Acetylcholine Receptors and Concanavalin A-binding Sites on Cultured Xenopus Muscle Cells: Electrophoresis, Diffusion, and Aggregation

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Abstract. Using digitally analyzed fluorescence videomicroscopy, we have examined the behavior of acetylcholine receptors and concanavalin A binding sites in response to externally applied electric fields. The distributions of these molecules on cultured Xenopus myoballs were used to test a simple model which assumes that electrophoresis and diffusion are the only important processes involved. The model describes the distribution of concanavalin A sites quite well over a fourfold range of electric field strengths; the results suggest an average diffusion constant of \( \sim 6 \times 10^{-10} \) cm\(^2\)/s. At higher electric field strengths, the asymmetry seen is substantially less than that predicted by the model.

Acetylcholine receptors subjected to electric fields show distributions substantially different from those predicted on the basis of simple electrophoresis and diffusion, and evidence a marked tendency to aggregate. Our results suggest that this aggregation is due to lateral migration of surface acetylcholine receptors, and is dependent on surface interactions, rather than the rearrangement of microfilaments or microtubules. The data are consistent with a diffusion-trap mechanism of receptor aggregation, and suggest that the event triggering receptor localization is a local increase in the concentration of acetylcholine receptors, or the electrophoretic concentration of some other molecular species. These observations suggest that, whatever mechanism(s) trigger initial clustering events in vivo, the accumulation of acetylcholine receptors can be substantially enhanced by passive, diffusion-mediated aggregation.

The migration of cell-surface molecules is a problem of general interest in developing biological systems. The cell membrane is a fluid matrix consisting of a heterogeneous lipid bilayer, peripheral and integral membrane proteins, and specialized molecular assemblies linking intracellular and membrane-bound components (Singer and Nicholson, 1972; Marchesi, 1985). Many proteins and lipids diffuse freely in the plane of the membrane, and show a globally uniform distribution early in development (Frye and Edidin, 1970; Poo and Cone, 1974; Edidin, 1975). At later stages some of these molecules are concentrated in local regions of the plasma membrane by mechanisms not fully understood. An important example in neurobiology is the induction of acetylcholine receptor (AChR) clusters at the developing neuromuscular junction (see Schuetze and Role, 1987, for review). In this case it is clear that at least some pre-existing, dispersed AChRs are recruited into the forming aggregate (Anderson et al., 1977; Kuromi and Kidokoro, 1984; Ziskind-Conhaim et al., 1984; Role et al., 1985).

A useful technique for the study of macromolecular migration is the in situ electrophoresis of membrane components in response to externally applied electric fields (Jaffe, 1977; Poo and Robinson, 1977; Poo et al., 1978). This technique perturbs molecular distributions independently of cell contacts, and permits quantitative analyses of important migration parameters. In particular, myoball (spherical muscle cell) cultures have been used to advantage in testing theoretical predictions concerning electrophoretic mobility, diffusion, and aggregate stability of concanavalin A (con A) ligand binding sites and AChRs (Orida and Poo, 1978, 1980, 1981; Poo et al., 1979). Because myoballs are nearly spherical, quantitation of component concentration about a two-dimensional perimeter is sufficient to characterize the distribution of the component in three dimensions. In the above-mentioned experiments, electrophysiological and microfluorometric techniques were used to analyze the distribution of cell surface components after experimental manipulations.

The increased availability of sophisticated computing and video-acquisition hardware has made possible a more detailed analysis of the distribution of cell surface molecules labeled with fluorescent probes. We report here the use of digitally analyzed videomicroscopy to extend the accuracy and resolution of data describing the distribution of con...
A-binding sites and AChRs after exposure to electric fields. The use of these techniques permits rigorous comparison to theoretical predictions based on electrophoresis and diffusion. The approach quantitatively demonstrates significant aggregation of AChRs over and above that caused by electric fields; this phenomenon is of great interest in light of the dramatic accumulation of AChRs at the developing neuromuscular junction. Additional experiments examining the requirements for the aggregation phenomenon are also presented.

Materials and Methods

Reagents

Leibovitz's L-15 medium and fetal bovine serum were obtained from Gibco (Grand Island, NY). Collagenase came from CooperBiomedical (Malvern, PA), and fluorescein-labeled concanavalin A (FLR-con A) from Vector Laboratories (Burlingame, CA). Colchicine and cytochalasin B were purchased from Sigma Chemical Co. (St. Louis, MO).

Purified alpha-Bungarotoxin (α-Bgt) was a gift from Drs. Darwin Berg and Stanley Halvesen. Tetra-methyl-rodamine–labeled α-Bgt (TMR-α-Bgt) was prepared by the method of Ravdin and Axelrod (1977). In brief the α-Bgt was incubated with tetra-methyl-rodamine-5-(6)-isothiocyanate (Molecular Probes, Inc., Eugene, OR) in 0.1 M Na HCO₃, pH 9.0. The TMR-α-Bgt conjugate was separated and fractionated using Sephadex G-25 and Sephadex CM C-50 chromatographic resins. Peak IV from this latter column was used for all experiments reported here (see Ravdin and Axelrod, 1977).

Culture System

Xenopus laevis embryos were obtained by in vitro fertilization of eggs from a gravid female (O'Rourke and Fraser, 1986), and staged in accordance with Nieuwkoop and Faber (1962). Stage 17-19 embryos were removed from jelly coats and transferred to sterile Steinberg's solution (58.2 mM NaCl, 0.7 mM KCl, 0.4 mM Ca(NO₃)₂, 4.6 mM Tris, 50 μg/ml Gentamycin, pH 7.8) containing 1 mg/ml collagenase. The surrounding ectoderm was removed from the dorsal third of the embryo using a tungsten needle, after which the exposed dorsal portion was excised from the rest of the embryo, and left to incubate in the collagenase-Steinberg's solution for ~5 min. Somites were then dissected free of the dorsal aorta, notochord, and neural tube, and transferred to Ca²⁺/Mg²⁺-free Steinberg's solution (58.2 mM NaCl, 0.7 mM KCl, 4.6 mM Tris, 0.3 mM EDTA, 50 μg/ml Gentamycin, pH 7.8). After ~5 min of incubation the cells were collected in a fire-drawn pipette, and transferred to a small volume (e.g., 0.5 ml) of culture medium (85% Steinberg's solution, 10% Leibovitz's L-15 medium, 5% FCS, 50 μg/ml Gentamycin, pH 7.8). The suspension was mixed gently and placed in 2-3-drop aliquots onto clean 24 x 60-ram glass coverslips (Coming Glass-works, Coming, NY) which had been scrubbed in 95 % ethanol and air dried. The cultures were kept in 100 x 15-mm plastic petri dishes at 22-24°C in a humidified, darkened chamber for 1-2 d before the experiments.

Electrophoresis

The electrophoresis chambers were modeled after those described previously (Peng and Jaffe, 1976; Poo et al., 1978; see Fig. 1). Two 7 x 60-mm coverslip runnels were cut and cemented along the long edges of 1 x 3-in glass slides (Sargent-Welch, Skokie, IL). Coverslips on which myoballs had been seeded were cemented to the glass slides with modified 100 x 15-mm petri dish lids. The apparatus permitted delivery and monitoring of 6 different fields to 6 chambers.

Fluorescent Labeling

After the electrophoresis (and in some cases a specified post-field period), the chambers were rapidly chilled and maintained on ice. The cells were incubated for 10 min with Steinberg's solution containing (a) 25 μg/ml FLR-con A, (b) 300 nM TMR-α-Bgt with 25 μg/ml unlabeled con A, or (c) 300 nM TMR-α-Bgt with 25 μg/ml FLR-con A (double label experiments). The cultures were then rinsed three times with 300 μl Steinberg's solution, and left on ice until commencement of videomicroscopy. As a control for autofluorescence and non-saturable binding, several cultures were labeled in the presence of excess unlabeled ligand. For both FLR-con A and TMR-α-Bgt, the nonspecific signal obtained was less than 10% of the total signal seen in sister cultures. A specific nonspecific signals have been subtracted from experimental data.

Videomicroscopy

Cells were imaged through a Zeiss Universal microscope using a Neofluor 40× objective with epi-illumination for fluorescence, and filter sets appropriate to excitation and emission spectra for fluorescein and rhodamine. Occasionally cultures in which cells did not appear robust and phase-bright after electrophoresis were not used for quantitative analysis, nor were individual cells deviating substantially from sphericity. Optical images were gathered by a SIT video camera (RCA; Lancaster, PA model No. TC1030) and stored on video cassettes (Sony U-matic video cassette recorder [Tokyo, Japan]; models VO-5600 and VO-5800).

Image Digitization and Acquisition

Analog videotape images were converted to digital images using a Digisector DS-88 board (Microworks, Del Mar, CA) modified by the addition of a 10-turn potentiometer to permit decreased contrast settings. The video image was digitized with a resolution of 256 x 256 pixels per screen, and a grey scale of 0-255. At the magnification used in these experiments, each pixel represented ~0.5 μm. The DS-88 board was used in conjunction with an American Micro Technology computer (model No. AMT 286; Santa Ana, CA), which performed all image analyses. To minimize deviations in response over the video field (see below), all scans were confined to a 100 x 100 pixel region in the center of the screen. All images were acquired as the average of 10 scans.

Spatial aberrations in illumination and viewing optics, and in camera sensitivity, were corrected by use of three "flatfield" images gathered in the absence of cells. One image was taken with the light path closed (camera noise only), while the other two were views of low and high concentrations (e.g. 5 μg/ml and 25 μg/ml) of FLR-con A in the electrophoresis chambers. The flatfield images were acquired separately for each experiment.

Correction of cell images were made on a pixel-by-pixel basis assuming...
Figure 2. Schematic illustrating the geometrical analysis of a cell image. Anode and cathode designate the orientation of the cell with respect to the electric field. The dashed-line circle indicates the boundary of the cell image. The solid circles represent the tolerance (±5% of the radius) within which pixels must lie to be accepted. For each pixel within this tolerance, the angle from the anode (θ) is found and the corrected intensity (less background) is sorted into one of 16 sectors representing θ. Note that although the sectors comprise equal angles (II. 25°) in two dimensions, the regions on a sphere represented by the sectors vary considerably in area. For simplicity the description and schematic assume the circularity of the image. In fact the software made the more general approximation of an ellipse with axes oriented horizontally and vertically.

Geometrical Analysis of Image

Before digital conversion of an image the left, right, top, and bottom boundaries of the cell were specified via cursor positioning on the video monitor. A rectangular scan was then performed to include the cell image with a 7-pixel margin along each border. Given the boundaries of the cell, the software identified those pixels falling within ±5% of the cell radius, and calculated the angle relative to the applied field subtended by each of them (see Fig. 2). The intensity scored at each pixel was then corrected, and the (corrected) background intensity found along the left and right margins of the image was subtracted. The resulting estimates of light intensity were summed into the appropriate sectors (16 in all) representing different angles (see Fig. 2). When all pixel intensities within the ±5% annulus were summed, the intensity corresponding to each sector was divided by the number of pixels within it to give the average intensity of the sector. This average was used as the estimate of binding site concentration within the region of the sphere’s surface sampled by the sector. These regions are bounded by concentric circles (lines of latitude) perpendicular to the electric field, and have areas ranging from ~1% (sectors 1 and 16) to 10% (sectors 8 and 9) of the total surface area of the sphere.

The total fluorescent label on each cell was approximated by the sum of the cell’s sector intensities weighted by the cell-surface area represented by each sector. To facilitate comparison of distributions between cells, this total label value was used to normalize each cell’s sector intensities such that the normalized total label on each cell was the same. Analysis of the cells on a given slide was completed by finding the average value (over n cells) of the normalized sector intensity for each sector. These means were used as the estimates of ligand site concentration at each sector, and expressed as a percent of the average concentration of sites.

To compare the extent of redistribution under different conditions, a simplifying analytical representation called the Asymmetry index (A) was used (Orida and Poo, 1977; Poo et al., 1979). The Asymmetry index summarizes the relative concentrations at the anode (C0) and cathode (C180) facing poles of a cell, and is defined as $A = (C_{180} - C_0)/(C_{180} + C_0)$. The index was calculated using intensities at the 1st and 16th sectors as approximations to C0 and C180, respectively (see Fig. 2).

Statistics and Curve-fitting

In tabulating data for the figures, the means of each data point for each experiment were averaged, and the standard error of each grand average (n = 3–8 experiments) was taken to account for possible increased variability between experiments.

Unless otherwise indicated, curves were fit to data by the Levenberg-Marquardt method; error estimates on fit parameters are not true standard errors, but rather corresponding estimates derived from the fitting procedure (cf. Press et al., 1986). Chi-square values are reported in the format $\chi^2$ (degrees of freedom) = value, as an objective measure of the correspondence between curves and data.

The Model

The basic model under test in these experiments is one put forward by Jaffe (1977) and elaborated on by Poo and collaborators (Poo et al., 1979; McLaughlin and Poo, 1981). The simplifying assumptions are (a) that the cell is approximated by a non-conducting sphere, and (b) that the two processes dominating the molecular distribution are electrophoresis and diffusion. Given these assumptions, the cell radius (r), the initial molecular concentration (C0), the field strength (E), the pseudo-electrophoretic mobility constant (m), and the diffusion constant (D), the steady-state concentration as a function of $\theta$ (θ) is

$$C_\theta = a \cdot \exp(-\beta \cdot \cos \theta),$$

where

$$\beta = \beta \cdot C/sin(\beta),$$

and

An additional term ($f_n$) is introduced to represent the fraction of sites which are mobile in the plane of the membrane. The equation describing steady-state distributions is therefore

$$C_\theta = f_n \cdot a \cdot \exp(-\beta \cdot \cos \theta) + (1-f_n) \cdot C_\theta,$$

where ($m/D$) and $f_n$ can be allowed to vary in fitting the data (all other parameters being measured quantities).

Results

Verification of Techniques

The data presented below depend on the validity of using intensity estimates from video images as a measure of relative ligand binding site concentrations. To test the estimation procedure, a range of FLR-con A concentrations were prepared, placed in electrophoresis chambers, and videotaped under the same conditions used in actual data collection. These records were analyzed with the described software as if an average-size (10 μm radius) cell image was present. Fig 3 shows the average corrected intensity found along the “cell perimeter” as a function of the concentration of dye used. The corrected value is seen to be proportional to the FLR-con A concentration up to ~60 μg/ml, after which it begins to plateau.

The proportional relationship between average corrected intensity and dye concentration does not rule out systematic deviations in sensitivity over the spatial field. Such variation, masked in the averages, would hamper interpretation of data.
The development of con A-binding site asymmetry, expressed as Asymmetry index vs. time in the electric field, is shown in Fig. 4. The approach to steady-state is well fit by an exponential with $\tau \approx 12$ min. On the basis of these data, 40 minutes was selected as the standard field duration for steady-state experiments (see below). Assuming the general model under consideration (see Materials and Methods), Poo (1981) has shown that the approach to steady-state is approximated by an exponential with time constant $\tau = r^2 (1 - 0.1 \cdot \beta^2)/2 \cdot D$. Given $m/D = 128 \, V^{-1}$ (see below), $r = \ldots$

Figure 4. Development of concanavalin A-binding site asymmetry. Cells were subjected to an electric field of 8 V/cm for varying amounts of time, and analyzed for asymmetry as described. Data represent means \pm standard errors of three experiments, each consisting of 8-10 scanned cells. The curve is the least-squares fit of an exponential to the data. The time constant is $12 \pm 4$ min; $\chi^2_{(df)} = 0.7$. A similar time course for development of asymmetry was seen at 4 V/cm.
The relaxation of con A-binding sites after exposure to electric fields is depicted in Fig. 5. Cultured cells were subjected to fields of 4 and 8 V/cm for 40 min, and then given varying amounts of time to relax. Given the assumptions of the model, it can be shown that the decay of asymmetry is approximated by an exponential with \( \tau = r^2/2 \cdot D \) (Huang, 1973). The relaxation from 4 V/cm shows \( \tau \approx 14 \) min, which yields \( D \approx 6 \times 10^{-10} \text{ cm}^2/\text{s} \), in good agreement with the estimate of \( D \) obtained from the development of asymmetry during exposure to fields. Relaxation from asymmetries induced at 8 V/cm were markedly incomplete.

Representative video images of cells showing steady-state distributions of con A sites are shown in Fig. 6. Con A-binding sites accumulated at the cathode end of cells, and they displayed a continuous gradation in concentration around the cell perimeter. A critical prediction by the model under consideration is that the same values for the parameters \( m/D \) and \( f_m \) should fit data for the distributions of sites at different voltage gradients. Fig. 7 shows the data and fitted curves for steady-state distributions at field strengths of 0, 1, 2, and 4 V/cm. As the data are fit well with the same parameter values, the results support the theoretical model. The experiments indicate estimated parameter values of \( m/D = 128 \text{ V}^{-1} \), \( f_m = 0.48 \) (solid curves). The dashed curves represent the best fit to \( m/D \) assuming all of the binding sites are mobile (\( f_m = 1.0 \)). The similarities between the solid and dashed curves, and the insubstantial change in the corresponding \( \chi^2 \) values (see legend to Figure 7), suggest that these data cannot be used to estimate \( f_m \) with any precision.

Steady-state experiments were also carried out at 8 V/cm (Fig. 8). Under these conditions the discrepancy between the data and the family of curves from Fig. 7 is conspicuous. At this higher field strength the solid and dashed curves, generated from the measurements in Fig. 7, are noticeably different from each other. However, neither curve is an adequate approximation to the data of Fig. 8. Under these conditions the data and the family of curves from Fig. 7 are noticeably different from each other. However, neither curve is an adequate approximation to the data of Fig. 8. Under these conditions the data and the family of curves from Fig. 7 are noticeably different from each other. However, neither curve is an adequate approximation to the data of Fig. 8. Under these conditions the data and the family of curves from Fig. 7 are noticeably different from each other. However, neither curve is an adequate approximation to the data of Fig. 8. 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field-"cells treated as in (a) and then given 40 min at room temperature for post-field redistribution. In either case the experiment was terminated by chilling and labeling the cultures with FLR-con A and TMR-α-Bgt, and analyzed using filter sets selective for the respective dyes. Control experiments demonstrated that the signal from either dye was insignificant when using filters appropriate to the other (data not shown).

Figure 7. Steady-state distributions of concanavalin A-binding sites after electrophoresis at 0–4 V/cm. Cultures were subjected to the indicated electric fields for 40 min and analyzed for the distribution of con A-binding sites as described. Data represent means ± standard errors for 3–6 experiments, each consisting of 7-14 scanned cells. Solid curves shown are the least-squares fit of the predicted distribution to the combined data from B, C, and D (see Materials and Methods, The Model). The fit parameters are m/D = 128 ± 15 V⁻¹ and f_m = 0.48 ± 0.05; χ²(45) = 67. Dashed curves represent the least-squares fit to the data assuming the mobile fraction f_m = 1.0. The fit parameter m/D is then 68 ± 1 V⁻¹; χ²(46) = 81. (A) 0 V/cm. (B) 1 V/cm. (C) 2 V/cm. (D) 4 V/cm.

Figure 8. Steady-state distribution of concanavalin A-binding sites after electrophoresis at 8 V/cm. Cultures were exposed to electric fields of 8 V/cm for 40 min and analyzed for the distribution of con A-binding sites as described. Data represent means ± standard errors for eight experiments, each consisting of 7-12 scanned cells. The solid and dashed curves represent the predicted distribution from the model given the measurements from Fig. 7 (solid; m/D = 128 V⁻¹, f_m = 0.48, χ²(10) = 524; dashed; m/D = 68 V⁻¹, f_m = 1.0, χ²(10) = 867). The dotted curve is the 2-parameter least-squares fit to this data, yielding m/D = 68 ± 7 V⁻¹ and f_m = 0.56 ± 0.05, χ²(10) = 14.0. Note that the scale of this graph is different from those of Figs. 7 and 8.

Postfield accumulation of AChRs was seen in almost all cells from control experiments. Typical videomicrographs illustrating the phenomenon are shown in Fig. 10 along with views of the same cells depicting con A site distributions. The AChRs continue to aggregate at the cathode-facing pole during the postfield period (cf. Fig. 10 f and e), while the con A sites relax back towards symmetry over the same interval (cf. Fig. 10 c and b). Quantitation of the asymmetries in TMR-α-Bgt and con A-binding sites under the field/postfield paradigm are shown in Figs. 11 and 12, respectively.

Pre-labeling and Cross-linking Experiments

Two kinds of experiments were undertaken to establish whether the observed postfield increase in asymmetry was in fact due to aggregation of preexisting AChRs, rather than to preferential expression of new AChRs. In the first set of experiments, cells were labeled with TMR-α-Bgt and then subjected to the standard experimental protocol. As seen in Fig. 11, pre-labeled AChRs show both field and post-field asymmetries indistinguishable from controls. In another set of experiments, cells were incubated with con A to cross-link existing AChRs, after which the cells were subjected to the protocol. Fig. 11 shows negligible electrophoresis or postfield accumulation of AChRs under these conditions, indicating that the accumulation phenomenon depends on the mobility of pre-existing AChRs. Fig. 12 demonstrates a substantial decrease in field-induced asymmetry of con A sites when the cells are labeled before the field.

Trypsin Experiments

Three sets of experiments were undertaken to analyze the effects of trypsin during, before, and after the field/postfield protocol. Cells treated with trypsin for 1 h before, and throughout the experiment, showed drastically reduced AChR asymmetries in both field and postfield conditions (Fig. 11). To ascertain whether this reduction depends on the presence of trypsin during the accumulation of AChRs, cells were incubated with trypsin, rinsed extensively, and then
Figure 10. Representative video images of cells subjected to electric fields with and without subsequent relaxation. Cultured cells were subjected to the indicated protocol and labeled with both FLR-con A and TMR-α-Bgt. (A–C) cells viewed with fluorescein filters to reveal con A-binding sites. (D–F) The same cells as A–C viewed with rhodamine filters to reveal TMR-α-Bgt-binding sites. (A and D) No field. (B and E) 4 V/cm for 20 min. (C and F) 4 V/cm for 20 min followed by 40 min postfield relaxation. Individual micrographs reveal cell-to-cell variation in overall brightness, and occasional bright foci unrelated to experimentally induced migration. These effects are minimized by the normalization and averaging procedures in the quantitative analysis of multiple images (see Materials and Methods). The fluorescent areas inside the cell perimeters in D–F are due to yolk granules within the cells; these exhibit more autofluorescence under the illumination conditions for rhodamine than under those for fluorescein. The prominent dark spots result from flaws in the camera tube; these along with other faulty pixels were rejected in the analysis (see Material and Methods). In all cases the field cathode is to the right. Bar, 10 μm.

subjected to the field/postfield protocol. Under these conditions the AChR accumulation was similarly reduced (Fig. 11). In the final set of experiments cells were subjected to the field, and then incubated with trypsin during the postfield period. These cultures showed a significant decrease in postfield asymmetry when compared to controls (Fig. 11). The effects of trypsin do not result from a loss of TMR-α-Bgt-binding sites because (a) analysis of the total amount of labeling shows no such decrease and (b) trypsin-treated cultures displayed a twitch response to acetylcholine, which could be blocked by α-Bgt (data not shown). The various trypsin treatments had no significant effect on con A site distributions under the field/post-field paradigm (Fig. 12).

The Role of Calcium

To investigate the possible role of calcium in the field-induced accumulation of AChRs, cells were incubated for 1 h before experimentation, and maintained throughout the experiment in Ca⁺⁺-free Steinberg's solution. As seen in Fig. 11, cells treated in this manner showed a small decrease in the field-induced accumulation of AChRs, and a substantial reduction in the postfield accumulation. Ca⁺⁺-free Steinberg's solution had no effect on the con A sites under these conditions (Fig. 12). To determine whether the effect on AChR accumulation is reversible, cells were incubated for 1 h in Ca⁺⁺-free Steinberg's solution, rinsed and incubated 1 h in Steinberg's solution, and then subjected to the experimental protocol. Fig. 11 shows a significant increase in the postfield accumulation as compared with cultures in Ca⁺⁺-free buffer throughout the experiment. Thus the effects of low calcium are substantially reversed within 1–2 h.

The Role of Microtubules and Microfilaments

To determine whether cytoskeletal elements are necessary for the postfield aggregation of AChRs, cells were treated with drugs to disrupt microtubules and microfilaments. Cultures were incubated with 20 μM colchicine or 20 μM cytochalasin B for 2 h before and throughout the course of experiments. Neither drug had significant effects on the redistribution of AChRs (Fig. 11) or con A sites (Fig. 12) in response to the field/postfield protocol.

Discussion

Methodological Considerations

The results presented in this paper rest on the assumption that corrected intensity estimates accurately reflect the redistribution of preexisting ligand binding sites over the cell surface. This in turn is based on linearity of the assay for ligand concentration. As shown in Fig. 3, the assay is quite linear over a substantial range of ligand concentrations. Analysis of experiments was performed only when the data were within that linear range. Under some conditions a multivalent ligand, such as con A, may bind nonlinearly with respect to site density. In this case the total number of sites would appear to change as the distribution of sites increased in asymmetry. As a check on this possibility, total con A binding (the
Changes in site density seen in these experiments (e.g., Fig. 7 a-d), cause a systematic bias in the selection of pixels representing the data tend to be low and high in regions around 60° and 120°, but systematic deviation from the predicted distributions; the group (p < 0.005) and the -Ca condition (p < .025, T tests). The control group field and postfield values are significantly different from those in the trypsin vs. 0.355 ± .023; p < .001, T test). The control group field and postfield asymmetry values are significantly different (0.247 ± .009 vs. 0.160 ± .013 vs. 0.055 ± .008; p < .0001, T test). The mean asymmetry of the control group field after the field is significantly different from the mean for pre-labeled FLR-con A (p < 0.001, T test).

Redistribution of FLR-Con A-binding Sites

Estimates of the diffusion constant for the "average" con A site were made in two different ways: (a) from the time course of asymmetry development during exposure to fields (Fig. 4), and (b) from the time course of postfield relaxation (Fig. 5). The two methods are in good agreement, yielding D = 6 × 10⁻¹⁰ cm²/s. This value is in the range reported (5-50 × 10⁻¹⁰ cm²/s for con A site diffusion measured via postfield relaxation in Xenopus myoball cultures (Poo et al., 1978, 1979), and is comparable to the value found by the same technique in several cell lines (1-3 × 10⁻¹⁰ cm²/s; Zagansky and Jard, 1979). The range of previously reported values is considerable, and probably due in part to differences in the developmental state of the cultured cells. In the related issue of AChR diffusion, for example, Orida and Poo (1980) have shown a decrease in mobility with age in culture.

The steady-state model describing the electrophoretic distribution of ideal (noninteracting) sites put forth by Jaffe (1977) describes the experimental distribution of con A sites quite well over a fourfold range of field strengths (Fig. 7). In fact the behavior of con A-binding sites at or below 4 V/cm appears ideal both in terms of steady-state distribution and relaxation to near symmetry (Fig. 5). Taking D = 6 × 10⁻¹⁰ cm²/s, and m/D = 128 V⁻¹ (Fig. 7), we arrive at an estimate of m = 7.7 × 10⁻⁸ cm²/V · s. Values for m reported by Poo et al. (1978, 1979) are 2 × 10⁻⁷ cm²/V · s, or more than a factor of two larger. This discrepancy is not serious given the variability in measured values for D, as dis-
Furthermore the value for \( m \) will depend critically on assumptions about \( f_m \), the fraction of sites that are free to move. This value cannot be rigorously estimated by the present techniques, as the predicted distributions over the range of ideal behavior are quite similar (see legend to Fig. 7). It has been argued that \( f_m \) can be estimated from the maximum asymmetry seen with increasing field strengths (Poo et al., 1979). However, our data suggest that the behavior of con A sites at higher field strengths is nonideal (see Fig. 8, and below), and accordingly that such an estimate may be misleading. Given the multiplicity of factors required for rigorous estimation of \( m \) and \( D \), the estimated values are less noteworthy than the observation that the same parameter values can successfully describe the experimental distributions over a significant range of field strengths.

The lack of correspondence between theory and observation at 8 V/cm is deserving of further comment. Two lines of evidence suggest non-ideal behavior under these conditions. First, and as seen by Poo et al. (1978), relaxation is incomplete after exposure to such a field (Fig. 5). Second is the finding that parameters characterizing steady-state distributions from lesser voltage gradients do not adequately describe the results seen at 8 V/cm under steady-state conditions; the asymmetry at 8 V/cm is less than would be expected (Fig. 8). Using different techniques to assay FLR-con A binding, Poo et al. (1979) concluded that the distributions of FLR-con A sites correspond to theory reasonably well in a range of fields up to 10 V/cm.

The apparent discrepancy documented here could in principle arise from nonlinear ligand binding, or concentration-dependent quenching effects, revealed at the higher binding-site concentrations found in the 8 V/cm condition. Inspection of Fig. 8, however, shows that even within the range of intensities found at 4 V/cm (\( \theta = 50-150^\circ \)), the 8 V/cm data do not correspond to the theoretical expectation. Thus, although such effects cannot be ruled out, neither can they account for the differences seen between the two experimental conditions.

There are several possible biophysical explanations for the reduction of experimental asymmetry at higher field strengths. One possibility is that under these conditions the mobility of the con A sites is reduced. The time course at 8 V/cm (Fig. 4) contradicts a simple version of this proposal, but an accumulation-dependent decrease in mobility could lead to an underestimate of the time required to reach steady-state. A second explanation is that electrostatic interactions, not accounted for in the model, play a role when asymmetries become large. There are reasons to believe such effects would be small under most conditions (e.g., a Debye length of \( \sim 1 \) nm in physiological saline4), but without knowing the concentrations and mobilities of all charged species involved, the possibility cannot be ruled out. A third argument challenges the assumption, inherent in the model, that the cell is closely approximated by a nonconducting sphere. At 8 V/cm, an iso-potential cell of radius 10 \( \mu \)m will experience 8 mV hyper- and depolarizations at the anode and cathode ends, respectively. These may lead to changes in membrane conductance due to the presence of voltage-sensitive channels, and thereby violate the validity of the approximation. Although predictions about the resulting distribution of sites at steady-state would require precise knowledge of the densities and responses of such channels (which might themselves migrate in a field) the influence would be in the observed direction, that is, it would lead to a decrease in the observed asymmetry. Finally, as noted in footnote 2, the driving force may be electroosmotic. A consequence of this is that redistribution of surface charge could diminish the driving force experienced by sites at the pole to which they are driven. A related effect is the possible establishment of a pH gradient on the cell surface, leading to a change in the charge carried by con A sites near the poles.

**Redistribution of AChRs**

In contrast to the case for the heterogeneous population of con A-binding sites, there are no conditions under which the AChR shows ideal behavior via the proposed model. Evidence for interaction among AChRs subjected to electrophoresis comes from the nonexponential rise in asymmetry with time (data not shown), the distribution after exposure to a field (Fig. 9), and the continued increase in AChR asymmetry after removal from the electric field (see below).

Two points emerge from analysis of the data in Fig. 9, which shows the distribution of AChRs seen after 80 min in an 8 V/cm field. First, the steady-state family of distributions from the model under consideration cannot adequately represent the observed distribution; to achieve even a poor fit the \( m/D \) parameter has to become quite large, and \( f_m \) is forced to be very small. A second point is that the minimum AChR density (at \( \theta = 100-130^\circ \)) is adjacent to the region of AChR accumulation, whereas the steady-state model predicts the minimum should be at the anode-facing pole. The location of this minimum suggests the operation of a diffusion-trap mechanism (Frye and Edidin, 1970), in which immobilization of AChRs at the cathode-facing pole leads to depletion in neighboring regions via diffusion-mediated migration. The location of this minimum would not be expected from a cell-wide cytoskeletal rearrangement of sites, although a locally confined mechanism of this sort cannot be ruled out.

In any event, it is clear that the distribution seen in Fig. 9 results from more than just the ideal behavior assumed by the model. The contribution made to the distribution of Fig. 9 by this ideal behavior can be roughly estimated by assuming that the effect accounts for 100% of the decrease in AChR density seen at the anode-facing pole. The dashed-line curve in Fig. 9 was fitted by eye with this in mind, and serves to illustrate graphically the minor role of electrophoretic migration per se under these conditions. This is in agreement with results showing similarity between asymmetries from experiments at 8 V/cm for 80 min (Fig. 9: \( A = 0.42 \pm .06 \)), and at 8 V/cm for 20 min + 60 min relaxation (data not shown): \( A = 0.42 \pm .05 \). Thus, the mechanism(s) underlying the postfield accumulation, discussed below, can dominate the distribution even in the presence of an electric field.

A field/postfield experimental protocol was devised to isolate the postfield aggregation phenomenon for further study. Analysis of con A site distribution under this paradigm shows the expected decrease in asymmetry during the postfield period; none of the applied treatments significantly altered the decrease (Fig. 12). In contrast, the results shown

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4. Note however, that the charges within or very near the membrane will interact significantly at distances greater than the aqueous phase Debye length.
in Fig. 10 and II demonstrate an increased accumulation of AChRs during the postfield period, as had been suggested by the data of Orida and Poo (1981).

That this aggregation is due to recruitment of AChRs already expressed on the cell surface is shown by experiments in which pre-existing AChRs were labeled with TMR-α-Bgt, and then analyzed with the field/postfield protocol (Fig. II). These results rule out the possibility that the effect is solely due to preferential insertion of AChRs at the cathode. Experiments in which cells were preincubated with con A and then treated with electric fields show no evidence of localized expression of new AChRs during the hour-long course of these experiments (Fig. II).

Previous experiments have shown that trypsin inhibits field-induced AChR aggregation, and disperses preexisting accumulations (Orida and Poo, 1980, 1981). Accordingly, experiments were undertaken to analyze the effects of trypsin on the postfield accumulation of AChRs. Trypsin digestion throughout the experiment, as a pretreatment, or at the start of the postfield period greatly reduced the asymmetry seen after the postfield period (Fig. II). In the first two paradigms the asymmetries seen immediately after the field were also significantly reduced, in agreement with the analysis (above), suggesting that a field-independent aggregation process dominates during exposure to fields. Although it is difficult to rule out indirect intracellular effects, or effects on AChR electrophoretic mobility, the most likely mechanism for the action of trypsin is a direct disruption of adhesion or cohesion events stabilizing AChR clusters.

The effects of low calcium on AChR accumulation are less dramatic and more difficult to interpret in a mechanistic sense. Cells incubated in Ca**+-free Steinberg's solution show a decreased asymmetry in both field and postfield conditions compared with control cultures (Fig. II). Low calcium can cause differences in charge density at the surface of the membrane (McDaniel and McLaughlin, 1985), and thus affect the electrophoretic mobility of AChRs. However, the observed asymmetry in low calcium experiments is still greater than the estimated electrophoretic contribution, suggesting that calcium must in part affect the aggregation phenomenon. The partial reversal of the calcium effect, seen in both field and postfield protocols, is consistent with this in light of the argument (above) that aggregation dominates the distribution even in the field (only) protocol. The observed effects could be based on calcium-dependent intracellular events, or calcium-mediated adhesion events (cf. Takeichi, 1977; Grunwald et al., 1980; Brackenbury et al., 1981). In the context of the effects of trypsin the latter interpretation is simplest, though the role of calcium in regulating myriad intracellular events prevents exclusion of this set of possibilities. Leaving aside mechanistic questions, the observation of reduced accumulation is in accord with the observed effects of low calcium on AChR clustering in neural and aneural Xenopus muscle cultures (Henderson et al., 1984; Davey and Cohen, 1986), as well as clustering induced by latex beads (Peng, 1984).

The results of experiments with cytoskeleton-disrupting drugs indicate that rearrangement of microtubules and microfilaments is not required for the aggregation effect documented here (Fig. II). In contrast colchicine, but not cytochalasin B, decreased AChR cluster number and reduced recovery of cluster number following treatment with azide, in a 6-h assay on cultured rat myotubes (Bloch, 1979). Experiments by Connolly (1984) demonstrated that cytochalasins disrupt preexisting AChR clusters in aneural chick muscle cultures over a period of 24 h, and that both cytochalasins and Colcemid prevent an increase in cluster number during the 6 h after addition of brain extract. Others have suggested cytoskeletal anchoring of AChR clusters as evidenced by the protection of clustered AChRs from detergent extraction (Prives et al., 1980, 1982; Stya and Axelrod, 1983).

Methodological differences may account for these apparent discrepancies. In the works of Bloch (1979) and Connolly (1984) it is cluster number that is being assayed, whereas our results speak directly to the question of AChR accumulation. In this context, Bloch (1979) has noted that drugs inhibiting re-clustering of AChRs seem to block the initial formation of foci, and that after foci formation, clustering proceeded in the presence of drugs. Most clusters in aneural cultures are found in regions of close contact between cell and substrate (Bloch, 1980). Thus, it may be that cytoskeletal involvement is required for such contacts, which serve to trigger foci formation (see Connolly, 1984). In contrast to these kinds of experiments, the accumulation event reported here is removed from regions of cell attachment. Put in more general terms, it may be that any of several unrelated in vitro manipulations can trigger accumulation. Under these circumstances, interpretation regarding in vivo mechanisms is difficult (see Fraser and Poo, 1982). A second point is the difference in time-scale of the experiments under discussion. It is possible that early events in AChR aggregation, themselves independent of the cytoskeleton, may lead to secondary stabilization via microtubules and/or microfilaments. Thus, experiments may give rise to different interpretations depending on how long after initial cluster formation the study is carried out. Finally, different species were used in these experiments. Though it is tempting to think that underlying mechanisms for phenomenologically similar events would be conserved, this need not be the case.

Taken together, our data are consistent with a simple interpretation of the events required for initial AChR accumulation: the AChR migrates in response to the electric field, and the local increase in AChR concentration at the cathode-facing pole activates a diffusion-trap mechanism (Frye and Edidin, 1970; Chao et al., 1981; Weaver, 1983). The trapping mechanism is calcium sensitive and trypsin labile, and is insensitive to colchicine and cytochalasin B. If the triggering event is simply the increase in local AChR concentration, the average concentration of AChRs before experimental manipulation is probably near threshold; shown in Fig. 9 (dashed line) is a crude estimate of the increase in AChR concentration due to the field alone. An interesting alternative view, indistinguishable from the above given the present data, is that electrophoretic migration of some other species results in the accumulation of components necessary for the trapping mechanism. Work underway in several laboratories is directed toward characterization of factors which may play a role in AChR clustering (for review see Schuetze and Role, 1987); factors such as these may interact with membrane components responsible for the accumulation documented here.

Is the diffusion of AChRs sufficiently rapid to account for the observed rate of aggregation via a diffusion-trap mechanism? The AChR diffusion constant was measured in Xen-
pus cultures by the recovery of sensitivity after local inactivation with α-Bgt, and found to be $2.6 \times 10^{-9} \text{ cm}^2/\text{s}$ (Poo, 1982). Two other parameters critical to estimation of trapping rate must be assumed; these are the trap size and the trapping efficiency (see Chao et al., 1981 and Weaver, 1983). Following Weaver (1983), and with the simple assumptions that the trap is 100% efficient and the trap size is 3 sectors ($\theta = 33.75^\circ$—see Fig. 9), we find the mean trapping time (i.e., the time after which half of the AChRs would be trapped) to be $\sim 11 \text{ min}$. Analysis of the data of Fig. 9 shows that, taking sectors 14–16 to represent the trap, only $\sim 9\%$ of the sites from the rest of the cell surface have been confined to the trap region after 80 min. Thus, diffusion of AChRs is easily fast enough to account for the observed aggregation. This conclusion is in accordance with results from Kuromi et al. (1985), in which the accumulation of AChRs at neuromuscular contacts was characterized using *Xenopus* cultures. It should be noted that use of fluorescence photobleaching recovery techniques to measure the AChR diffusion constant in *Xenopus* cultures gives a result $\sim 10\text{-fold}$ lower than that used in this calculation (see Kuromi et al., 1985, for discussion of intrinsic differences between these methods). However, even using this much lower estimate, the rate of diffusion is $\sim 5\text{-fold}$ greater than that needed to account for the observed accumulation. The fact that the experimental aggregation is considerably slower than that predicted in this calculation implies that, if the diffusion-trap mechanism is operative, the trap is less than 100% efficient, or the trap size is less than 33.75°, or both.

A further test of the diffusion-trap proposal would be to examine whether the aggregation can occur in the absence of metabolic energy sources. Attempts were made to test this by administration of drugs interfering with ATP synthesis. Unfortunately, the time required for consistent, complete loss of acetylcholine-induced twitch response was $\sim 1.5 \text{ h}$, and the cells began to lyse shortly thereafter. Thus, conclusive testing of this prediction could not be performed.

**General Conclusions**

Our experimental findings suggest that the data analysis techniques presented here constitute a valid and powerful tool for the examination of factors contributing to the distribution and migration of membrane components. We find support for a model based on electrophoresis and diffusion (Jaffe, 1977) in the behavior of con A-binding sites over a fourfold range of electric field strengths. At a higher field strength the description of con A-binding sites over a fourfold range of electric field strengths. At a higher field strength the description of con A site behavior breaks down, presumably due to the failure of assumptions on which the model is based.

The AChR is not well described by this model under any of the conditions examined, apparently because of interactions between the AChRs (and possibly other surface components). These interactions are probably mediated by a local increase in the concentration of AChRs or other components, which triggers an aggregation event independent of microtubule or microfilament rearrangements. It is possible that multiple in vitro manipulations can trigger the accumulation of AChRs. Our data are consistent with a description of the aggregation phenomenon as one driven by a passive, diffusion-trap mechanism. The aggregation behavior reported here is suggestive in light of the in vivo accumulation of AChRs at the developing neuromuscular junction, and may play a significant role in the initial events responsible for AChR clustering.

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