ELECTROPHORESIS AND DIFFUSION IN THE PLANE OF THE CELL MEMBRANE

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ABSTRACT Electrophoretic and diffusional movements of concanavalin A (Con A) receptors and acetylcholine (ACh) receptors in the plane of the plasma membrane of mononucleate, spherical *Xenopus* myoblasts were studied by microfluorimetry and iontophoresis. We found that (*a*) a uniform electric field of 10 V/cm applied along the cell surface produces a partial accumulation of both types of receptors toward the cathodal pole of the cell within 30 min; (*b*) post-field relaxation of the culture results in the complete recovery of the uniform distribution of the Con A receptors within 10 min; and (*c*) in contrast to the Con A receptor in general, accumulation of ACh receptors by the electric field results in the formation of stable, localized receptor aggregates. Theoretical analyses were carried out for the distribution of charged membrane receptors at equilibrium between electrophoresis and diffusion, and for the rate of back diffusion after the removal of the field. These analyses indicated that, at 22°C, the average electrophoretic mobility of the electrophoretically mobile population of the Con A receptors is about 1.9×10^{-3} μm/s per V/cm, while their average diffusion coefficient is 5.1×10^{-9} cm^2/s.

INTRODUCTION

The most convincing evidence that the cell membrane is basically fluid in structure comes from studies that showed that many macromolecular components undergo long-range movement in the plane of the cell membrane. One such movement, the translational diffusion of membrane proteins and cell surface receptors, has been demonstrated in a number of cell types (1–11). Recently, we have shown that concanavalin A (Con A) receptors, presumably cell surface glycoproteins, can be redistributed along the cell surface by an externally applied electric field in a manner consistent with the notion that they undergo passive electrophoretic movement (12, 13). In these studies, the redistribution process of the Con A receptors was examined by scoring the number of cells showing asymmetrical staining with fluorescently labeled Con A. The methodology entailed visual comparison of fluorescence intensities on two sides of the cell (which faced the two poles of the electric field) before and after exposure to the electric field. In the present report, redistribution of the Con A receptors, as revealed by post-field fluorescence labeling, was studied by microfluorimetry. This approach enabled a more quantitative assessment of the distribution of Con A receptors on the cell surface. The theoretical analysis given in this report also provides a framework for more accurately determining the average electrophoretic mobility and diffusion coefficient of these

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receptors. In addition, we describe an electrophysiological method for studying the movement of a specific membrane protein or protein complex, the acetylcholine (ACh) receptor, in the embryonic muscle cell membrane. The distribution of ACh receptors was examined by mapping the sensitivity of various points of the cell surface to ACh ejected from a micropipette. The findings indicate that the ACh receptors also undergo electrophoretic redistribution in the plane of the cell membrane. However, in contrast to Con A receptors in general, the accumulated ACh receptors did not back-diffuse after the field was removed. Instead, they remained localized as stable receptor aggregates.

METHODS

Cell Culture and Electrophoresis Apparatus

Embryonic muscle cells were obtained by dissociating the neural tube region of 1-d-old *Xenopus laevis* embryos. Cells were plated as a monolayer on clean glass electrophoresis chambers (Fig. 1) and were used for experiments after 2–3.5 d in culture. Culture medium contained 85% Steinberg's saline (14), 10% Leibovitz medium (L-15, Grand Island Biological Co., Grand Island, N.Y.), and 5% fetal calf serum (Grand Island Biological Co.); pH of the medium was 7.8. After 2 d in culture, embryonic myoblasts of two distinct morphologies were observed: extended, spindle shapes and spherical shapes. All experiments were carried out on the isolated, spherical, mononucleate cells (diameter 35 ± 5 μm SD, 150 cells measured) that adhered firmly to the culture substratum. Electrophoresis chambers were made from microscope slides and pieces of no. 1 cover glasses. A defined chamber geometry (60 × 10 × 0.2 mm) was obtained by sealing the sides of the chamber permanently with Silastic sealant (Dow Corning Corp., Midland, Mich.) and the top cover glass with silicon grease during electrophoresis. The typical current of 1.5 mA, which produced a field of 10 V/cm (10.3 ± 1.6 V/cm, SD, 47 chambers measured) along the culture substratum, was delivered to the chamber through two agar bridges. Except where indicated, all experiments were carried out at room temperature (22 ± 1°C). For further details of the culture method and apparatus, see reference 13.

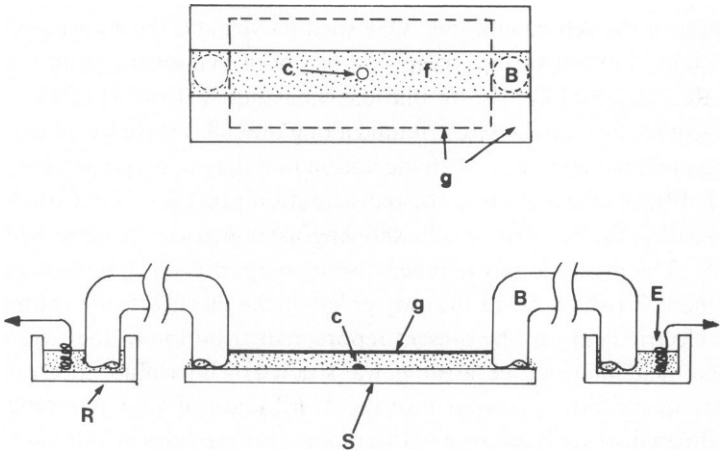

FIGURE 1 Electrophoresis apparatus (top and side view, not drawn to scale). *g*, No. 1 cover glasses; *S*, microscope slide, *f*, fluid-filling groove (60 × 10 × 0.2 mm); *c*, cultured spherical myoblasts adhered to the glass; *B*, glass agar bridges filled with saline gelled with 2% agar; *R*, saline reservoirs; *E*, Ag-AgCl current supplying electrodes.

Fluorescence Labeling and Microfluorimetry

Fluorescence labeling of cell surface Con A receptors was carried out at 0°–4°C for 15 min, with Steinberg's saline containing 50 μg/ml of Con A conjugated with tetramethylrhodamine isothiocyanate (TMR-Con A; Vector General Inc., Woodland Hills, Calif.). When labeling was completed, cells were fixed immediately with cold acetone (0 to −4°C) and preserved in 100% glycerol. The TMR-Con A labeling was specific, since it was completely blocked by the presence of 0.1 M α-methyl-D-mannoside, one of the sugars that specifically binds Con A. Microfluorimetry was carried out on a Zeiss universal fluorescence microscope fitted with a PM1 photometer (Carl Zeiss, Inc., New York). Segments of the fluorescent ring stain were sampled through a 8-μm-diameter aperture at various points around the cell perimeter. Fluorescence intensity collected by the photomultiplier was recorded on a digital indicator. The ring stain on the spherical cells was sampled either at the two poles of the cells facing the cathode (180°) and the anode (0°) of the applied field or at 30° intervals around the perimeter. Background fluorescence intensity, measured by shifting the measuring aperture to the adjacent cell-free region, was subtracted from all ring stain intensity measurements. Since we were concerned only with the relative fluorescence intensities over the surface of each cell, the intensity at 180° pole was arbitrarily set at 100%. Intensities at other positions, therefore, are expressed as a percentage of the 180° pole reading. The asymmetry of fluorescence stain on each cell was normalized by determining the asymmetry index (A) defined in the following formula:

$$A = (I_{180} - I_0)/(I_{180} + I_0), \tag{1}$$

where I_{180} and I_0 are intensities measured at the 180° and 0° poles, respectively. This formula gives an asymmetry index of 0.33 when the ratio of absolute intensities at two poles is 2:1.

Iontophoretic Mapping of ACh Receptors

Intracellular recording used glass microelectrodes filled with 3 M potassium acetate (resistances 120–200 MΩ). Micropipettes for iontophoretic application of ACh were filled with 3 M acetylcholine chloride (Sigma Chemical Co., St. Louis, Mo.), had resistances from 200 to 350 MΩ, and required braking currents between 1.0 and 2.0 nA to prevent ACh leakage. Currents ejected from the ACh pipettes were measured with a current-to-voltage converter that kept the bath at virtual ground. All electrophysiological recording was done in pure Steinberg's saline supplemented with an additional 10 mM $CaCl_2$ to stabilize electrode penetrations. To determine the ACh sensitivity of a particular area on the cell surface, the ACh pipette was positioned by approaching the cell perimeter until a slight dimple appeared at the desired site of mapping. The ACh electrode was then backed up until the dimple disappeared. Under this condition, current pulses of 0.5 ms duration and graded amplitudes delivered from the ACh pipette produced graded ACh potentials with rise times between 6 and 15 ms (av. ≈9 ms). Peak depolarizations of the ACh-produced potentials were plotted against the number of coulombs ejected from the ACh pipette. The linear portions of the curves connecting the data points were used to express ACh sensitivity in volts per nanocoulomb. The measured volt per nanocoulomb sensitivities were quite reproducible for each cell. For example, for 17 determinations of the 0° pole of a control cell made through repeated repositioning, the volt per nanocoulomb values ranged from 5.0 to 8.3 V/nC and averaged 6.75 ± 0.42 V/nC (95% confidence limits).

THEORETICAL ANALYSIS

The Model

The present analysis of the electrophoresis and diffusion of membrane-bound charged molecules is based on a single model depicted schematically in Fig. 2 A. The charged portion of the molecule that senses the external field is represented by a rigid sphere possessing a uniformly distributed surface charge density, arbitrarily chosen as positive. The hydrophobic

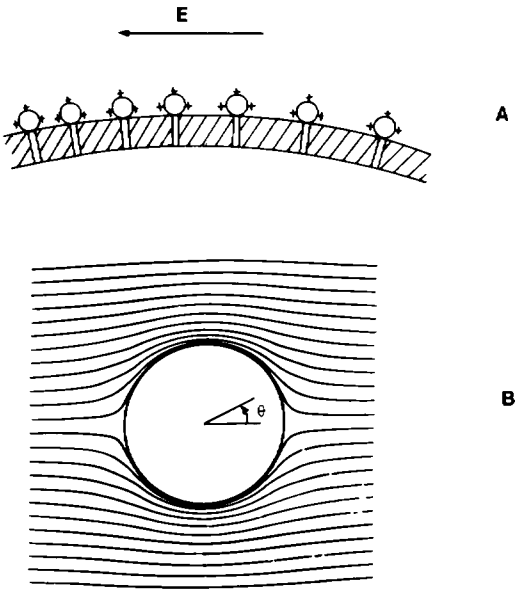

FIGURE 2 Simple model for electrophoresis in the plane of cell membrane. A. Charged membrane-bound molecules migrate in the plane of cell membrane in response to an external electric field tangent to the cell surface. Equilibrium distribution of molecules on the cell surface is achieved when the fluxes of electrophoretic migration (to the left) and back-diffusional transport (to the right) become equal. Charges on the molecules were arbitrarily chosen as positive. B. Field lines around a nonconducting sphere placed in uniform electric field (modified from Cole, reference 15). Tangential field at the cell surface is related to the uniform field E_0 by Eq. 2.

segment of the molecule is pictured as a bar deeply embedded in the membrane. The interaction between the adjacent molecules will not be considered (see Discussion).

A uniform electric field E_0, when applied to a spherical cell in conducting fluid, will be slightly distorted by the cell (Fig. 2 B). The tangential field E_θ at the cell surface, which produces the effective electrophoretic force, is

$$\mathbf{E}_\theta = fE_0 \sin\theta \cdot \hat{\mathbf{e}}_\theta, \tag{2}$$

where f is a numerical factor ($f = 1.5$ for a nonconducting sphere, see reference 15), θ is the polar angle, $\hat{\mathbf{e}}_\theta$ is the unit polar vector, and E_0 is the magnitude of the field applied. We further assume that the adhesion of the cell to the culture chamber substratum does not distort significantly the spherical shape of the cell; hence the processes of electrophoresis and diffusion can be considered to proceed with azimuthal symmetry.

Equilibrium Analysis

Under the influence of a steady electric field E_0, the distribution of the molecules at the cell surface reaches an equilibrium state when the fluxes of electrophoretic migration and back diffusion become equal, as described by the equation

$$m\mathbf{E}_\theta C_\theta(\theta) = D\nabla C_e(\theta)$$

or

$$\frac{\partial C_e(\theta)}{\partial \theta} - \frac{mfE_0 r}{D} \sin\theta\, C_e(\theta) = 0, \tag{3}$$

where $C_e(\theta)$ = surface density distribution at equilibrium, m and D are the electrophoretic mobility and diffusion coefficient of the molecule, respectively, and r is the cell's radius. Solving Eq. 3, we obtain

$$C_e(\theta) = \alpha \exp[-\beta(1 + \cos\theta)], \tag{4}$$

where $\beta = mfE_0 r/D$ and α is a constant determined by the boundary condition: $\int_0^\pi C_e(\theta) \cdot 2\pi r^2 \sin\theta\, d\theta = 4\pi r^2 C_0$, where C_0 is the uniform surface density of the molecules before the start of the electrophoresis. Similar analysis of the equilibrium distribution of charged membrane-bound molecules under the influence of external field has been reported previously (16).

Back-Diffusion after the Field is Removed

When the field is removed after the equilibrium distribution has been reached, the back diffusion process is described by the diffusion equation $\partial C/\partial t = D\nabla^2 C$,

or

$$\frac{\partial C(\theta, t)}{\partial t} = \frac{D}{r^2 \sin\theta} \frac{\partial}{\partial \theta}\left[\sin\theta \frac{\partial C(\theta, t)}{\partial \theta}\right]$$

The solution to the above equation can be written as

$$C(\theta, t) = \sum_{l=0}^{\infty} \alpha K_l P_l(\cos\theta) \exp[-Dl(l + 1)t/r^2],$$

where $P_l(\cos\theta)$ is the Legendre polynomial of l^{th} order, K_l's are constant coefficients, and $C(\theta, t)$ satisfies the initial condition of equilibrium distribution described by Eq. 4, i.e., $C(\theta, 0) = C_e(\theta)$,

or

$$\sum_{l=0}^{\infty} K_l P_l(\cos\theta) = \exp[-\beta(1 + \cos\theta)],$$

and the coefficients K_l's can be obtained by integration

$$K_l = \frac{2l + 1}{2} \int_{-1}^{1} P_l(x) \cdot \exp[-\beta(1 + x)]\,dx, \tag{5}$$

(where $x = \cos\theta$). The decay of asymmetry index during the back diffusion process can be predicted by

$$A(t) = [C(180°, t) - C(0°, t)]/(C(180°, t) + C(0°, t)]$$

$$= -\sum_{l=\text{odd}} K_l \exp[-Dl(l + 1)t/r^2] / \sum_{l=\text{even}} K_l \exp[-Dl(l + 1)t/r^2], \tag{6}$$

since $P_l(1) = 1$ and $P_l(-1) = (-1)^l$.

Summary

The above simplified calculations provide a mathematical framework for a first-order estimate of the electrophoretic mobility (m) and diffusion coefficient (D), based on information on the equilibrium distribution of receptors and the post-field decay of the asymmetry index. The value of β which best fits the equilibrium distribution data can be used in Eq. 6 to determine D. The D value obtained can then be used to calculate m from β.

RESULTS

Electrophoretic Accumulation of Con A Receptor

When cells were exposed to a field of 10 V/cm (corresponding to a potential drop of 35 mV across a cell 35 μm in diameter) for 10 min and labeled with TMR-Con A after the field was removed, a definite accumulation of stain was observed on the side facing the cathode of the field (180° pole). Cells exposed longer in the same field (1.5 h) had a stronger accumulation. Up to a 2:1 difference in fluorescence intensity was observed on two poles of the cells exposed to this field for 4.5 h. To assay the degree of accumulation produced by the field, we determined the asymmetry index by the formula shown in Eq. 1. Fig. 3 shows the asymmetry index plotted against duration of the field (10 V/cm) obtained from experiments on 25 separate cultures (circles). Production of an asymmetric distribution of Con A receptors was rapid, as shown by the rise of the asymmetry index, which reached a plateau of about 0.2 after 30 min in the field.

The asymmetric distribution of TMR-Con A staining after exposure to the field was not induced by Con A binding or cross-linking. When the cells were fixed with 1.5% glutaraldehyde (Ladd Research Industries, Burlington, Vt.) immediately after the removal of the field but before TMR-Con A labeling, the same accumulation was observed (Fig. 3, filled square). In addition, the following evidence strongly suggested that the accumulation of fluorescence stain was due to the redistribution of preexisting Con A receptors on the cell surface, rather than to preferential synthesis-insertion and/or selective degradation of the receptors. First, pre-field fixation of the culture with 1.5% glutaraldehyde for 15 min completely prevented the post-field asymmetric staining (Fig. 3, open square). Secondly, no accumulation of fluorescence stain was observed when the cultures were labeled with TMR-Con A before the application of the field (Fig. 3, filled circles). It is known that Con A binding immobilizes cell surface receptors (5, 7, 8), presumably through the cross-linking of its receptors. These results suggest that immobilization of the Con A receptors either by glutaraldehyde fixation or by Con A binding prevented the redistribution of the preexisting Con A receptors by the field. Furthermore, pre-field treatment of the culture with neuraminidase (Sigma, grade VI, 0.1 U/ml) for 1 hr at pH 6.6 resulted in a reversal of the accumulation polarity. Post-field labeling now showed asymmetric accumulation of stain toward the anodal pole of the field (Fig. 3, open triangles). Finally, we have previously shown (13) that treatment of the muscle cells with metabolic inhibitors had no effect on the accumulation of the Con A receptors. Cell locomotion and rotation were also not involved in the field-induced asymmetry of Con A receptor distribution (see Fig. 11). All the above findings are consistent with the notion that Con A receptors undergo passive electrophoretic movement in the plane of the cell membrane in the presence of external electric field. Immobilization of receptors prevents

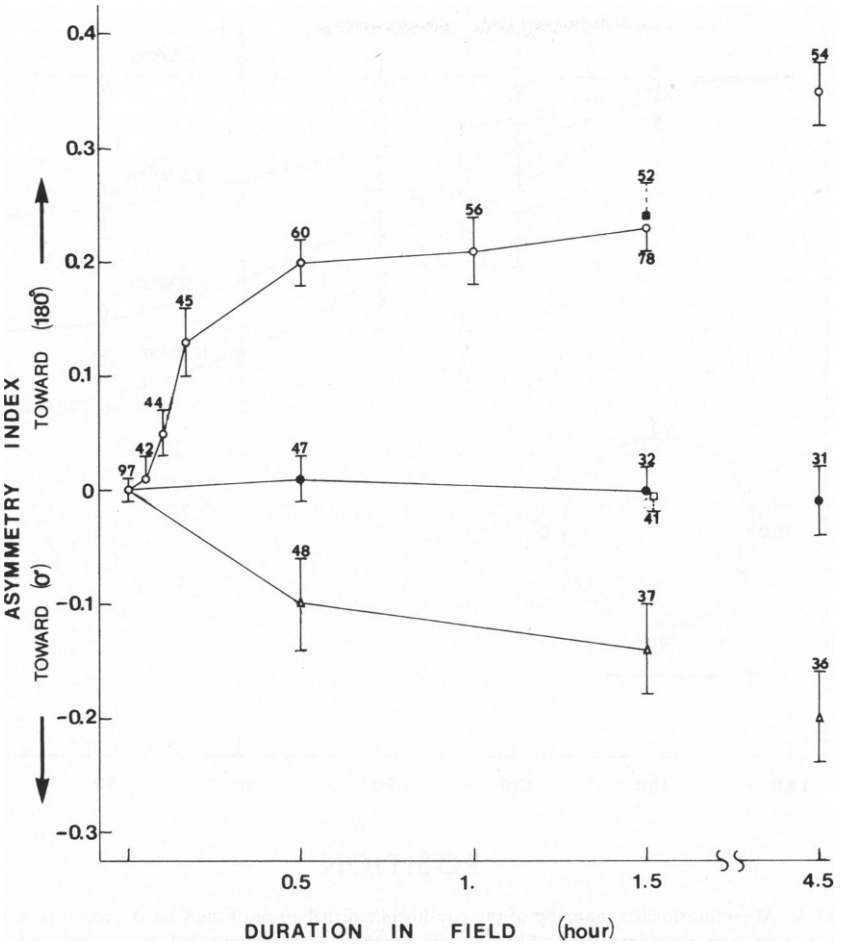

FIGURE 3 Asymmetric accumulation of the Con A receptor by fields of different durations. Field strength was 10 V/cm. Asymmetry indices were determined from pairs of measurements at 180° (cathodal) and 0° (anodal) poles for each cell by the formula in Eq. 1. The data points represent average indices for all cells of the same duration. Error bars represent 95% confidence limits, and the number of cells examined is shown at the top of each error bar. —○—, no treatment; —●—, cells preincubated with Con A; —△—, cell preincubated with neuraminidase; ■, cells fixed with glutaraldehyde before post-field labeling; □, cells prefixed with glutaraldehyde.

field-induced movement, while modification of the surface charge by removing the sialic acid from the cell surface (17) greatly influences the direction of movement.

Equilibrium Distribution under Various Field Strengths

Fig. 3 shows that the accumulation of the Con A receptors reached a plateau value after 0.5 h in the field, indicating that the redistribution has reached equilibrium state. If the redistribution was electrophoretic in nature, the topography of receptor distribution over the cell surface should agree with the characteristics of equilibrium distribution predicted by theoretical analysis given in the previous section. Fig. 4 depicts results from microfluorometric measurements of relative intensities of the TMR-Con A ring stain at 30° intervals around the

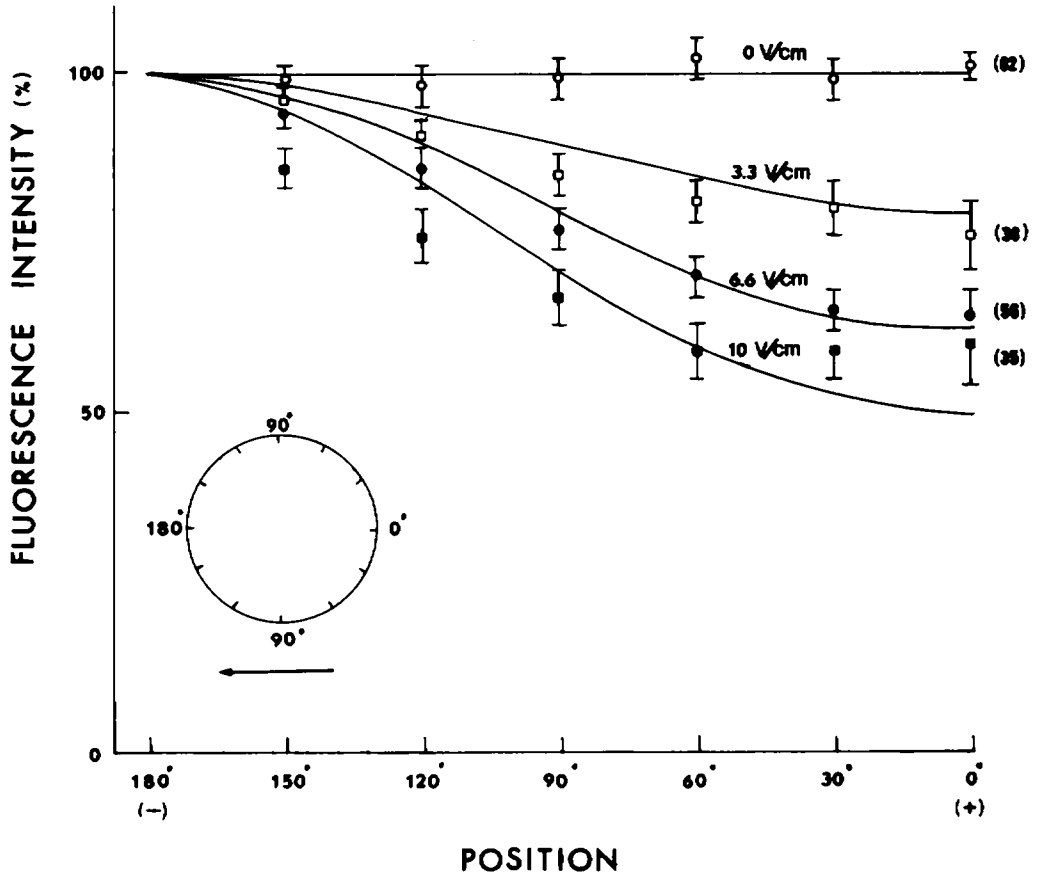

FIGURE 4 Microfluorimetric mapping of the equilibrium distribution of the Con A receptors after exposure to fields of various intensities. Fluorescence intensities were monitored by a photomultiplier (Hamamatsu R446, Hamamatsu Corp., Middlesex, N.J.) with 8-μm-diameter aperture focused on the perimeter of the cells. Ring-staining by TMR-Con A labeling was measured at 30° spacing around the whole perimeter. Data at corresponding angles with respect to the chamber and field axis (0°–180°) were grouped together. The sequence of measurements around the perimeter of each cell was performed at random to minimize the error introduced by slight bleaching during the examination. The intensities measured were normalized for each cell by setting the 180° measurement to be 100%. Each data point represents average of measurements for at least 35 cells from 3 or more separate cultures. Error bars indicate 95% confidence level. The cells were exposed to a field of 0, 3.3, 6.6, and 10 V/cm, respectively. The arrow indicates the direction of the field. Note that the downward shift of relative intensities does not represent the decrease of total number of receptors. The number of cells examined is shown in parenthesis associated with each curve. The curves were drawn using Eq. 7 in the text. No immobile Con A receptors was assumed ($B = 0$). $\beta = 0$, 0.12, 0.23, and 0.35 for 0, 3.3, 6.6, and 10 V/cm fields, respectively.

perimeter of the spherical muscle cells. For control cells not exposed to the field, the intensities were essentially equal around the perimeter. For cells exposed to a field of 3.3, 6.6, or 10 V/cm for 1.5 h, the intensity distribution became progressively more asymmetric. To compare the results with the theoretical predictions, the equilibrium distribution described by Eq. 4 is normalized into the following form:

$$I_m(\theta) = I_c(\theta) + I_B = K[C_c(\theta) + C_B],$$

or

$$I_m(\theta) = (100 - B)\exp[-\beta(1 + \cos\theta)] + B, \quad (7)$$

where $I_m(\theta)$ is the measured fluorescence intensity at position θ; $I_e(\theta)$ and I_B are the fluorescence intensity due to the electrophoretically mobile and immobile fractions of the Con A receptors, respectively; K is a constant relating the fluorescence intensity to the surface concentration of the receptors, and B ($0 \leq B \leq 100$) is the percent background intensity measured at 180° pole due to the immobile Con A receptors. The value B can be estimated from the maximum asymmetry index (A_m) produced by an electric field, by using Eq. 1 and assuming the remaining I_0 is due exclusively to immobile fraction of Con A receptors, i.e., $A_m = (100 - B)/(100 + B)$. We found that the maximum asymmetry index (A_m) produced by fields of 10 V/cm or higher strengths was about 0.35 (Lam and Poo, unpublished observations; see also Fig. 3). This gave a B value of about 50, i.e., there exists about a 50% constant background fluorescence for all positions measured.

Fig. 4 shows an example of curve fitting with Eq. 7, in which no immobile fraction of the receptors is assumed ($B = 0$). It is clear that the prediction fits the data poorly. Two noticeable difficulties are the spacing between the curves for various field strengths and the curvature near 0°. Fig. 5 shows an example of curve fitting using $B = 50$ and $\beta = 0$, 0.32, 0.64, and 0.96 for data of 0, 3.3, 6.6, and 10 V/cm field exposure, respectively. Except for the intensity near the pole of the cell where receptors were accumulated by 10 V/cm field, the data agreed reasonably well with the theoretical prediction. This agreement strongly suggests the electrophoretic nature of Con A receptor migration and the existence of an appreciable fraction of electrophoretically immobile Con A receptors.

Back Diffusion of Con A Receptors

Our previous study (13) demonstrated that the accumulation of the Con A receptors was reversible after mild accumulation was produced by a short-duration field. After removing the field, if we allowed the cells to remain in regular saline for 30 min before fluorescent Con A labeling, uniform distribution of the Con A receptors was observed. This recovery movement of the receptors was also shown to be a passive process, independent of the cellular metabolism. In the present study, the asymmetry indices were determined for cultures allowed to relax for different durations from the time of field removal to the time of TMR-Con A labeling. All cultures were initially exposed to a field of 10 V/cm for 30 min at 22°C, then incubated at three different temperatures (22°, 10°, and 0°C) for various periods during post-field relaxation. The results are shown in Fig. 6. The decay of asymmetry indices demonstrates the recovery of uniform receptor distribution. The rate of recovery showed strong temperature dependence. For cultures relaxed at room temperature (22°C), the cells recovered their uniform distribution of the Con A receptors within 10 min. Much slower recovery was observed for cultures relaxed at 10°C, and insignificant recovery occurred for cultures relaxed at 0°C.

From the asymmetry index decay rates, one can estimate the average diffusion coefficient of the electrophoretically accumulated Con A receptors. Eq. 6, from the theoretical analysis, predicts a time dependency of asymmetry index. The asymmetry index, taking into account the contribution of fluorescence intensity by the immobile population of the Con A re-

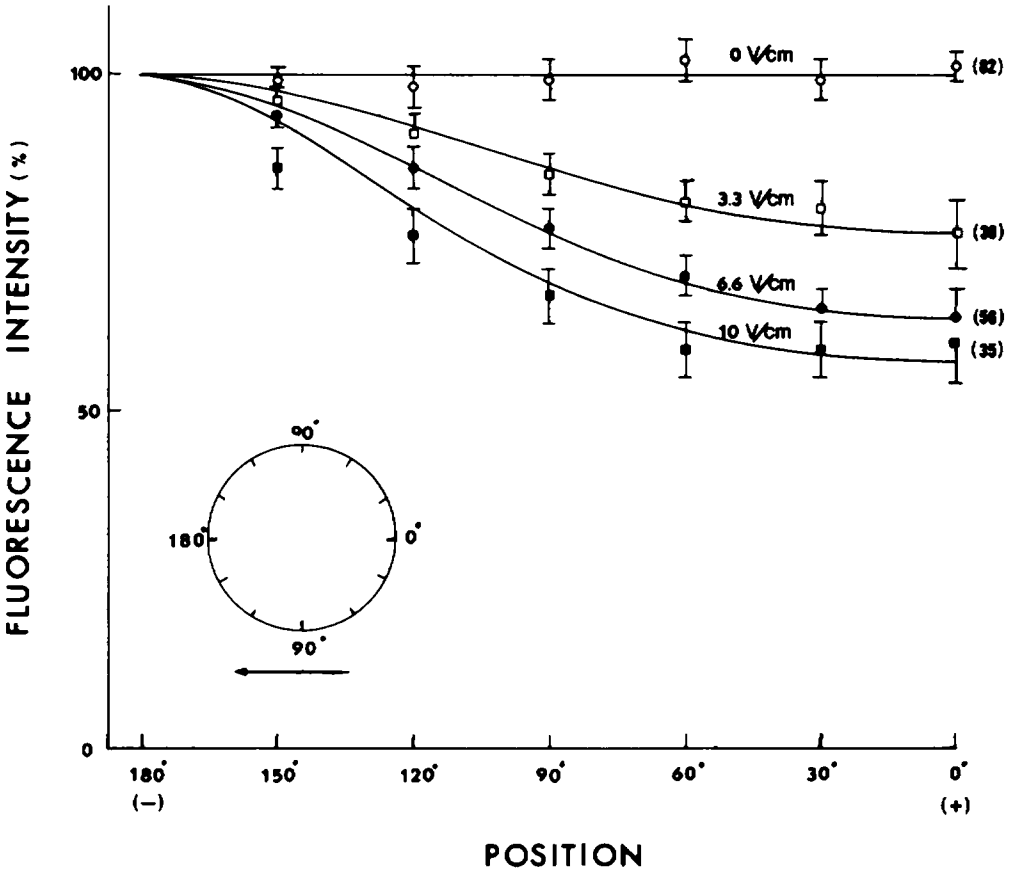

FIGURE 5 The same microfluorimetric data as in Fig. 4. Curve fitting used Eq. 7. 50% of the measured intensity at 180° was assumed to be due to electrophoretically immobile Con A receptors ($B = 50$). $\beta = 0$, 0.32, 0.64, and 0.96 for 0, 3.3, 6.6, and 10 V/cm fields, respectively.

ceptors, becomes

$$A(t) = \frac{-(100 - B)\sum_{l=\text{odd}} K_l \exp(-l(l+1)Dt/r^2)}{(100 - B)\sum_{l=\text{even}} K_l \exp[-l(l+1)Dt/r^2] + B}, \tag{8}$$

where K's are given by Eq. 5, and B ($0 \leq B \leq 100$) is the percent intensity measured at 180° pole due to immobile Con A receptors; $r = 17.5 \pm 2.5$ μm (SD, $n = 150$) is the cell radius; $\beta = 0.96$ and $B = 50$ obtained in the analysis of equilibrium distribution (see previous section) was used to compute K_l's and $A(t)$. Numerical integration of Eq. 5 was carried out for $l = 0$ to $l = 7$. K's obtained, together with values of B and r, were used to calculate $A(t)$ from Eq. 8 for various estimates of diffusion coefficient D. $A(t)$ obtained was then used to fit data shown in Fig. 6. The solid curves (a) were drawn by assuming $D = 5.1 \times 10^{-9}$ cm^2/s. The dashed curves (b) and (c) were drawn with D having values

of 3.4 and 8.5 $\times$ 10^{-9} cm^2/s, respectively, chosen as limits of D that provided acceptable curve fits. The value of 5.1 $\times$ 10^{-9} cm^2/s for D, when substituted into the equation $\beta = mfrE_0/D = 0.96$, yields an electrophoretic mobility m of about 1.9 $\times$ 10^{-3} $\mu m/s$ per V/cm, if the cells are nonconducting spheres ($f = 1.5$). As will be discussed later, the interpretation of this electrophoretic mobility depends crucially on further knowledge of the charge properties of the Con A receptors yet to be elucidated.

In the present study of back diffusion, the initial accumulation of the Con A receptors was produced by brief fields (30 min). When stronger accumulation was produced by longer-lasting fields, the post-field recovery of uniform receptor distribution was incomplete or even absent (13). In the latter situation, electrostatic and electrodynamic forces among charged particles in close apposition (18) may complicate the accumulation and recovery processes. The following will focus on a specific receptor of these muscle cells, the ACh receptor, which is probably a specific subpopulation of the heterogeneous Con A receptors (19). We will demonstrate that the accumulation of the ACh receptors by electric field results in the formation of stable receptor aggregates which persist against back diffusion during the post-field relaxation.

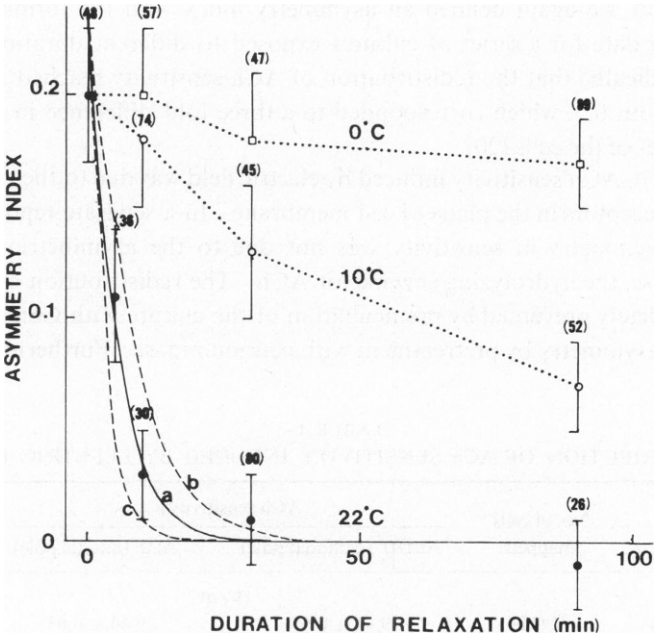

FIGURE 6 Back diffusion of field-induced accumulation of Con A receptors. All cells were exposed to a field of 10 V/cm for 30 min and were then allowed to relax at different temperatures (22°, 10°, and 0°C) for various durations before TMR-Con A labeling. The field was removed at $t = 0$. Rapid decay of asymmetry index was observed for cells relaxed at room temperature (22°C $\pm$ 1°C). Only a slight decay was seen for cultures relaxed at 0°C. Data points represent averages and error bars indicate 95% confidence limits. Numbers associated with each bar indicate total number of cells examined. Dotted curves for 0° and 10°C connect average asymmetry indices observed for various relaxation periods. The curve for 22°C was drawn with Eq. 8, shown in the text. Solid curve (*a*) represents a D (diffusion coefficient) value of 5.1 $\times$ 10^{-9} cm^2/s, dashed curves (*b* and *c*) represent D values of 3.4 $\times$ 10^{-9} and 8.5 $\times$ 10^{-9} cm^2/s, respectively.

Electrophoretic Accumulation of ACh Receptors

In a culture not exposed to electric field, iontophoretic mapping of ACh sensitivity around the perimeter of spherical muscle cell indicated that ACh receptors are uniformly distributed over the cell surface. For 72 cells mapped in 11 separate cultures, the sensitivities were 9.45 ± 0.65 and 9.44 ± 0.63 V/nC for 180° and 0° poles, respectively (Table I). Fig. 7 plots data collected for sensitivity determination on two control cells (circles). The slopes of the curves in Fig. 7 indicate nearly equal sensitivities at two poles of the cells. Perimeter mappings at regular intervals around the half perimeters of six control cells show nearly equal ACh sensitivity distribution over the cell surface (Fig. 8). When a field of 10 V/cm was applied for 30 min to the muscle cells and the ACh sensitivity mapped post-field, we found that ACh sensitivity over the cell surface became asymmetric. For 18 cells mapped in 3 separate cultures, the sensitivities at the cathodal pole (180°) averaged 18.4 ± 3.8 V/nC, while that at the anodal pole (0°) averaged 9.1 ± 1.5 V/nC. Higher asymmetry in sensitivities was found when longer-lasting fields were applied (see Fig. 7 and Table I). The increased ACh sensitivity was always confined to the vicinity of the 180° pole of the cells. This is shown in Fig. 9, which depicts representative perimeter mappings of three cells in a culture exposed for a field of 10 V/cm for 3 h. To assess the degree of asymmetry in ACh sensitivity induced by the field, we again defined an asymmetry index with the formula described in Table I. Mapping data for a series of cultures exposed to different durations of the same field (10 V/cm) indicated that the redistribution of ACh sensitivity reached a plateau asymmetry index of about 0.5, which corresponded to a three-fold difference in the sensitivities at 180° and 0° poles of the cells (20).

The asymmetry in ACh sensitivity induced by electric field was due to the redistribution of preexisting ACh receptors in the plane of cell membrane. In a separate report (20), we have shown that the asymmetry in sensitivity was not due to the asymmetric distribution of acetylcholinesterase, the hydrolyzing enzyme for ACh. The redistribution of sensitivity was found to be completely prevented by preincubation of the culture with Con A, and reversed in the polarity of asymmetry by pretreatment with neuraminidase. Furthermore, the process

TABLE I
REDISTRIBUTION OF ACh SENSITIVITY INDUCED BY ELECTRIC FIELD

Duration of field exposure (10 V/cm)	No. of cells mapped	ACh sensitivity‡		Asymmetry index§
		At 180° (cathodal pole)	At 0° (anodal pole)	
		V/nC		
Control (no treatment)	72 (11)	9.45 ± 0.65	9.44 ± 0.63	0.01 ± 0.02
30 min	18 (3)	18.4 ± 3.8	9.1 ± 1.5	0.33 ± 0.07
1.5 h	42 (5)	18.9 ± 2.3	6.3 ± 1.0	0.48 ± 0.04
3.0 h	16 (4)	16.0 ± 2.4	6.0 ± 1.4	0.44 ± 0.12

*All isolated, spherical cells mapped successfully (membrane potential $|E_m| \geq 80$ mV, $|\Delta E_m| \leq 10$ mV during mapping) were included. Data pooled from separate cultures (number of cultures shown in parenthesis). Since there was no relaxation of the asymmetry after the removal of field (see text and Fig. 10), data were pooled regardless of the exact time the mapping was carried out.

‡Sensitivity presented as (average) ± 95% confidence limits.

§Asymmetry index = (sensitivity at 180° − sensitivity at 0°)/(sensitivity at 180° + sensitivity at 0°), was calculated for each cell before averaging, and presented as (average) ± 95% confidence limits.

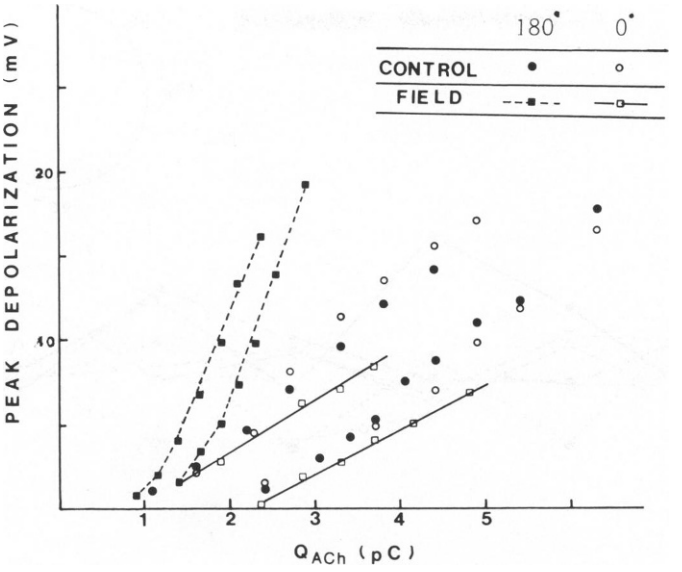

FIGURE 7 Dose-response curves of the muscle cell membrane to applied ACh. Peak depolarizations of ACh potentials were plotted against the picocoulombs of charge ejected from the ACh pipette placed on the cell surface. Lines were drawn by eye through data points from each mapping site. Slopes of the linear portions of the lines were used to obtain the ACh sensitivity at each particular point of mapping in terms of volts per nanocoulomb. The sensitivities were nearly equal at 0° and 180° poles for two control cells (circles), while they became grossly asymmetric for two other cells exposed to a field of 10 V/cm for 1.5 h (squares). $|E_m| \geq 80$ mV, $|\Delta E_m| \leq 10$ mV during the course of mapping for all four cells shown.

of sensitivity redistribution was independent of cell metabolism and was not affected by treatment with cytochalasin B and colchicine, drugs that are known to disrupt cytoskeletal structures. Our recent studies[1] using fluorescently labeled α-bungarotoxin (α-BGT) have clearly demonstrated a field-induced gross redistribution of toxin-binding sites over the surface of these muscle cells. It was also found that the toxin binding did not significantly affect the rate of receptor redistribution. Ligand-receptor complexes on cells prelabeled with fluorescent α-BGT were observed to move during exposure to the field.

Formation of ACh Receptor Aggregates

Unlike Con A receptors in general, ACh receptors appeared to possess an aggregate-forming property. Accumulation of ACh receptors by the field always lead to the formation of stable aggregates resistant against back diffusion. One consistent finding during the ACh sensitivity mapping was that the asymmetry in ACh sensitivity always persisted after the field was removed. Fig. 10 depicts the asymmetry indices for cells mapped at various times after the termination of a 1.5-h field (10 V/cm). Data from four separate cultures were plotted. Weak asymmetry observed for cultures exposed to the same field for only 15 min also persisted after field removal (data not shown). It was further demonstrated that the stability of

[1] Poo, M-m., and W-j. H. Poo. Electrophoresis of α-bungarotoxin receptors in embryonic muscle cell membrane. Submitted for publication.

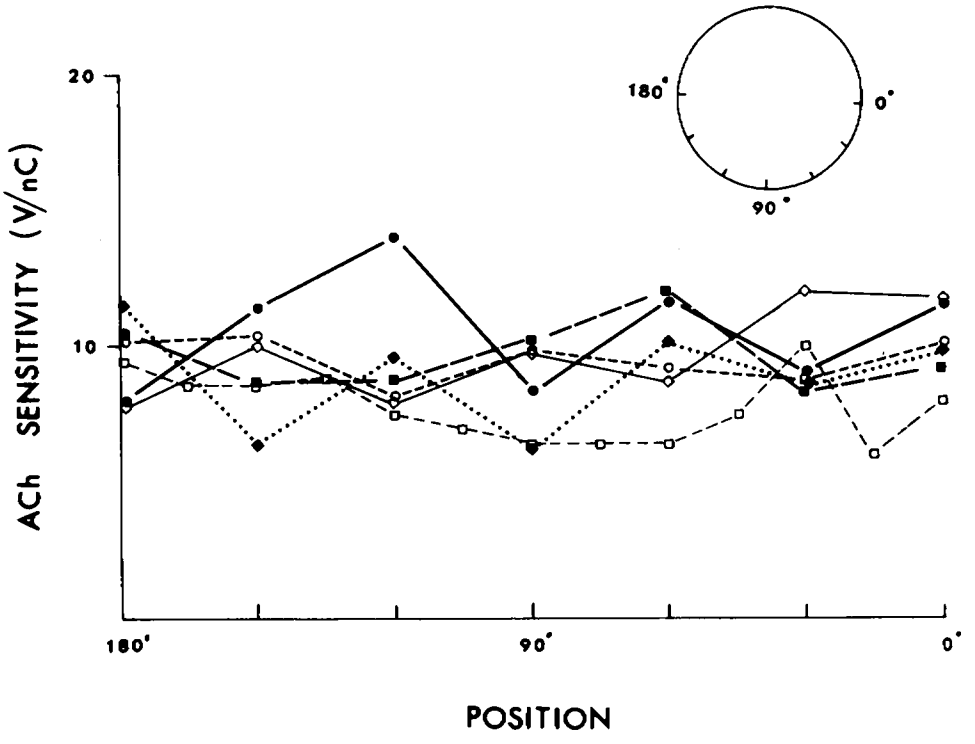

FIGURE 8 Distribution of ACh sensitivity at various points along the half-perimeter of six control cells (not exposed to the field). Different symbols depict data from different cells.

field-induced ACh receptor aggregates was affected by neither depletion of cellular energy nor by treatment with cytochalasin B and colchicine (20). Most recently, experiments using fluorescent α-BGT[1] have shown that accumulation of ACh receptors by the field resulted in the formation of "hot patches" (up to 15 μm in length) at the cathodal pole of the cells (see Fig. 11 E, F), which remained localized at the pole for as long as observations were made (up to 12 h).

Effect of Extracellular Medium Flow

One possible cause for the field-induced receptor redistribution is the physical action of electro-osmotic flow over the cell surface during the exposure to the electric field. The following experiments were performed to determine if the asymmetric distribution of the Con A receptors arose from flow conditions produced by the field. We first examined field-induced particle movement in the extracellular medium near the cell surface. We found that latex beads (0.794 $\pm$ 0.004 μm [SD], Sigma Chemical Co., St. Louis, Mo.) suspended in the medium flowed over the cell surface at average speeds of 3.2 $\pm$ 0.2, 5.0 $\pm$ 0.5, and 10.7 $\pm$ 0.9 μm/s (95% confidence limits, n = 50 each) toward the anode in a 6.6, 10, and 13.3 V/cm field, respectively. Since latex beads themselves are negatively charged, their movement toward the anode may represent a net velocity resultant from a combination of electro-osmotic flow and electrophoresis. However, this observation indicates that the velocity of electro-osmotic flow near the cell surface must be in the same order of magnitude as or much

smaller than 5 μm/s. We then carried out two separate experiments in which flows of different velocities were introduced into the culture chamber in the presence or absence of the field. The results are shown in Table II. In the absence of the field, the cells were exposed for 30 min to medium flowing toward one side of the chamber (by slight elevation of medium reservior on one side). In the same focal plane as the cell perimeter, the latex beads suspended in the medium were observed to move at a speed of either 3.4 $\pm$ 0.8 or 5.4 $\pm$ 0.5 μm/s (rows 1 and 2, Table II). In the absence of imposed flow, a field of 10 V/cm produced a migration of latex beads toward anode at a speed of 5.0 $\pm$ 0.5 or 4.1 $\pm$ 0.6 μm/s, respectively, in two experiments (rows 3 and 4, Table II). Finally, in the presence of both field and imposed flow of various velocities, the resultant velocities of latex beads range from 2.9 $\pm$ 0.2 to -8.5 ± 0.8 μm/s (rows 5–8, Table II). The cultures from these experi-

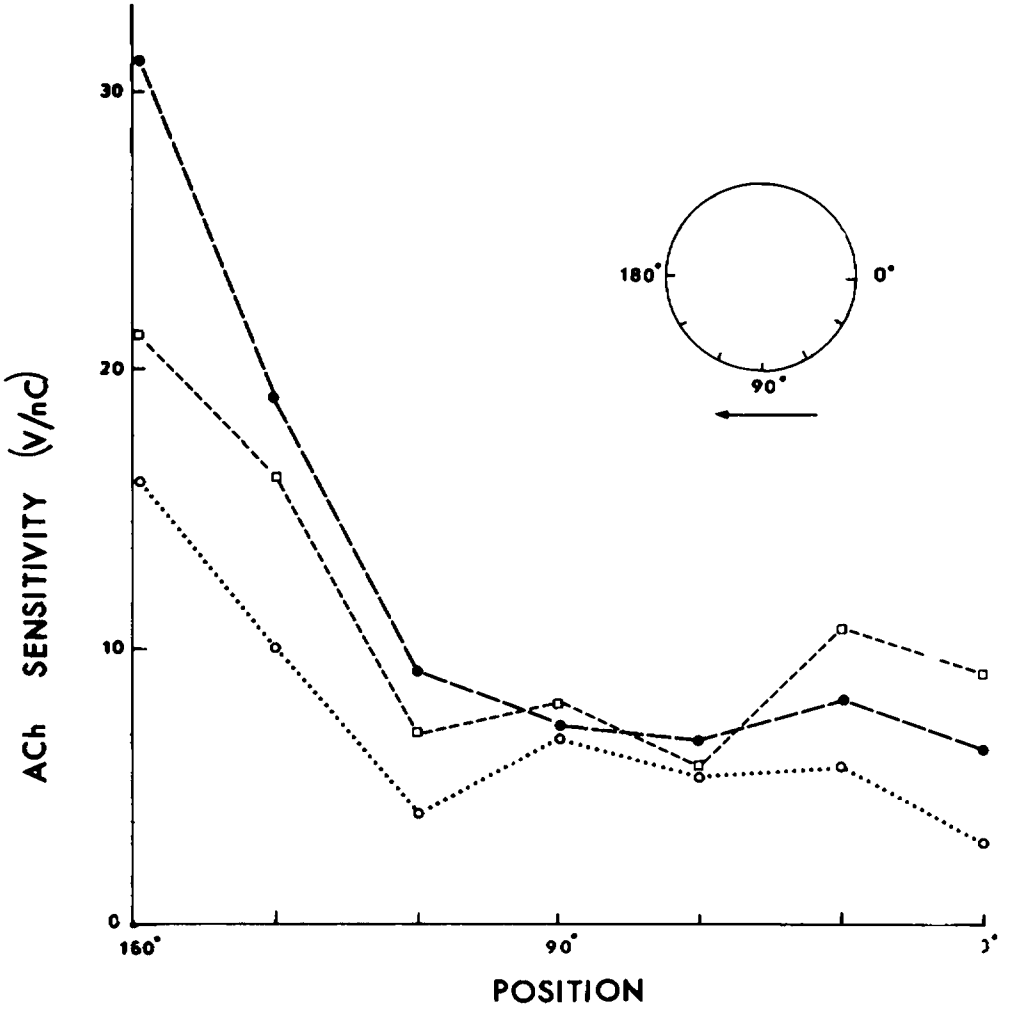

FIGURE 9 Redistribution of ACh sensitivity at various points along the half-perimeter of three cells exposed to an electric field of 10 V/cm for 3 h. Different symbols depict data from different cells. Arrow indicates the direction of the field.

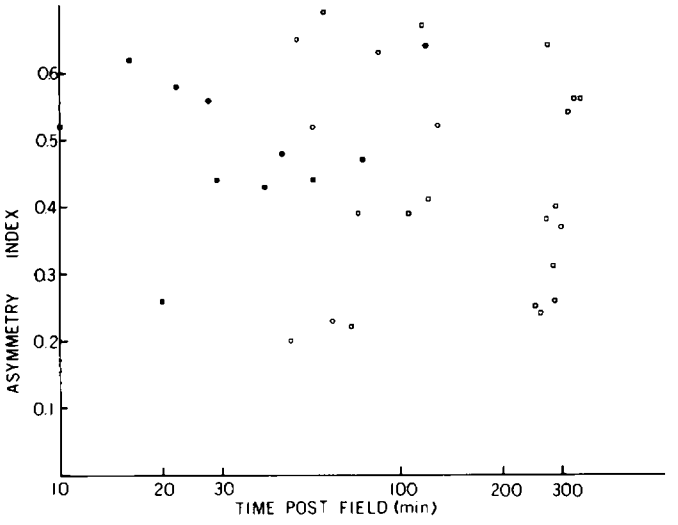

FIGURE 10 Persistence of asymmetric distribution of ACh sensitivity in cells of four separate cultures after removal of electric field at $t = 0$. The asymmetry index, defined by formula shown in Table I, indicated that the asymmetric distribution of sensitivity remained throughout the time of post-field mapping. Different symbols depict data from different cultures.

ments were then labeled with TMR-Con A and the asymmetry of fluorescence ring stain was measured by microfluorimetry. The asymmetry indices obtained are shown in the last column of Table II. The data indicate that medium flow alone failed to produce any significant redistribution, and that gross alteration of the medium flow produced no significant change in the field-induced asymmetric distribution of Con A receptors.

Study of the Cell Locomotion and Morphology

Does the electric field cause the muscle cells to migrate or rotate preferentially? Is there a gross membrane alteration induced by the field? Fig. 11 A and B are representative photographs of a culture taken before and after the exposure to a field of 10 V/cm for 1.5 h. Comparison of the two photographs indicates that, unlike the fibroblast in the culture (broad arrow, Fig. 11 A, B), embryonic muscle cells hardly moved or rotated during exposure to the field. The morphologies of fine processes on the spindle-shaped cells were preserved. The relative disposition of extracellular and intracellular yolk granules was also unchanged. Furthermore, scanning electron microscope studies (Orida and Poo, unpublished observations) have demonstrated that the surface of these muscle cells is remarkably smooth and free of the microvilli frequently seen on other cultured cells. Moreover, no surface alteration was observed on cells after exposure to a field of 10 V/cm for 1.5 h.

DISCUSSION

Heterogeneity of Con A Receptors

Con A receptors on the surface of these muscle cells probably comprise a heterogeneous population of glycoproteins and glycolipids. The maximum value of asymmetry indices induced by electric field was about 0.35 (see Fig. 3). This indicated that roughly only 30–40%

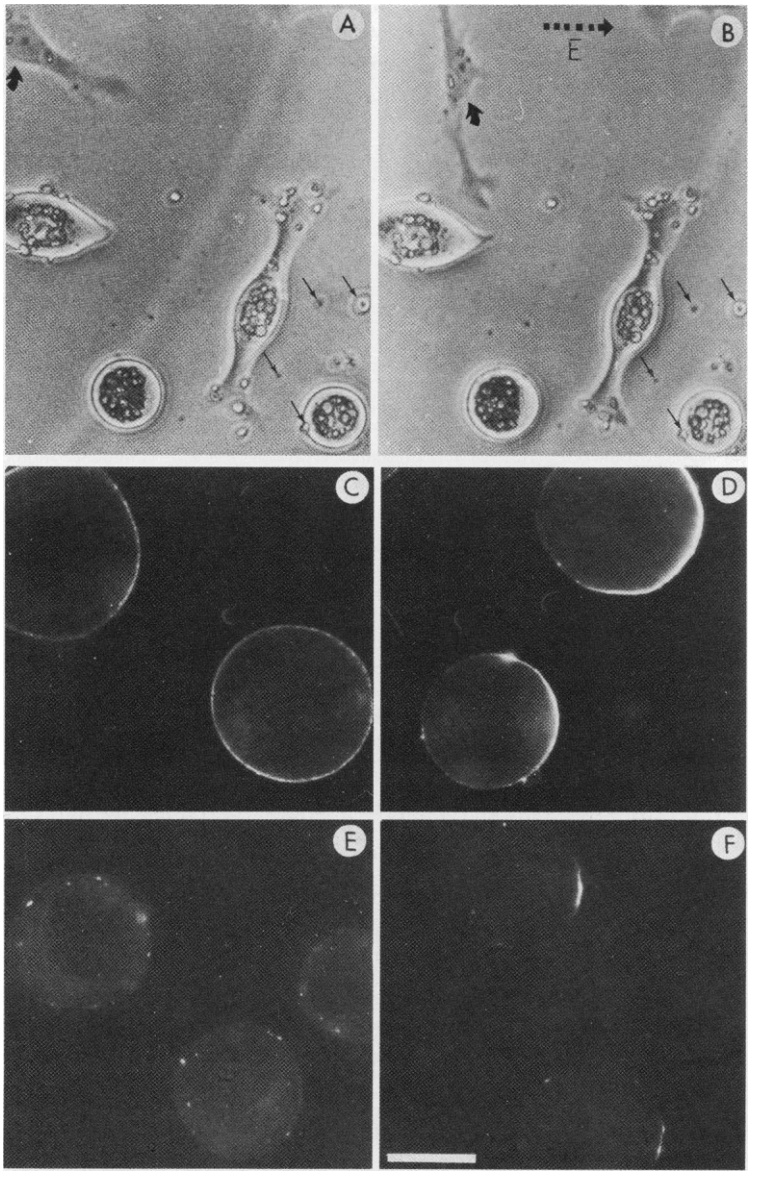

FIGURE 11 (A) Phase-contrast photograph of a *Xenopus* muscle culture before exposure to the electric field. (B) Photograph of the same culture as in A after 1.5-h exposure to a field of 10 V/cm. Dashed arrow indicates the direction of the field. Thin arrows indicate markers on the culture substratum and on the cell surface. Broad arrows indicate the positions of a motile fibroblast before and after the exposure to the field. Comparison of A and B indicates the lack of motility and rotation of both spindle-shaped and spherical muscle cells. (C and E) Fluorescence photographs of control cultures (not exposed to the field) labeled with either TMR-Con A (C) or TMR-α-BGT (E). (D and F) Fluorescence photographs of cultures exposed to a field of 10 V/cm for 1.5 h and subsequently labeled with either TMR-Con A (D) or TMR-α-BGT (F). Note the characteristic ring stain of the majority of the Con A receptors, and the patchy appearance of α-BGT binding sites (presumably the ACh receptors). Field-induced accumulation of Con A receptor showed smooth gradient of intensity along the cell surface, while the accumulation of ACh receptors resulted the formation of discrete patches of receptor aggregates (for details see footnote 1). Bar represents 25 μm.

TABLE II
EFFECT OF EXTRACELLULAR FLOW ON CON A RECEPTOR DISTRIBUTION

No.	Condition	Field strength (30 min exposure)	Particle velocity*	Asymmetry index of fluorescent Con A stain‡
		V/cm	μm/s	
1	Flow only (exp. 1)	0	3.4 ± 0.8 (n = 42)	−0.01 ± 0.01 (n = 42)
2	Flow only (exp. 2)	0	5.4 ± 0.5 (n = 72)	−0.02 ± 0.06 (n = 10)
3	Field only (exp. 1)	10	5.0 ± 0.5 (n = 50)	0.21 ± 0.04 (n = 25)
4	Field only (exp. 2)	10	4.1 ± 0.6 (n = 50)	0.19 ± 0.03 (n = 40)
5	Field and flow (exp. 1)	10	2.9 ± 0.2 (n = 50)	0.18 ± 0.01 (n = 63)
6	Field and flow (exp. 1)	10	0.2 ± 0.3 (n = 50)	0.18 ± 0.02 (n = 50)
7	Field and flow (exp. 2)	10	0.0 ± 0.3 (n = 50)	0.26 ± 0.13 (n = 8)
8	Field and flow (exp. 2)	10	−8.5 ± 0.8 (n = 50)	0.23 ± 0.04 (n = 45)

*Velocities of latex beads near the cell perimeter were presented as (average) ± 95% confidence limits. Positive value indicate the direction of the movement was toward anode. n refers to the number of particles examined.
‡Fluorescence asymmetry indices were obtained by Eq. 1 shown in the text, and presented as (average) ± 95% confidence limits. n refers to the number of cells examined.

of the Con A receptors were redistributed by the field. Three possibilities may account for this finding. Firstly, a large fraction of Con A receptors on these cells is either immobile or has a zero net charge. In fact, that some Con A receptors are immobile in the cell membrane was also shown in two previous studies (5, 9). Secondly, the field induces the receptors of opposite charge to move toward the opposite poles of the cell and the observed asymmetry in fluorescence intensity represents the difference of preferential accumulation at two poles. Thirdly, the field may have immobilized some of the potentially mobile receptors. Equilibrium analysis of the intensity topography shown in Figs. 4 and 5 demonstrated that a constant residual intensity must be considered to best fit the theoretical predictions with the data. Which one or what combination of the above three possibilities accounts for our result remains to be elucidated. Finally, is it possible that the low fraction of electrophoretically mobile Con A receptors we observed consists of only charged glycolipids that bind Con A? This seems unlikely, since ACh receptors, known to be single proteins or protein complexes (19), were rapidly redistributed by the field.

The reversed asymmetry index for neuraminidase-treated cells (Fig. 3) may be accounted for by the heterogeneity of electrophoretically mobile cell surface receptors. The fact that Con A receptors accumulated toward the 180° pole of the cells not treated with neuraminidase may suggest that Con A receptors are positively or less negatively charged than other mobile components that do not bind Con A, the latter accumulated towards the 0° pole. It may be that Con A receptors are, in fact, negatively charged but contain fewer sialic acids than other more negatively charged components. After neuraminidase treatment, they became among the most negatively charged components that accumulated at the 0° pole.

It should be noted, however, that the details of field-induced redistribution of heterogeneous populations of membrane components with different mobilities and charge properties within the closed boundary of the cell surface involve additional factors outside the scope of this report. Such factors include the interaction among various components, the

competition for space between the molecules, and the effects of counterions near the charged moieties.

Interaction among the Membrane Components

The model used in the theoretical analysis assumes no interaction among the charged receptors. Two facts point out that this may be an oversimplification. First, ACh receptors readily form stable aggregates once they are accumulated (Fig. 10). We found that even a 15–30 min exposure to a 10 V/cm field results in persistent ACh receptor aggregates (Orida and Poo, unpublished). Secondly, Con A receptors in general show a lack of back diffusion when cells were exposed to a field of 10 V/cm for more than 1.5 h (13). Whether this attractive interaction during aggregate formation is due to the electrodynamic (van der Waals) force among the cell surface receptors or the establishment of possible extramembraneous "anchoring" organization remains to be elucidated. The electrostatic repulsive interaction during electrophoretic accumulation of identical charged components will be effectively shielded by the ionic atmosphere around the charged moieties until the proximity of receptors reaches electrical double-layer thickness on the order of 10 Å. Further theoretical analyses of electrophoresis and diffusion (Chao and Poo, in preparation) which take into account the electrostatic interaction among the molecules indicate that, at equilibrium, deviation from Eq. 4 is significant only for distribution at the poles of cell where extensive accumulation occurs. The decay of asymmetry during the back diffusion process upon removal of the field follows the same time-course as shown in Eq. 6. Thus, although the present simple theoretical analysis ignores interaction among the molecules, it suffices as a basis for interpreting the data on Con A receptors in this report.

Diffusion Coefficient of the Con A Receptors

The translational diffusion of cell surface receptors or membrane proteins in the plane of cell membrane has been studied in a number of cell types. The apparent diffusion coefficient (D) reported spans a wide range (from 5×10^{-9} to 10^{-12} cm^2/s, see references 1–11). The gross differences in the D values may result from: Differences in the fluidity of the lipid matrix; the size, configuration, and disposition of molecules in the membrane; the restriction of the molecular movement imposed by ligand binding or the association with extramembraneous structure; and, finally, the techniques used in various studies. Three methods have now been used in determining diffusion coefficients: "Sendai virus fusion" (1, 11), "photobleaching recovery" (3–10) and "post-field relaxation" (present report). Quantitative differences of D values obtained by these three methods are expected. First, except in the rare case when the molecule being studied contains a bleachable chromophore, the diffusion rates measured by the photobleaching technique are that of the molecule complexed with exogeneous-labeled ligand. Secondly, unlike Sendai virus fusion and photobleaching methods, the post-field relaxation technique measures only the diffusion rate of electrophoretically mobile fraction of the receptors. In fact, these two considerations may account for the difference between the diffusion coefficient of Con A receptors obtained in the present study and those obtained in the previous reports (5, 7, 8).

Our previous study (13) on the diffusion of Con A receptors on spindle-shaped *Xenopus* myoblast using the post-field relaxation method gave smaller D values ($4–7 \times 10^{-10}$ cm^2/s) than the present report ($3.4–8.5 \times 10^{-9}$ cm^2/s). Two possibilities may account for

this difference. First, the Con A receptors on the surface of extended spindle-shaped myoblasts (13) are more restricted in their mobility due to the adhesion to the substratum or the intrinsic changes of the Con A receptor topography associated with the cell shape and adhesion. Secondly, the cell-counting technique used in previous study (13) underestimated the diffusion rate of the Con A receptors. Quantitative measurement of receptor redistribution over the surfaces of individual cells, as in the present study, was clearly necessary for determining the diffusion coefficient accurately. We estimated that the uncertainty of D value determined in the present study is within a four-fold range. The measurements of the fluorescence asymmetry index and the cell's radius, as carried out on a large group of cells, were reasonably accurate with error bars (95% confidence limits) generally less than 20% of the average value measured. Most of the uncertainty came from the D value estimated during curve fitting. As shown in Fig. 6, acceptable curves always lay within a two-fold range from the best-fit curve. Moreover, the K_l value obtained from integration in Eq. 5 decayed rapidly as l increased. The contribution of higher-order decay to $A(t)$ became progressively insignificant, especially for fitting the slowly decaying portion of the data.

Alternative Mechanisms for Field-Induced Redistribution

The field-induced migration of the Con A and ACh receptors and the recovery of uniform Con A receptor distribution after removal of the field are consistent with the processes of electrophoresis and diffusion, respectively, in the plane of the cell membrane. We have attempted to rule out some alternative interpretations to these observations. First, studies on the effect of extracellular medium flow suggest that electro-osmotic flow over the cell surface is not the cause of field-induced migration of Con A receptors. Secondly, migration or redistribution of the receptors was induced neither by the movement or rotation of the cells in the field nor by gross membrane alteration. We are, however, unable to resolve the relative contribution of direct electrophoresis and effect of the flow of other membrane components in the field-induced migration of Con A and ACh receptors. It is reasonable to expect that electrophoretic accumulation of some charged molecules may result in migration of other charged and/or neutral membrane components, simply because of the limited space available for redistribution within the closed, two-dimensional layer of the cell membrane.

Conclusion

We have presented evidence for electrophoretic accumulation, diffusional recovery, and aggregate formation of cell surface receptors/membrane proteins in the plane of the cell membrane. These phenomena appear to be analogous to those exhibited by soluble proteins in aqueous solution, namely, electrophoresis, diffusion, and aggregation. Such analogous properties of cell surface receptors support the view that the cell membrane is a two-dimensional solution and suggest that interactions among membrane proteins are similar to those among charged colloidal particles in solution.

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