membrane of the cells was measured by FRAP (Fig. 1). The apparatus has been described (Bloom & Webb, 1983; Wolf, 1989) but had a number of unusual features as detailed below.

Electro-optic modulator: The intensity of the 488 nm line of a 10 W argon ion laser (Ar^+; Coherent Innova 100-10) was modulated by an electro-optic modulator (EOM), constructed from two crossed Glan-Taylor polarizing prisms and an LCD light valve. The first polarizer prism was positioned in the light path and set to allow maximum light throughput (i.e., parallel with the polarization of the laser). The second polarizer prism (analyzer) was placed in the light path and adjusted to give minimum light output. The light valve (LV050AC; Displaytech)

was positioned between the two crossed polarizers and adjusted to give minimum light output (i.e., 90° orientation from the first). The light valve was driven using a waveform which exhibited a ±15 V switching transient, which decayed to a ±5 V hold voltage in 300–600 μsec. This device allowed rapid switching between the monitoring and bleach beam intensities, the latter being approximately 300-fold more intense than the monitoring beam.

The microscope (Nikon Optiphot) was arranged with two epi-illumination attachments to permit fluorescence illumination from two separate light sources. This made it possible to switch between full field fluorescence illumination to allow labeled cell location and point illumination for the photobleaching experiments. Full field illumination was provided by the beam output from a 488 nm air-cooled argon ion laser (Cyonics; model 2101). This was delivered via a fiber optic attachment into one epi-illumination port, through a ground glass diffusion filter and delivered to the sample via a dichroic mirror (DM2; DM510 Nikon) and barrier filter. Note it was necessary to remove DM1 from the light path for this form of illumination.

The modulated laser beam was guided into the microscope through a beam defining aperture (PH1) located at the image plane of the second epi-illumination attachment. The latter was equipped with a dichroic mirror (DM1; DM500). The beam was focused to a spot on the cell membrane using a 40× (Nikon 0.4-NA) long working distance objective. The spot intensity profile was determined using a BeamScan (Model 2180; Photon) and was found to be Gaussian. The spot radii ($1/e^2$) was 2.5 μm. The emitted fluorescence gathered by the objective lens was transmitted by the dichroic mirror (DM1) and a 500 nm barrier filter, and focused on a second aperture (PH2) located at the image plane of the photomultiplier port. Fluorescence intensity was detected by a photoncounting photomultiplier tube (PMT; Thorn-EMI). The detector tube was protected by a HV electronic gate and a mechanical shutter (MS). It is worth noting that during a photobleaching data collect sequence, DM2 and prism 3 were withdrawn from the light path. The stability of the input laser beam was monitored using a beam splitter (BS) and photodiode (PD) and this signal was used to electronically compensate for minor laser fluctuations. System timing and control, data acquisition, and data analysis were performed using a VME microcomputer (Motorola 68020).

FRAP data were analyzed by nonlinear least-squares fitting to an expression defining the time dependence of fluorescence recovery observed with a circular beam of Gaussian cross-sectional intensity and had the form (Yguerabide, Schmidt & Yguerabide, 1982):

$$F(t) = \frac{F(0) + F(\infty)(t/\beta\tau_D)}{1 + (t/\beta\tau_D)} \tag{1}$$

Where $F(t)$ was observed fluorescence as a function of time, $F(0)$ was the intensity of the fluorescence immediately after the bleach pulse, $F(\infty)$ was the fluorescence at infinite time after the bleach pulse, β was the depth of bleach parameter and τ_D was the diffusion time. The lateral diffusion coefficient, D, is given by $D = \omega^2/4\tau_D$, where ω is the half-width at $1/e^2$ height of the laser beam at its point of focus on the membrane. Mobile fraction ($\%R$) is given by

$$\%R = \frac{F(\infty) - F(0)}{F(t < 0) - F(0)} \tag{2}$$

where F ($t < 0$) is the prebleach fluorescence (Wolf, 1989).

Single FRAP recovery curves were collected from ten separate cells for each experimental condition and then averaged before analysis.

Steady-State Fluorescence Anisotropy

Membrane fluidity was measured using the fluorescence anisotropy properties of the probe trimethylammoniumdiphenylhexatriene (TMA-DPH) under steady-state conditions using a spectrofluorimeter (Perkin-Elmer LS-5) coupled with a L-format polarization unit. TMA-DPH (Molecular Probes), prepared as a 0.5 mmol/liter stock solution in dimethylformamide, was stored refrigerated and protected from light. Equal volumes (0.5 ml) of cheek cells and TMA-DPH dispersion (1.0 μmol/liter) in PBS buffer were mixed and incubated at room temperature for 5 min. Sample temperature was equilibrated to 37°C in the fluorimeter and fluorescence emission obtained at 435 nm with 385 nm excitation was measured with constant stirring.

Temperature was monitored continuously using a digital thermometer with an accuracy of ±0.1°C. The anisotropy (r) was calculated using the expression:

$$r = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}} \tag{3}$$

where I refers to the observed fluorescence intensity and the subscript denotes the orientation of the excitation and emission polarizers (V = vertical and H = horizontal). The instrumental correction factor (G) (Lakowicz, 1983) is given by

$$G = \frac{I_{HV}}{I_{HH}} \tag{4}$$

Thus, anisotropy measurements were always collected with vertical excitation, whereas the correction factor involved horizontal excitation. The absorbance of the sample at the excitation wavelength was below 0.2 units and the results were corrected for light scattering (Kuhry et al., 1985; Deguercy et al., 1986).

Statistical Significance

Statistical analyses were performed using ANOVA and testing significance by the Tukey method.

Results

The plasma membrane fluidity properties of human cheek cells were measured by FRAP using ODAF as fluorescent probe. Prior to the FRAP measurement, fluorescent-labeled cheek cells were inspected by fluorescence microscopy with full field illumination. The cheek cells were of a normal polyhedral appearance indicating that labeling did not alter their morphology (Fig. 2*a*). The FRAP results are summarized in the Table. The data from cells obtained from several subjects returned lateral diffusion coefficients within the range of expected values for mammalian cells at 37°C. However, the proportion of fluorescence recovery observed under these conditions was considered abnormally low, amounting to only approximately 50% of the prebleach intensity as shown by the data curves in Fig. 3. Experiments were also performed following preincubation of the sample at 37°C to establish whether the sample's temperature history influenced the extent of fluorescence recovery. However, no

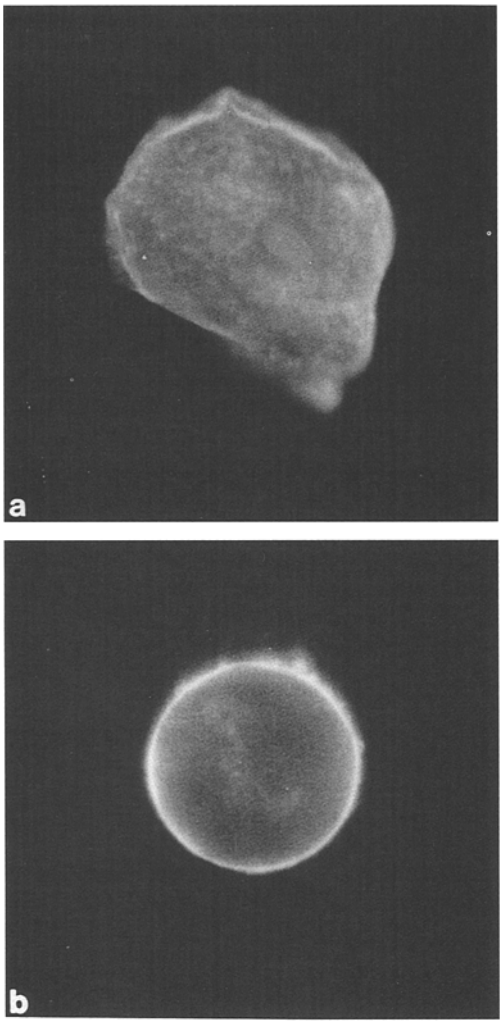

Fig. 2. Effect of trypsin on the morphology of cheek cells. Photograph (*a*) shows control cells and (*b*) trypsin-treated cells both fluorescently labeled with ODAF.

Table 1. Effect of trypsin on the membrane fluidity of human cheek cells measured by FRAP and fluorescence anisotropy

Treatment	FRAP method		Fluorescence polarization
	Diffusion coefficient (D) ($D \times 10^{-9}$ cm^2/sec)	Recovery (R) (%)	Anisotropy (r)
Control[a]	2.01 ± 0.151	54.25 ± 3.46	0.270 ± 0.0037
37°C[b]	2.01 ± 0.169	51.15 ± 3.57	0.270 ± 0.0065
EDTA	2.08 ± 0.204	55.78 ± 2.43	0.275 ± 0.0058
Trypsin-EDTA	6.40 ± 0.667*	72.15 ± 2.62*	0.270 ± 0.0025

Data values represent mean ± SE of four determinations.
[a] Control sample was stored on ice prior to equilibration at 37°C for the measurements. [b]The 37°C was preincubated at 37°C for 30 min in the absence of trypsin prior to the measurements at 37°C. *Data values which are significantly different from control values $P < 0.05$.

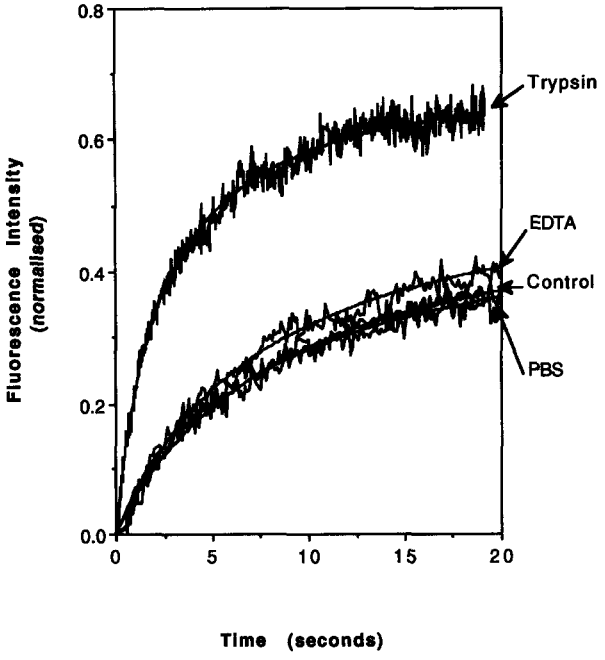

Fig. 3. Effect of trypsin on the lateral diffusion of lipids in cheek cell plasma membrane. The results from typical FRAP experiments are displayed on this graph. Data points (640) were collected for each treatment. Data were averaged and analyzed as described in the text. The unbroken line (–) is the computer-generated best-fit recovery curve.

significant alteration in either the lateral diffusion coefficient or the extent of recovery was observed.

It was unclear whether the polyhedral morphology derived from properties of the cytoskeleton or from a proteinaceous ECM. It was conceivable that the presence of an ECM could compartmentalize the membrane inhibiting complete redistribution of the photobleached fluorophore by lateral diffusion. A mild trypsin-EDTA treatment was devised to test the latter hypothesis. The progress of trypsinolysis could be monitored by fluorescence microscopy, since exposure to trypsin caused the cheek cells to adopt a spherical appearance (Fig. 2*b*).

FRAP measurements were performed on the trypsin-treated cells and the results are shown in the Table. A threefold increase in the measured diffusion coefficient was observed along with a 29% increase in the extent of recovery as shown by the data curves in Fig. 3. Both these changes were determined to be significant ($P < 0.05$). In contrast, there were no significant changes detected in either diffusion coefficient or percentage recovery following treatment of the cells with EDTA alone.

The fluidity properties of the plasma membranes of cheek cell samples were also assessed by steady-state

anisotropy measurements. The steady-state fluorescence anisotropy measured with TMA-DPH in human cheek cells at 37°C was 0.270. Surprisingly, few anisotropy measurements with the same probe, performed on intact cells at this physiological temperature, appear in the literature. However, a value of 0.249 has been reported for platelets (Kitagawa et al., 1991) and 0.220 for isolated hepatocytes (Benedetti et al., 1989). Most measurements have been carried out at lower temperatures. For example, the anisotropy at 25°C for L929 cells was 0.289 (Kuhry et al., 1985), and for erythrocyte membranes at the same temperature was 0.302 (Deguercy et al., 1986). The comparatively small difference between these observed anisotropy values at the lower temperature would seem to indicate that cheek cell membranes may not be as fluid as those of other cell types. The data from steady-state fluorescence anisotropy showed no significant change in membrane fluidity across all the conditions tested.

Discussion

Detection of only partial recovery of fluorescence following photobleaching of the fluorescent lipid analogue in the plasma membrane of control human cheek cells indicated the presence of two lipid fractions in the membrane, one mobile, the other immobile. We have demonstrated that the barrier to diffusion is proteinaceous in nature, responsible for the maintenance of cell shape, and at least partially accessible to digestion and elimination by trypsin in the extracellular suspension medium.

The changes in membrane properties were only detectable by the FRAP technique and not by steady-state fluorescence anisotropy. The explanation for this lies in the fact that TMA-DPH steady-state fluorescence anisotropy is sensitive to changes in the packing (Pottel, Meer & Herreman, 1983) of lipid molecules in the membrane, whereas FRAP measures the freedom of molecules to diffuse laterally in the membrane (Bloom & Webb, 1983). Therefore, trypsin treatment only increases the diffusion of lipid molecules in the plane of the membrane without affecting the lipid-lipid interactions in the membrane.

Incomplete recovery of fluorescence detected by the FRAP technique applied to the measurement of lipid diffusion has been attributed to plasma membrane regionalization (Gumbiner & Louvard, 1985; Yechiel & Edidin, 1987; Tocanne et al., 1989; Edidin, 1990; Edidin, 1992; Tocanne, Dupou-Cezanne & Lopez, 1994). The mechanisms of regionalization vary between cell type but the most likely cause in the cheek cell is the interaction of the extracellular matrix (Wade & Coleman, 1989) molecules with the membrane. Numerous studies have shown that the cell shape, D and $\%R$ can be modulated by the exogenous ECM components (Packard et al., 1984; Nakache et al., 1985; Tournier, Lopez & Tocanne, 1989).

Invariably, detachment of the cell from the extracellular matrix causes rounding-up, as observed in the present study following trypsin digestion. Thus, this protease appears to destroy the interactions between the extracellular matrix and the membrane in the cheek cell system.

The effects of ECM components on D and $\%R$ in FRAP measurements of membrane lipid properties depend on cell type. Packard et al. (1984) demonstrated that when MDCK cells were cultured on substrata consisting of collagen, fibronectin or laminin, $\%R$ for the lipid probe used was reduced by 45.7, 57.6 or 42%, respectively. However, D for the lipid probe increased by 65 or 66% for cells grown on collagen or fibronectin but remained unchanged for cells grown on laminin matrix. In contrast, Nakache et al. (1985) using primary cultures of bovine pulmonary artery endothelial cells, showed no change in D and $\%R$ of a lipid probe in membranes of cells grown on collagen or fibronectin-coated coverslips. However, cells grown on glass coverslips coated with ECM extract showed an eightfold decrease in D.

The removal of barriers to lipid diffusion by enzyme digestion has been attempted for sperm, keratocytes and yeast. Wolf, Lipscomb and Maynard (1988) demonstrated that when ram sperm were treated with a nonspecific protease (pronase), no change in the D or $\%R$ of the lipid probe was observed. They ascribed the immobile lipid fraction to lateral phase separation of the lipid in the membrane. Similarly, keratocytes (Lee et al., 1993) treated with an enzyme that removes components of the glycocalyx (heparinase) also showed no change in D or $\%R$. In contrast, trypsin-treated yeast plasma membrane (Greenberg & Axelrod, 1993) did show an increase in D and $\%R$ of the lipid probe which is in keeping with the findings of this study on human cheek cells. However, prior to FRAP measurements on the yeast plasma membrane, the cell wall was removed by zymolase digestion followed by trypsin treatment of the exposed plasma membrane. This treatment resulted in increased lateral diffusion in the membrane of the yeast but the reasons for this were unclear. The most likely explanation was the removal of membrane protein, but the possibility that cell wall fragments remain after zymolase treatment which are further disrupted by trypsin treatment cannot be ruled out.

In conclusion, cheek cells isolated by the method of McMurchie et al. (1984) have surface structures which hinder lateral diffusion. This is an important finding that will need to be taken into account in future studies of the influence of dietary lipid on the biophysical properties of cheek cell plasma membrane, since changes caused by lipid manipulation may be masked by the effect of the ECM.

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