LATERAL ELECTROPHORESIS AND DIFFUSION OF CONCANAVALIN A RECEPTORS IN THE MEMBRANE OF EMBRYONIC MUSCLE CELL

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ABSTRACT

A uniform electric field of 10 V/cm applied across the surface of embryonic toad *Xenopus* muscle cells results in the asymmetric accumulation of concanavalin A (Con A) receptors toward one side of the cells within 10 min, as visualized by postfield fluorescent Con A labeling. This field produces an extracellular voltage difference of 20 mV across these 20-μm wide cells. The effect is reversible in two respects: (*a*) Additional exposure of the cell to the same field of opposite polarity for 10 min completely reverses the asymmetric accumulation to the other side of the cell. (*b*) Relaxation occurs after the removal of the field and results in complete recovery of the uniform distribution in 30 min. Both the accumulation and the recovery movements are independent of cell metabolism, and appear to be electrophoretic and diffusional in nature. The threshold field required to induce a detectable accumulation by the present method is between 1.0 and 1.5 V/cm (corresponding to a voltage difference of 2-3 mV across a 20-μm wide cell). The electrophoretic mobility of the most mobile population of nonliganded Con A receptors is estimated to be about 2×10^{-3} μm/s per V/cm, while their diffusion coefficient is in the range of 4-7×10^{-10} cm^2/s. Extensive accumulation of the Con A receptors by an electric field results in the formation of immobile aggregates. The Con A receptors appear to consist of a heterogeneous population of membrane components different in their charge properties, mobility, and capability in forming aggregates.

KEY WORDS   electrophoresis · diffusion · concanavalin A · receptor mobility · myoblast

The cell membrane is a fluid matrix of lipids and proteins (23, 5). Some of the lipids and proteins bear electrical charges under physiological conditions (4, 13). It is reasonable to expect that an external electric field applied along the surface of the membrane will electrophoretically segregate charged membrane components. Indeed, it has been demonstrated recently that cell surface concanavalin A (Con A) receptors can be electrophoresed *in situ* (21). Fluorescent Con A labeling showed that a uniform external field of about 4 V/cm applied along the cell surface grossly redistributed Con A receptors along the membrane of living *Xenopus* muscle cells within a few hours. The Con A receptors (presumably glycoproteins) accumulated on the side of the cells nearer the negative pole in a way that is consistent with a

passive electrophoretic mechanism (13, 21).

Several attempts have been made in recent years to measure the mobility of membrane proteins or cell surface receptors in the plane of membrane (1, 6, 7, 12, 19-22, 25). Most previous measurements employed the technique of photobleaching in which the chromophores or fluorophores specifically bound to membrane proteins are bleached locally on the cell surface, and the recovery of unbleached chromophores at the bleached region is used to infer the diffusional mobility of the membrane proteins. In the present report, we generate an inhomogeneity in the distribution of charged cell surface receptors by externally applying an electric field along the membrane surface. The approach to the inhomogeneous distribution was used to infer the electrophoretic mobility of the receptors, while the relaxation of the inhomogeneous distribution after the removal of electric field provided an estimate of the diffusion coefficient of the receptors. In addition, we present evidence which suggests that extensive passive electrophoretic accumulation of the surface receptors may result in the formation of immobile aggregates of the receptors.

MATERIALS AND METHODS

Monolayer Culture of Amphibian Muscle Cells

Embryos of the toad *Xenopus laevis* were obtained by conventional breeding procedures and staged by the criteria of Nieuwkoop and Faber (17). Embryonic muscle cells were obtained by dissociating mesodermal tissue of stage 17-19 embryos in Ca^{++}, Mg^{++}-free Steinberg's saline containing 0.4 mM EDTA (14). When the cells became dissociated, they were picked up with a fine glass pipette (inner diameter 100-150 μm) and plated onto a clean glass culture chamber (see apparatus). Approximately 10^3 cells from the same embryo were plated on each chamber. The cells were cultured at room temperature (22 $\pm$ 1°C) in Steinberg's solution supplemented with 10% Leibovitz medium (L15, Grand Island Biological Co. [GIBCO] Grand Island, N. Y.) and 5% fetal calf serum (GIBCO). The pH of the medium was 7.80 $\pm$ 0.05. After 2 days in culture, the predominant cells in this culture were fibroblasts and embryonic muscle cells. The latter were mostly spindle-shaped and developed clear striations visible under the phase-contrast microscope. Experiments were carried out on these isolated, mononucleated, spindle-shaped muscle cells. Muscle cells that retained their smooth spherical shape were also studied in two experiments. Both spindle-shaped and spherical muscle cells respond to bath-applied acetylcholine (1 mM) by instantaneous contraction into spheres of irregular surface.

Electrophoresis Apparatus

A simple and precise electrophoresis apparatus was designed for applying uniform electric field to monolayer culture. A similar apparatus was previously described by Peng and Jaffe (18). As depicted in Fig. 1, it consists of a shallow rectangular culture chamber made from a microscope slide and two pieces of no. 1 cover glasses, a pair of agar bridges (8-10 cm long) filled with Steinberg's saline gelled by 2% agar, a pair of Ag-AgCl electrodes, and a pair of electrode baths containing Steinberg's saline. Electrical current was supplied by a regulated power supply (Buchler 3-1155, Buchler Instruments Div., Searle Analytic Inc., Fort Lee, N. J.) and was measured by an electrometer (Keithley 602, Keithley Instruments, Inc., Cleveland, Ohio). A defined chamber geometry (60 × 10 × 0.2 mm) was obtained by sealing the sides of the chamber permanently with silastic sealant and the top cover by silicon grease during electrophoresis. The thickness (0.20 $\pm$ 0.03 mm SD, n = 47) of the chamber, which refers to the fluid-filling space, was obtained from micrometer measurements of the slide, cover, and sealant thickness. 47 slide chambers were used and recycled during the course of this study. The agar bridges isolated the culture from contamination by the Ag-AgCl electrodes. Culture medium was substituted with plain Steinberg's saline (pH 7.8) before the onset of electrophoresis. The field strength along the culture substratum was calculated from the total current flow through the chamber, the chamber's cross-sectional area, and the conductivity of the Steinberg's saline (138 $\pm$ 3 Ω cm), as measured by a conductivity bridge (YSI 31). A typical current of 1.5 mA produced a field of 10 V/cm (10.3 $\pm$ 1.6 V/cm) along the culture substratum. All experiments were carried out at room temperature (22 $\pm$ 1°C).

Fluorescence Labeling and Microscopy
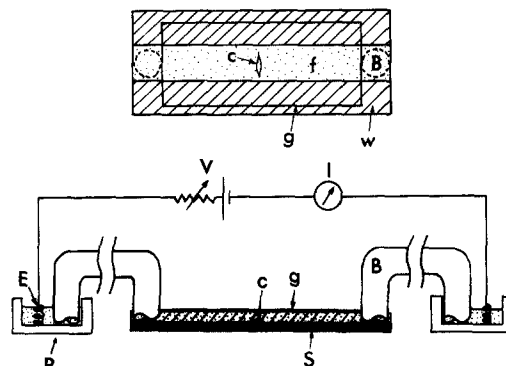
Fluorescence labeling of the cell surface was carried

FIGURE 1 Electrophoresis apparatus (top and side view, not drawn to scale). *g*: cover glass, *f*: fluid-filling groove (60 × 10 × 0.2 mm), *c*: cultured cell, *w*: groove wall, *S*: microscope slide, *B*: glass agar bridge, *E*: Ag-AgCl electrode, *R*: saline reservoir, *V*: regulated power supply, *I*: current meter. (See text for details.)

out at 0°-4°C for 15 min after removal of the electric field (postfield labeling). Labeling solution was Steinberg's solution containing 140 μg/ml fluorescein isothiocyanate conjugated Con A (FITC-Con A, Miles Laboratories Inc., Miles Research Products, Elkhart, Ind.). When the labeling was completed, the cells were immediately fixed with cold acetone (0° to −5°C). The labeling was specific, since it was completely blocked by 0.1 M α-methyl-D-mannoside, one of the sugars for which Con A is specific. In some control experiments, 3% glutaraldehyde (Ladd Research Industries, Inc., Burlington, Vt.) fixation was used. The autofluorescence caused by glutaraldehyde can be greatly reduced by the use of fresh glutaraldehyde and FITC selective filters.

Fluorescence microscopy was done with a universal Zeiss microscope fitted with both dark-field and epi-illumination (III RS) optics. The light source was an HBO 200 W mercury lamp (Wotan Inc., Germany). The exciting light passed through a heat-absorbing filter and a blue interference filter (455–490 nm). The emitted light was filtered by a green interference filter (520–560 nm). This filter set is specific for studying FITC fluorescence.

Cell Scoring

In the control culture when no electric field was applied, FITC-Con A labeling showed uniform "ring"

staining (with random patches) around the periphery of the cell (Figs. 3 A and 13 a). Estimates of electrophoretic accumulation of the Con A receptors were done by scoring the number of cells with asymmetric accumulation of their ring staining on one side of the cell periphery. Since these spindle-shaped muscle cells are more uniform in their width than in their length, we scored the number of cells with asymmetry in staining along the width of the cell. All isolated spindle-shaped muscle cells which had their long axis lying within 45° with respect to the direction normal to the field direction were scored by visual comparison of the fluorescence stain on two sides of the cells. Three categories of cells were counted: cells showing definite asymmetric accumulation of stain toward the negative pole (−) or toward the positive pole (+) of the field and cells showing no asymmetric accumulation toward either pole (indeterminant). All cell countings were carried out using × 40 oil immersion lens together with epi-illumination optics. 40 to 150 cells were counted for each electrophoresis chamber. The results were tabulated as those shown in Table I. We also defined "percent asymmetry" by the formula shown in Table I. This formula gives 100% asymmetry when all cells examined show asymmetric accumulation of stain toward one side of the cell, and 0% asymmetry when all cells examined are indetermi-

TABLE I

Accumulation of Con A Receptor by Electric Field

Culture no.	Field strength	Duration of exposure	No. of cells examined*	No. of cells showing asymmetry in the fluorescence ring staining			Percent asymmetry[‖]
				Toward (−) pole	Toward (+) pole	Indeterminant	
	V/cm						
1	0	0	49	5	4	40	2
2	10	1 min	73	16‡	1	56	21
3	10	10 min	93	83‡	2	8	87
4	10	0.5 h	66	57§	2	7	83
5	10	1.5 h	40	38§	0	2	95
6	3.4	1.5 h	150	90‡	6	54	56
7	1.0	1.5 h	28	2	0	26	7
8 (Prefixed)	10	1.5 h	45	5	5	35	0
9 (Fixed before labeling)	10	1.5 h	127	114§	2	11	88
10 (Prelabeled)	10	1.5 h	169	17	4	148	8

Cultures 1–7: Cells were labeled with 140 μg/ml FITC-Con A at 0°-4°C for 15 min immediately after removal of the electric field and then fixed with cold acetone at 0° to −5°C. Culture 8: Cells were fixed with 3% glutaraldehyde for 20 min before exposure to the field; FITC-Con A labeling was done immediately after removal of the field. Culture 9: The same as culture 5 except that cells were fixed with 3% glutaraldehyde for 20 min before FITC-Con A labeling. Culture 10: The same as Culture 5 except that cells were labeled with FITC-Con A before exposure to the field. Temperature: 22°C. All data are from the same experiment.

* All spinde-shaped muscle cells which had their long axis aligned within 45° with respect to the direction normal to the field direction were examined.

‡ Weak but definite asymmetry.

§ Striking asymmetry.

‖ Percent asymmetry = $\dfrac{\text{no. of cells toward } (-) - \text{no. of cells toward } (+)}{\text{total no. of cells examined}} \times 100\%$.

nant in their asymmetry. In 22 separate experiments carried out for the present report, the asymmetry of the control cultures (not exposed to the electric field) never exceeded 10%.

Several approaches were taken to eliminate the subjectivity that may occur in the asymmetry countings. The culture chambers being examined were coded in such a way that the examiner was not aware of the polarity, the magnitude of the field applied, and the duration of the field application. To strengthen the statistical relevance, cell counting data of cultures that had total cell counts <40 were discarded in plotting the figures. Finally, as a test of reproducibility, six cultures were randomly selected from the experiments in the present report and were counted by all three authors independently. In percent asymmetry, the results were: 9, 11, 19; 63, 65, 52; 62, 71, 80; 4, 0, 2; 2, 6, −1; and 45, 34, 42. We concluded that the cell counting technique introduces an uncertainty <20% in the present study.

RESULTS

Redistribution of Con A Receptors by an Electric Field

Table I shows the results from one of the experiments in which electric fields of various magnitudes and durations were applied to a group of muscle cultures. The distribution of Con A receptors was visualized by FITC-Con A labeling after removal of electric fields (postfield labeling). Cell scoring data were characterized by percent asymmetry as defined in the table. When a field of about 10 V/cm was applied to a culture for 10 min, nearly all cells examined showed definite accumulation of stain toward the negative pole of the field (no. 3, Table I; Fig. 3 D). Most extensive accumulation was observed for the culture treated with 10 V/cm field for 3 h. Fig. 2 shows three representative cells from this culture, as viewed by phase-contrast and dark-field fluorescence microscope.

Four types of control cultures were examined. First, when no electric field was applied, the cells showed uniform ring staining (no. 1 of Table 1, also Fig. 3A). Second, when cells were fixed with 3% glutaraldehyde (Ladd Research Industries, Inc.) for 20 min before applying the electric field, no asymmetric accumulation of stain was observed (no. 8, Table I). Third, when the same glutaraldehyde fixation was done after the application of field but before the FITC-Con A labeling, we observed definite accumulation of stain toward the negative pole of the field (no. 9, Table I, also

Fig. 3 C). Lastly, when FITC-Con A labeling was carried out before applying the field, no asymmetric accumulation of stain was observed (no. 10, Table I, also Fig. 3 B). These control experiments indicate that the asymmetric accumulation of the receptors observed was induced by the electric field, not by the labeling of FITC-Con A. Crosslinking of the receptors of either bifunctional glutaraldehyde or tetravalent Con A results in the immobilization of the Con A receptors. The first type of control (zero field) was repeated in all experiments described in this report; the percent asymmetry never exceeded ± 10%. Second and third control experiments (glutaraldehyde fixation) have been repeated once. Results were similar to those shown in Table I. In five separate experiments, we applied an electric field of 10 V/cm to cells prelabeled with FITC-Con A for durations ranging from 30 min to 6 h. In no case did we observe any induced asymmetry in stain distribution above 10%. Duration of field exposure longer than 6 h was not studied, since extensive intracellular uptake of the FITC-Con A surface labels occurred and resulted in the disappearance of fluorescence ring stain. We concluded that FITC-Con A labeling essentially immobilized the Con A receptors.

Figs. 4 and Fig. 5 depict graphically the results of the cell scoring shown in Table I together with data from four other separate experiments. Data from cultures which had total cell counts <40 were discarded. Fig. 4 shows the percent asymmetry induced by electric fields ranging from 0.5 to 10 V/cm applied to cultures for 10 min (dashed line) or 1.5 h (solid line). Fig. 5 shows the percent asymmetry induced by a field of 1 (dashed line) or 10 V/cm (solid line) for various durations of field application. These results indicate that there was a gradual increase in the induced asymmetry when the field strength and duration of exposure were increased. However, a minimum field strength of about 1.5 V/cm was required to induce a detectable asymmetric accumulation (>10% asymmetry). This field corresponds to an extracellular potential difference of about 3 mV across a muscle cell 20 μm in width. It may be noticed that the estimate of percent asymmetry does not take into account the extent of asymmetry observed; thus in Fig. 5, 10 V/cm curves reach a plateau in 10 min, while the degree of asymmetry observed at 10 min is in fact much weaker than that observed for cultures exposed for longer durations.

As a physiological test of the viability of the muscle cells after the application of electric field, we examined their contractile response to bath-applied acetylcholine (ACh). Isolated spindle-shaped muscle cells in these 2-day-old cultures respond to ACh by spontaneous contraction into spheres. Plotted in Fig. 6 is the percent of spindle cells contracted vs. the concentration of ACh in Steinberg's solution. We observed no significant difference in the dose-response curves between control cultures (no electric field) and cultures exposed to a field of 10 V/cm for 3 h.

Very limited cell locomotion (10) was observed for these spindle muscle cells under the influence of an electric field of 10 V/cm. Fig. 7 shows the phase-contract micrographs of a culture before the field application (Fig. 7a), after 3 h in the field (Fig. 7b), and the post-field FITC-Con A fluorescence stain (Fig. 7c). The accumulation of Con A receptors observed thus seems to be unrelated to the surface movement that may be associated with cell locomotion (10).

Except in some control cultures described above, all FITC-Con A labeling was carried out at 0°−4°C, a condition known to prevent Con A-induced capping (15), and the cells were fixed with cold acetone immediately after removal of labeling solution. All samples were preserved in 90% glycerol before microscope examination. We noticed in general that many fluorescent micropatches were formed on the muscle cell surface after FITC-Con A labeling. Presumably, this was due to the cross-linking action of Con A, a phenomenon known as "patching" in other cell types (14). Furthermore, using FITC-labeled monovalent Con A (9), we found that the fluorescence staining of these muscle cells became relatively free of micropatches (M.-m. Poo and W.-j. Poo, unpublished observations).

Low-Field, Long-Duration Experiments

We have studied the redistribution of the Con A receptors induced by small electric fields (range from 0.5 to 1.5 V/cm) applied for a longer duration (24 h). Table II shows the results from two experiments. No asymmetric accumulation of the receptors was detectable for fields lower than 1 V/cm, while a definite asymmetry was observed for a field of 1.5 V/cm. We concluded that the threshold field strength needed to induce asymmetric accumulation within 24 h lies between 1.0 and 1.5 V/cm. This corresponds to a voltage gradient of about 2-3 mV across a muscle cell 20

μm in width. A lower value (1 mV across 20-μm-wide cell) was previously reported to give a detectable response (20). As will be discussed in a later section, the low-field result in the present report is consistent with the expectation that once the equilibrium state between electrophoretic and diffusional transport has been reached, no further increase in receptor accumulation can be achieved by applying the field for a longer duration.

Reversibility of Asymmetric Accumulation

We have studied the reversibility of asymmetric accumulation of Con A receptors induced by an electric field in two respects: (a) The reversal of asymmetric distribution by applying fields of opposite polarity; and (b) the recovery of the uniform distribution after removal of the electric field. In the first set of experiments, we first applied a field of 10 V/cm for 10 min to a group of six cultures. At the end of 10 min, the polarity of the field was reversed and applied for an additional 0, 2, 4, 6, 8, or 10 min. The resulting receptor distribution in these cultures was then studied by postfield FITC-Con A labeling. Fig. 8 depicts results from three separate experiments. Application of fields of opposite polarity progressively reversed the asymmetry induced by the original field. An additional 10-min application resulted in complete reversal of asymmetry in receptor distribution, with clear accumulation of stain toward the new negative pole of the field (Fig. 3 D and E). However, complete reversal of asymmetry was not observed when greater accumulation was induced by the original field applied for longer duration. In two separate experiments, a field of 10 V/cm was applied to four pairs of cultures for 10 min, 0.5 h, 1.5 h, and 3 h, respectively. One culture from each pair was labeled and fixed immediately at the end of each respective period, while the other culture in each pair was put under a 10 V/cm field of opposite polarity for an additional 10 min, 0.5, 1.5, and 3 h. The results from these experiments are shown in Fig. 9. Complete reversal was found only for the culture in which the field was applied for 10 min in each direction. Asymmetry induced by 3-h field application persisted toward the original negative pole (see also Fig. 12 C). This is not surprising, since extensive accumulation of receptors in one region of the cell membrane may result in the formation of
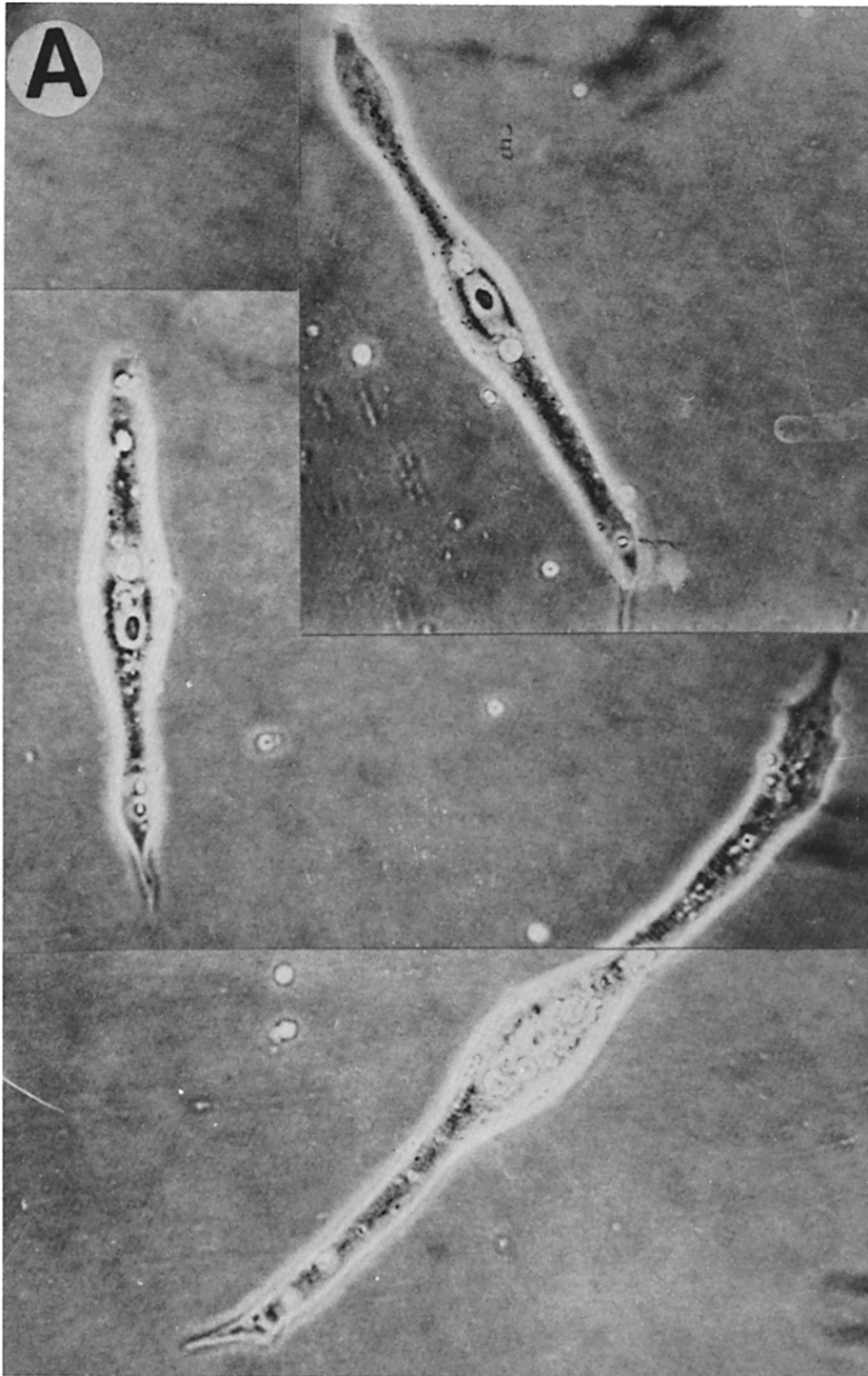
FIGURE 2 Electrophoretic accumulation of the Con A receptors. FITC-Con A labeled, living embryonic muscle cells observed by (A) phase-contrast and (B) dark-field fluorescence microscopy. Cells were exposed to a uniform electric field of 10 V/cm for 3 h before FITC-Con A labeling was carried out. Fluorescence ring stain showed strong asymmetry toward the negative pole of the field (to the right of the figure), indicating accumulation of the Con A receptors. All micrographs were taken of adjacent cells in the same culture (the same for later figures). Bar, 25 μm.

receptor clusters or aggregates which are not free to undergo electrophoretic redistribution by the reversed field.

For the other aspect of reversibility of asymmetry, namely, the recovery of uniform distribution after removal of the field, we carried out
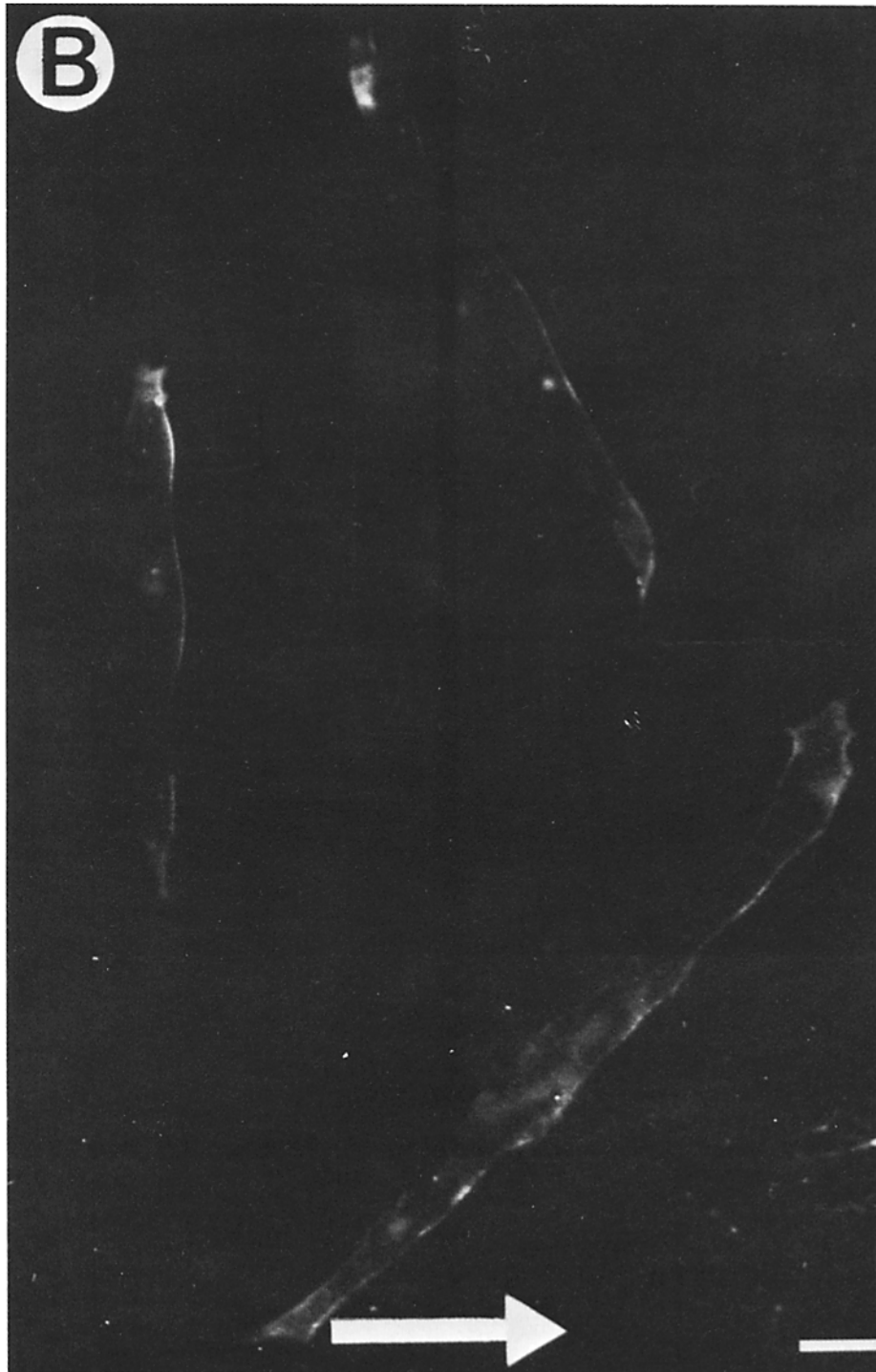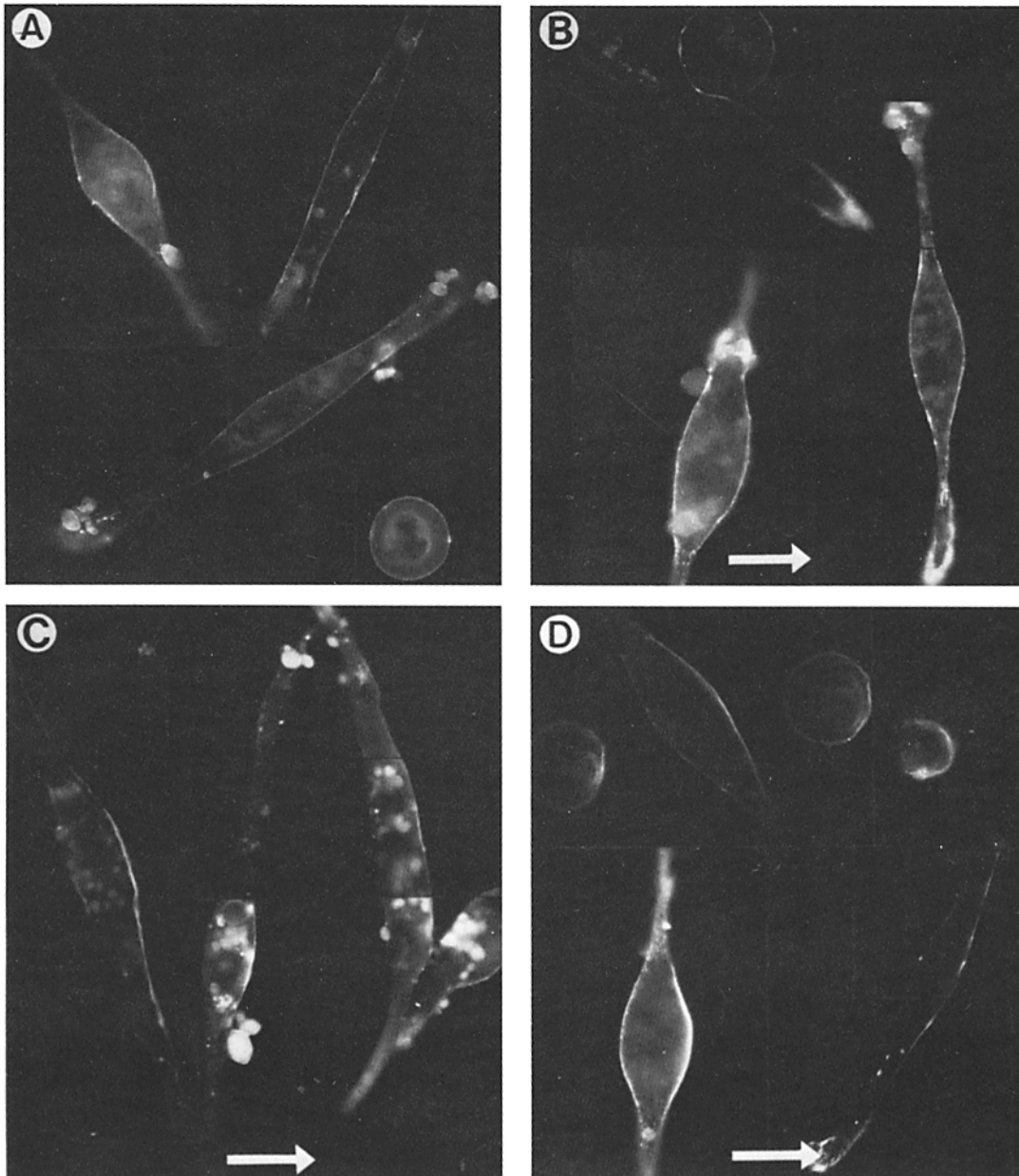
FIGURE 3   FITC-Con A labeled muscle cells observed by epi-illumination fluorescence microscopy. (A) Control cells not exposed to the electric field showed uniform ring staining. (B) Cells were labeled with FITC-Con A before exposure to a field of 10 V/cm for 1.5 h. No asymmetry of stain was observed, indicating that Con A labeling immobilized the receptors. (C) After exposure to an electric field of 10 V/cm for 1.5 h, these cells were fixed immediately by 3% glutaraldehyde, and FITC-Con A labeling was then carried out. Definite accumulation of stain was observed. The accumulation of the receptors was thus not due to labeling. (D) Cells exposed to a field of 10 V/cm for 10 min; definite accumulation of the Con A receptors was seen by post-field labeling. (E) Cells treated the same as those in Fig. 3D except that they were exposed to an additional field of same strength with opposite polarity for another 10 min before labeling was carried out. Definite accumulation of Con A receptors occurred at the side of the cells facing the negative pole of the second field. (F) Cells treated the same as those in Fig. 3D except that they were allowed to "relax" in saline for 30 min before FITC-Con A labeling was carried out. Ring stain was essentially uniform, suggesting the recovery of uniform distribution due to back diffusion. Except in Fig. 3C, all cells were fixed in cold acetone and preserved in 90% glycerol before observation was made. Spherical cells were also muscle cells. Bar, 25 μm.
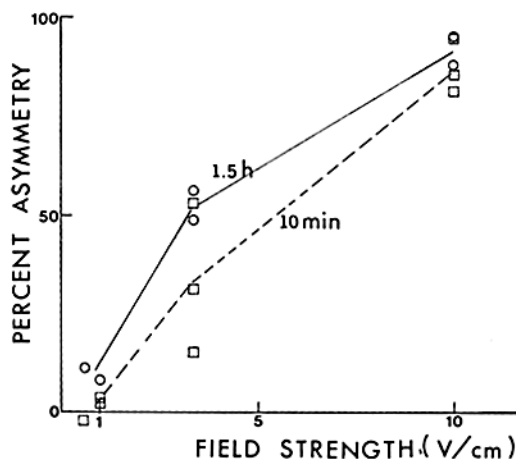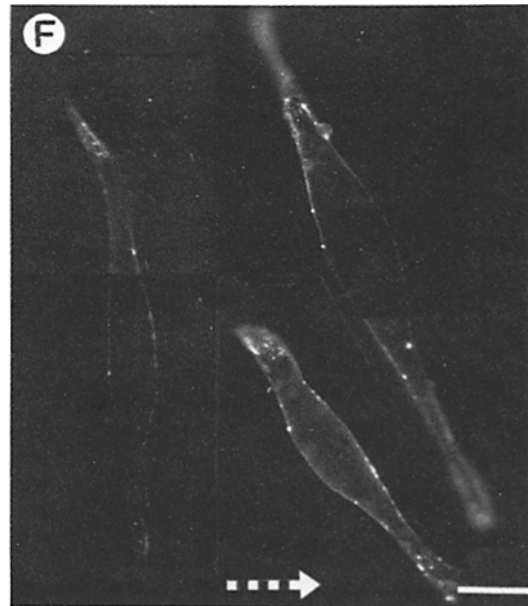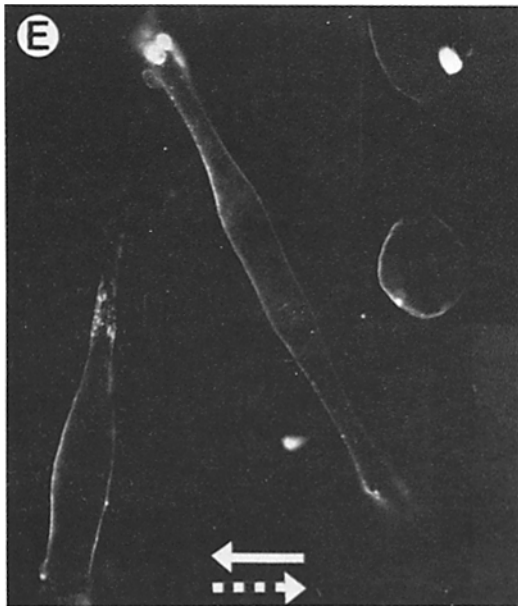
FIGURE 4 Asymmetric accumulation of the Con A receptors vs. the strength of the electric field applied. Receptor accumulation was characterized by the "percent asymmetry" defined in Table I. Data points were from three separate experiments, and each point represents cell scoring from one culture chamber. Fields ranging from 0.5 to 10 V/cm were applied to the cultures for either 1.5 h (—O—) or 10 min (—□—). Curves connect the average values of the data from three experiments.
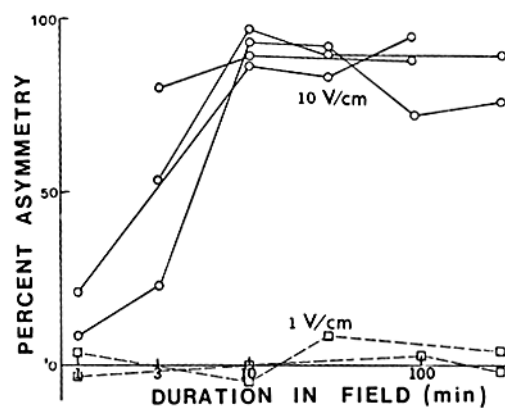
FIGURE 5 Asymmetric accumulation of the Con A receptors vs. the duration of field application. Data were from three separate experiments. Cultures were exposed to a field of either 10 V/cm (O) or 1 V/cm (□) for various durations (ranging from 1 min to 4.5 h). Each curve connects data from the same experiment. Although percent asymmetry appeared to reach a plateau after 10 min in 10 V/cm field, there was an increase in the extent of asymmetry in ring staining of individual cells as duration prolonged, which was not taken into account in the calculation of percent asymmetry.
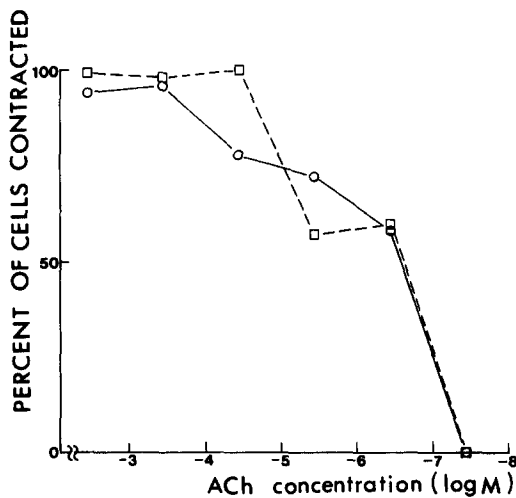
FIGURE 6 Contractile response to acetylcholine (ACh). Control cells not exposed to electric field (□) and cells exposed to a field of 10 V/cm for 3 h (O) were tested for their response to bath-applied ACh. Isolated spindle-shaped muscle cell responds to ACh by spontaneous contraction into a sphere. Percent of spindle-shaped cells that have contracted after the substitution of culture medium with ACh containing Steinberg's saline was taken as a measure of the response.

the following experiments. Groups of cultures that had been treated with an electric field of 10 V/cm for 10 min were allowed to relax after removal of the field for 0, 10, 30, and 90 min; the cells were then labeled and fixed. The results from three experiments are depicted in Fig. 10 (open circles). The asymmetry of stain induced by a 10-min field application disappeared in the culture that had relaxed for 30 min. Ring staining was essentially uniform (see also Fig. 3F). In other experiments, however, we found that when extensive accumulation was induced by fields applied for longer duration, there was a progressive loss of recovery. Fig. 11 shows results of two other relaxation experiments in which accumulation induced by a field of 10 V/cm applied for 0.5, 3, and 4.5 h was allowed to relax. In the last two cases, no complete recovery was observed. In fact, in one experiment a substantial asymmetry of 45% persisted after 2 days of relaxation. This result is consistent with the idea that extensive accumulation of receptors results in the formation of aggregates which are essentially immobile.

Movements Independent of Cell Metabolism

Evidence presented above is consistent with

the idea that the exposure to the electric field caused electrophoretic movement of the Con A receptors in the plane of the membrane, and that removal of the field allowed back diffusion of the accumulated receptors. It has been shown previously that the asymmetric accumulation of the Con A receptors by an electric field is a process independent of cell metabolism (21). We describe here our study on the effect of metabolic inhibition of both types of receptor movement shown in the present report. Table III shows results from the same experiment in which all cultures were preincubated for 50 min in a Steinberg's saline containing 10^{-2} M sodium arsenate, 10^{-3} M sodium fluoride, and 10^{-3} M dinitrophenol. This treatment blocked the contraction response of these cells toward bath-applied ACh (1 mM), indicating depletion of cellular energy supply. Cells so pretreated were then exposed to a field for 10 min (still in the presence of these drugs), and post-field labeling of FITC-Con A was carried out after 0, 10 min, 30 min, and 1.5 h of relaxation. The results of asymmetry scoring indicated that the relaxation of asymmetry was not prevented by the metabolic inhibition. Data from two separate experiments (including that shown in Table III) are plotted in Fig. 10 (filled circles). Distribution of the Con A receptors became essentially uniform after 10 min of relaxation. In these two experiments, the rate of relaxation seems to be more rapid than that in the absence of the drugs. Whether this is due to the action of the drugs on the fluidity of cell membrane or to the drug-induced disruption of metabolic energy-dependent "anchoring" mechanism for the Con A receptors awaits further investigation. A similar result was obtained in another experiment in which 10^{-2} M sodium azide and 10^{-3} M dinitrophenol were used as metabolic inhibitors. We concluded that both accumulation of receptors and recovery of uniform distribution are passive processes independent of cell metabolism. Since these muscle cells do not remain viable after prolonged treatment with these metabolic inhibitors (as indicated by the high intracellular stain of FITC-Con A), experiments requiring longer durations were not carried out.

Receptor Aggregates Induced by Extensive Electrophoretic Accumulation

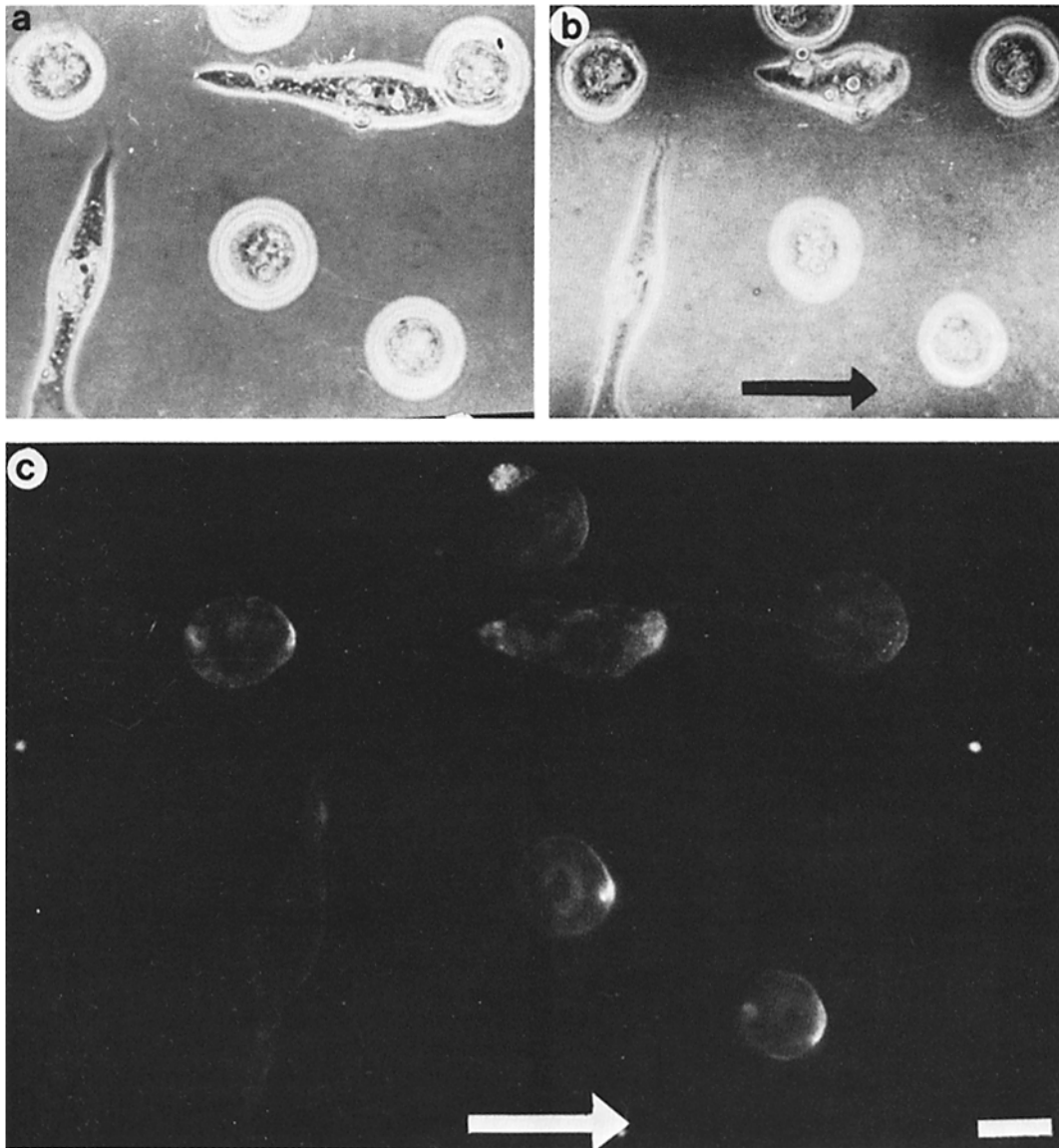Both types of reversal experiments shown in this report suggest that immobile aggregates of

FIGURE 7 Experiment showing no locomotion of the muscle cells in the presence of electric field. Phase-contrast micrograph of a group of spherical and spindle-shaped muscle cells before the start of the experiment (*a*) and after 3 h in a field of 10 V/cm (*b*). All cells in the culture hardly undergo any movement under the influence of the field. One spindle-shaped cell contracted during the 3-h period. Dark-field fluorescence micrograph (*c*) showed that in all cells Con A receptors accumulated toward one side of the cells. Negative pole of the field was on the right-hand side of the figure. Fluorescence FITC-Con A labeling was done immediately after removal of the field. Bar, 25 μm.

the Con A receptors may be formed as a result of extensive passive accumulation of the Con A receptors by an electric field. The phenomenon is illustrated in Fig. 12. Fig. 12*A* shows the postfield FITC-Con A stain of a culture that was exposed to a field of 10 V/cm for 3 h. Fig. 12*B* shows another culture treated the same as that

shown in Fig. 12*A*, but the postfield labeling was delayed for a period of 3 h. Asymmetry persisted for many of the cells in the culture (see also Fig. 11).

In comparison with Fig. 3*D* and *F* which depicts the complete recovery of the uniform distribution for asymmetric accumulation in-

TABLE II

Low Field, Long Duration Experiment

Culture no.	Field‡ strength	Duration of expo- sure	No. of cells exam- ined	No. of cells showing asymmetry in the fluores- cence ring staining			Percent asymme- try
				Toward (−) pole	Toward (+) pole	Indeterminant	
	V/cm						
1	0	0	82	2	5	75	−4
2	0.5	24 h	95	11	5	78	6
3*	0.5	24 h	49	0	0	49	0
4	1.0	24 h	117	19	4	94	13
5	1.0	24 h	127	20	8	99	9
6	1.5	24 h	82	44	7	31	45
7	1.5	24 h	104	63	10	31	51
8*	1.5	24 h	35	18	4	13	40

Except for cultures 3 and 8, the electric field was applied to the cells in culture medium (see Materials and Methods), and the field strength was corrected for the change in the medium conductivity. The criterion for cell counting and the calculation of percent asymmetry were the same as those described for Table I. The threshold field required to induce a detectable asymmetry was between 1.0 and 1.5 V/cm, corresponding to a voltage difference of about 2–3 mV across a muscle cell 20 μm in width. Data are from two separate experiments.

* Electric fields applied in pure Steinberg saline. All others were in supplemented culture medium.

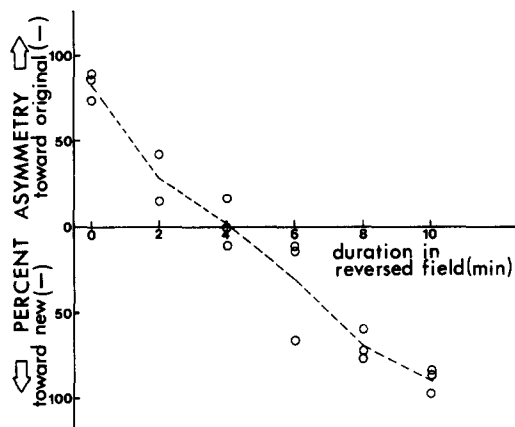‡ 1 V/cm corresponds to a voltage difference of 2 mV across a cell 20 μm wide.

FIGURE 8 Complete reversal of asymmetric accumulation by electric field of opposite polarity. Accumulation of the Con A receptors was first achieved by a field of 10 V/cm applied for 10 min to a group of cultures. Then the same field was reversed in its polarity and was applied to the cultures for an additional 0, 2, 4, 6, 8, or 10 min before postfield labeling was carried out. Asymmetry in receptor distribution progressively shifted toward the negative pole of the second field. Data were from three separate experiments. (See also Fig. 3 D and E). Curve connects average values of the data from three experiments.

duced by 10-V/cm field applied for 10 min, the persistence of accumulation in Fig. 12 B suggests immobilization of the accumulated receptors against back-diffusional transport.
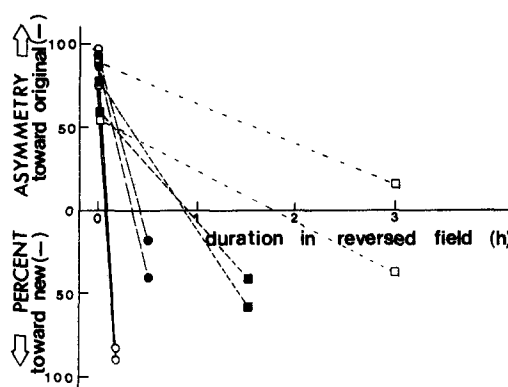
In the other study of asymmetry reversal,

FIGURE 9 Reversibility of Con A receptor distribution vs. the degree of accumulation. Complete reversal of asymmetry as that depicted in Fig. 8 was not found when higher degree of accumulation was achieved by applying the original field for longer duration. Field of 10 V/cm was applied to four pairs of cultures for 10 min (O), 0.5 h (●), 1.5 h (■), and 3 h (□), respectively. One culture of each pair was exposed for an additional 10 min, 0.5 h, 1.5 h, and 3 h in the reversed field of 10 V/cm. Complete reversal of asymmetry was found only for the 10-min pair (O——O). In one experiment, extensive accumulation resulting after 3 h in the original field did not reverse in its asymmetry (□——□). Curves connect data from the same experiment. Data from two separate experiments are shown.

namely, the reversal of asymmetry caused by a reversed field, the cell counts indicate that when the field was applied for a longer duration (3 h, Fig. 9), subsequent treatment of reversal field

● PREPOISENED CELLS

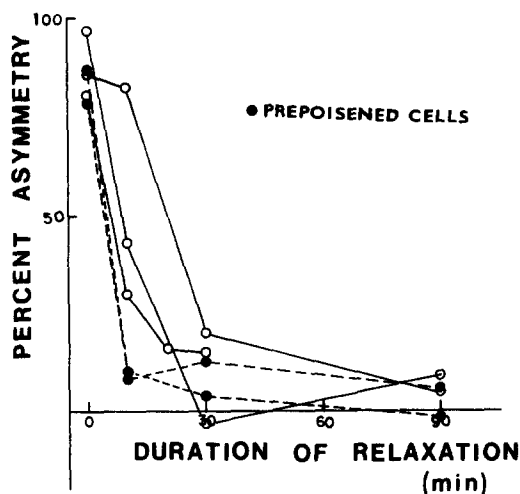PERCENT ASYMMETRY

DURATION OF RELAXATION (min)

FIGURE 10 Recovery of asymmetric distribution by relaxation. Asymmetric distribution of the Con A receptors was induced by applying a field of 10 V/cm for 10 min. After removal of the field, the cells were allowed to remain in saline for different periods of relaxation before FITC-Con A labeling and acetone fixation were carried out. Asymmetric accumulation of the receptors resulting from the field disappeared after 30 min of relaxation (O) (see also Fig. 3F). Two experiments with metabolic inhibitors (10^{-2} M sodium arsenate, 10^{-3} M sodium fluoride, and 10^{-3} M dinitrophenol, see text) are also shown (●), one set of the data was also shown in Table III. Recovery of uniform distribution was complete after 10 min of relaxation. Curves connect data from the same experiment.

for another 3 h did not result in complete reversal of the asymmetry (see also Fig. 12C). We observed that many spindle-shaped cells showed no asymmetry when the stain intensities on the two sides of the cells were compared. However, we noticed that there was an accumulation of receptors on both sides of the cells facing the original negative pole of the field and the negative pole of the reversed field. This effect was most clearly seen on spherical muscle cells. For
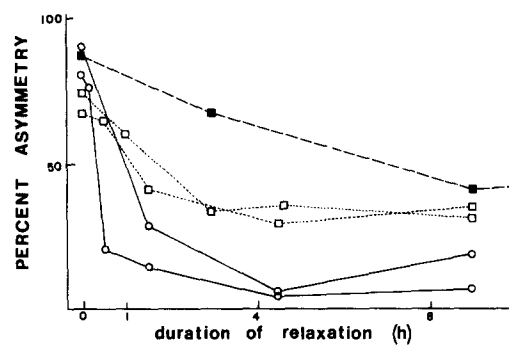

duration of relaxation (h)

FIGURE 11 Incomplete recovery of asymmetric distribution by relaxation. Procedure was the same as that of Fig. 10 except that the accumulation of the Con A receptors was achieved by applying a 10 V/cm for 0.5 h (O), 3 h (□) and 4.5 h (■). The characteristic relaxation time increases progressively. No complete recovery was observed when the accumulation was produced by the field for a duration above 3 h.

TABLE III

Metabolic Inhibition Experiment

Preincubation time	Duration of exposure	Duration of relaxation	No. of cells examined	No. of cells showing asymmetry in the fluorescence ring staining			Percent asymmetry
				Toward (−) pole	Toward (+) pole	Indeterminant	
min*	min‡	min§					
0	10	0	79	64	5	10	75
50	0	0	120	11	8	101	3
50	10	0	57	52	2	4	88
50	10	10	121	17	5	98	10
50	10	30	75	9	6	60	4
50	10	90	94	2	4	88	−2

Preincubation of the cells for 50 min blocked the metabolic energy supply of these cells (as tested by their contraction response to bath-applied ACh). Definite accumulation of the Con A receptors was observed after 10-min exposure to a field of 10 V/cm. Recovery of uniform distribution was complete after 10 min of relaxation. Data are from the same experiment.

* Preincubation medium: pure Steinberg's saline containing 10^{-2} M sodium arsenate, 10^{-3} M sodium fluoride, and 10^{-3} M dinitrophenol.

‡ A field of 10 V/cm was used for all cultures in this experiment except for zero field control. Field was applied in the preincubation medium.

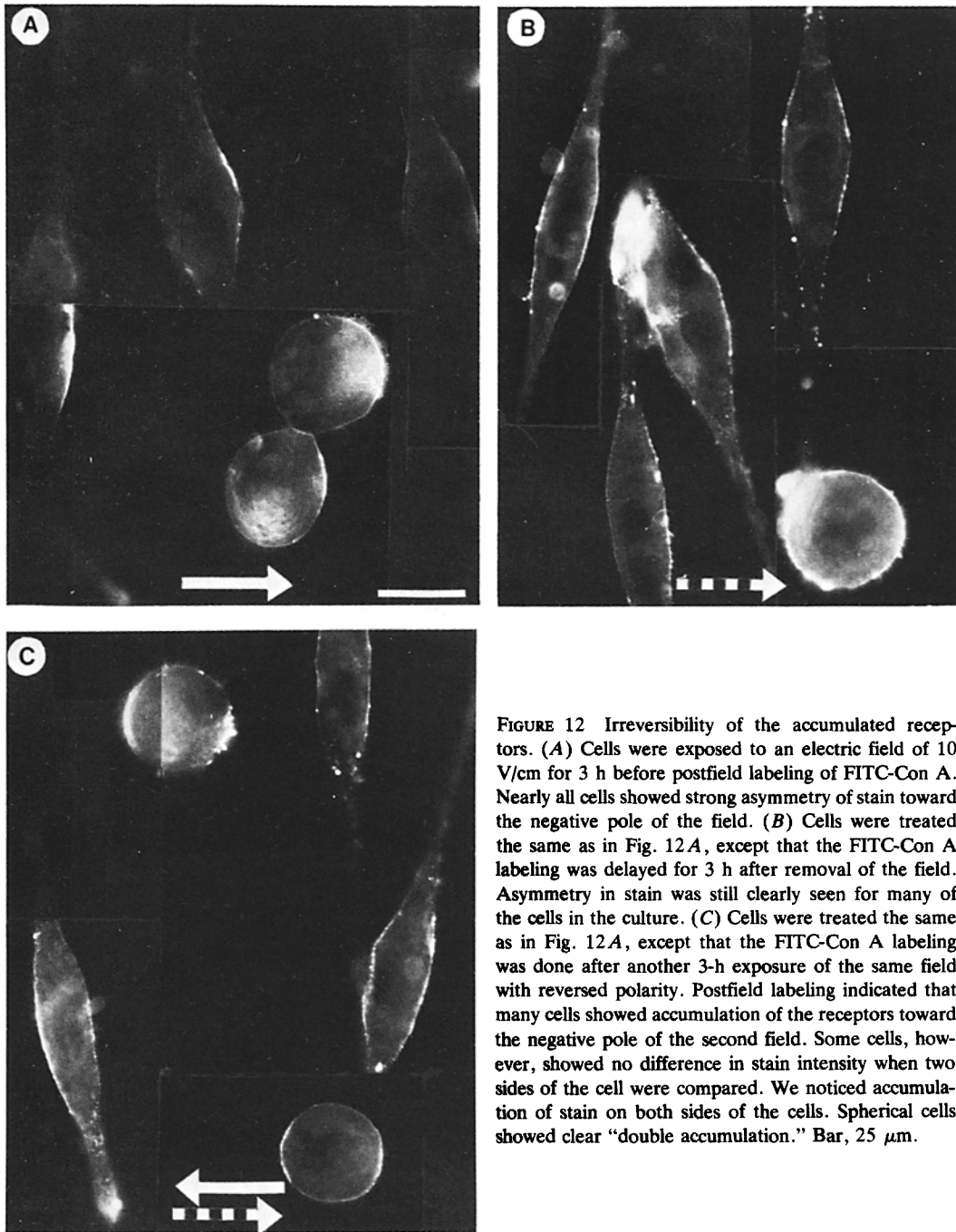§ Cells labeled with FITC-Con A immediately after relaxation period.

FIGURE 12 Irreversibility of the accumulated receptors. (A) Cells were exposed to an electric field of 10 V/cm for 3 h before postfield labeling of FITC-Con A. Nearly all cells showed strong asymmetry of stain toward the negative pole of the field. (B) Cells were treated the same as in Fig. 12A, except that the FITC-Con A labeling was delayed for 3 h after removal of the field. Asymmetry in stain was still clearly seen for many of the cells in the culture. (C) Cells were treated the same as in Fig. 12A, except that the FITC-Con A labeling was done after another 3-h exposure of the same field with reversed polarity. Postfield labeling indicated that many cells showed accumulation of the receptors toward the negative pole of the second field. Some cells, however, showed no difference in stain intensity when two sides of the cell were compared. We noticed accumulation of stain on both sides of the cells. Spherical cells showed clear "double accumulation." Bar, 25 µm.

the control cultures not exposed to the field, FITC-Con A labeling showed uniform ring staining (with random patches) (Fig. 13A and D). When the culture was exposed to a field of 10 V/cm for 3 h, postfield FITC-Con A labeling showed that in nearly all spherical cells the Con A receptors had accumulated toward the negative pole of the field (Fig. 13B and E). However, "double accumulation" of stain (Fig. 12C and F) was observed on those cells that had been

exposed to a reversed field of 10 V/cm for an additional 3 h. Apparently, the original field produced an accumulation of the Con A receptors in which a subpopulation of the receptors did not form aggregates, and thus can still be electrophoresed toward the new negative pole. Although no cell counting was carried out for these spherical cells in the present report, we noticed that the response of the Con A receptor distribution to the electric field was qualitatively similar to that of the spindle-shaped muscle cells.

DISCUSSION

Electrophoretic Mobility and Diffusion Coefficient

The cell scoring technique used in the present report provides an estimate of the receptor distribution over a population of cells, but it does not give a quantitative measurement for progressive electrophoretic accumulation on the same cell. The plot of percent asymmetry vs. duration thus cannot be used directly to calculate electrophoretic mobility. However, the reversal experiment shown in Fig. 8 provided us a crude estimate. Because complete reversal of asymmetric accumulation of receptors from one side of the muscle cell to the other side for nearly all cells (See Fig. 3 D and E, and Fig. 7) requires movement of the accumulated receptors across a distance larger than the half-width of the cell (11.6 $\pm$ 2.0 μm SD, n = 72), we concluded that the electrophoretic mobility of the most mobile population[1] of Con A receptors is about 12 μm/10 min per 10 V/cm, i.e. 2 $\times$ 10^{-3} μm/s per V/cm, a mobility that is 500 times slower than that for a typical globular protein (e.g. hemoglobin, reference 24) in aqueous solution.

Nothing is known about the charge properties of the Con A receptors on these embryonic muscle cells. However, if one simply assumes that the charges on these receptors are similar to the charge on a typical soluble protein, the above estimate of electrophoretic mobility of Con A receptors indicates that the membrane environment in which these Con A receptor molecules undergo electrophoretic movement is about 5 poise (since water's viscosity is 1 centipoise), a

[1] Con A receptors seem to be heterogeneous in their mobility (see Discussion); the receptors that were accumulated earlier by the electric field are presumably most mobile.

value consistent with other measurements of membrane viscosity (5).

The recovery of uniform distribution after removal of the electric field provides a basis for an estimate of the diffusion coefficient of the Con A receptors in the plane of the membrane. The characteristic $1/e$ time for the relaxation of asymmetry induced by a 10 V/cm field applied for 10 min is about 20 min (range: 15–25 min). The diffusion coefficient for the most mobile nonliganded receptors was thus estimated to be about 5 $\times$ 10^{-10} cm^2/s (range: 4–7 $\times$ 10^{-10} cm^2/s), since $D = r^2/2t$ where r is equal to the half width of the cell, and t is the $1/e$ relaxation time (11).

The diffusion coefficient of the Con A receptors has been measured in several other cell types. It ranges from 5 $\times$ 10^{-11} to 10 $\times$ 10^{-11} cm^2/s in the membrane of mouse 3T3 and SV3T3 cultured cells (12) and 8 $\times$ 10^{-12} to 3 $\times$ 10^{-11} cm^2/s on cultured rat embryo myoblasts (22). The higher diffusion coefficient obtained in the present report may be due to the fact that (a) we were measuring a certain selective population of charged mobile receptors and that (b) by the postfield FITC-Con A labeling technique we are studying the movements of the nonliganded receptors. The slower diffusion rate of the Con A receptors observed by Jacobson et al. (12) and Schlessinger et al. (22) may be due to some cross-linking of the receptors by succinylated Con A used in those studies.

The degree of the asymmetric accumulation of cell surface receptors is determined by not only the magnitude of the field applied along the membrane but also the ratio of electrophoretic mobility to diffusion coefficient. At equilibrium, the percent asymmetry (ϕ) induced by the field can be estimated by the equation:

$$\phi \approx (m/D) \cdot (V/6) \cdot 100\%,$$

where m is the electrophoretic mobility, D is the diffusion coefficient, and V is the voltage applied across the cell (13). The time required to reach the equilibrium state is of the order of the characteristic $1/e$ relaxation time for the back-diffusion of the receptors over the cell surface. These arguments lead to two predictions in the present study: First, with use of the m and D values estimated in the present study, the percent asymmetry produced by a field of 1 V/cm (corresponding to a voltage drop of 2 mV across a cell 20 μm in width) is about 13%, an insignificant asymmetry close to that of the control cultures (<10%). Second, when insignificant asymmetry is induced
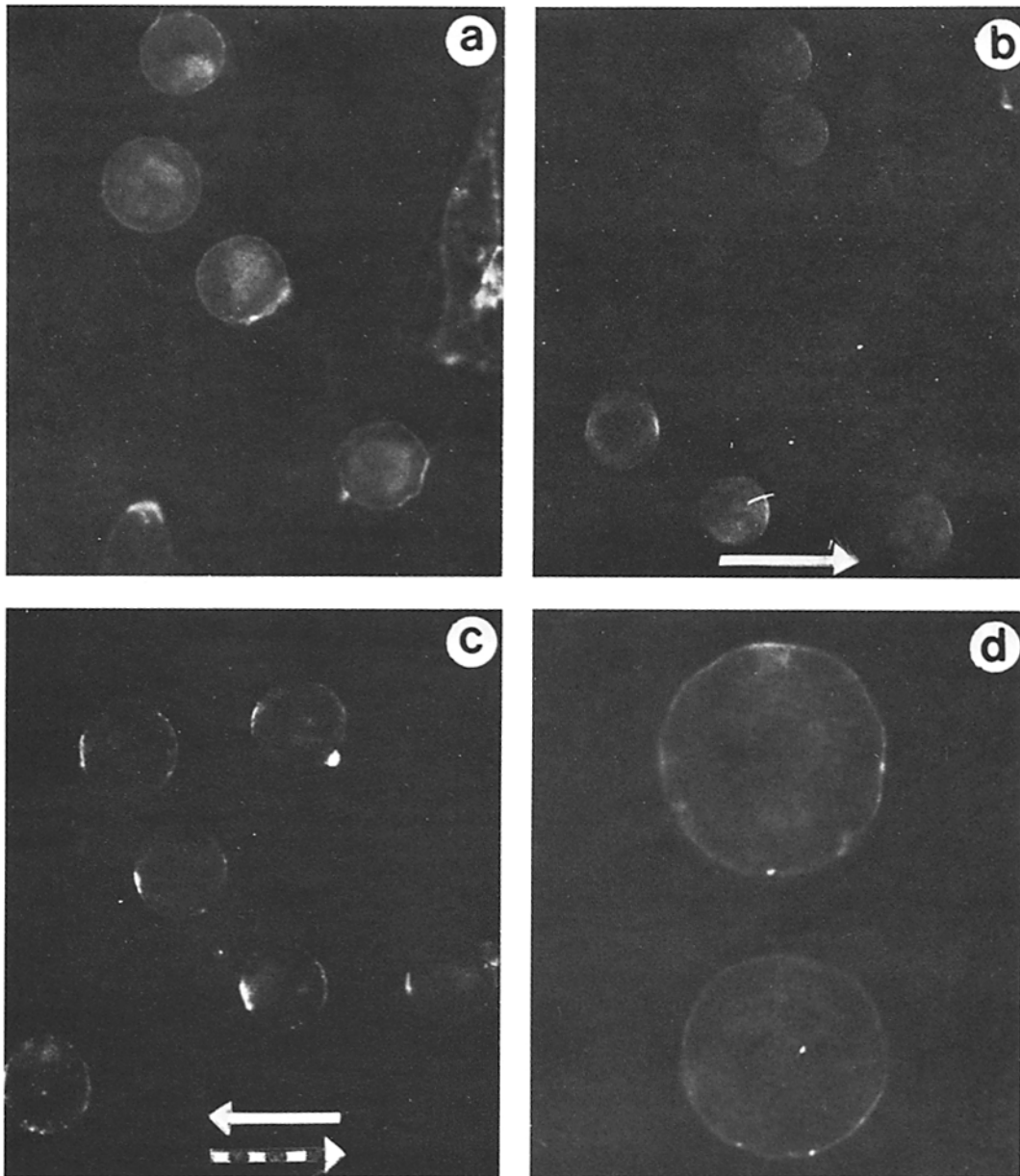
FIGURE 13   Accumulation of the Con A receptors on spherical muscle cells. (a and d) Control cells not exposed to the electric field. The ring staining of FITC-Con A was essentially uniform (with random micropatches). (b and e) The cells were exposed to a field of 10 V/cm for 3 h. Postfield labeling of FITC-Con A indicates that the Con A receptors accumulated toward the negative pole of the field on nearly all cells in the culture. (c and f) "Double accumulation" of the Con A receptors on two poles of the cells was observed when the cells were first exposed to a field of 10 V/cm for 3 h in one direction (toward right-hand side) and then to an additional field of 10 V/cm for another 3 h in the reverse direction (toward left-hand side). Postfield labeling was carried out immediately after removal of the latter field. Some stain persisted on the pole facing the negative pole of the first field, indicating formation of immobile aggregates which were not redistributed by the second field. (a-c) Dark-field fluorescence micrographs. (d-f) High-magnification micrographs taken using × 40 oil immersion objective and epi-illumination optics. Bar, 25 μm.
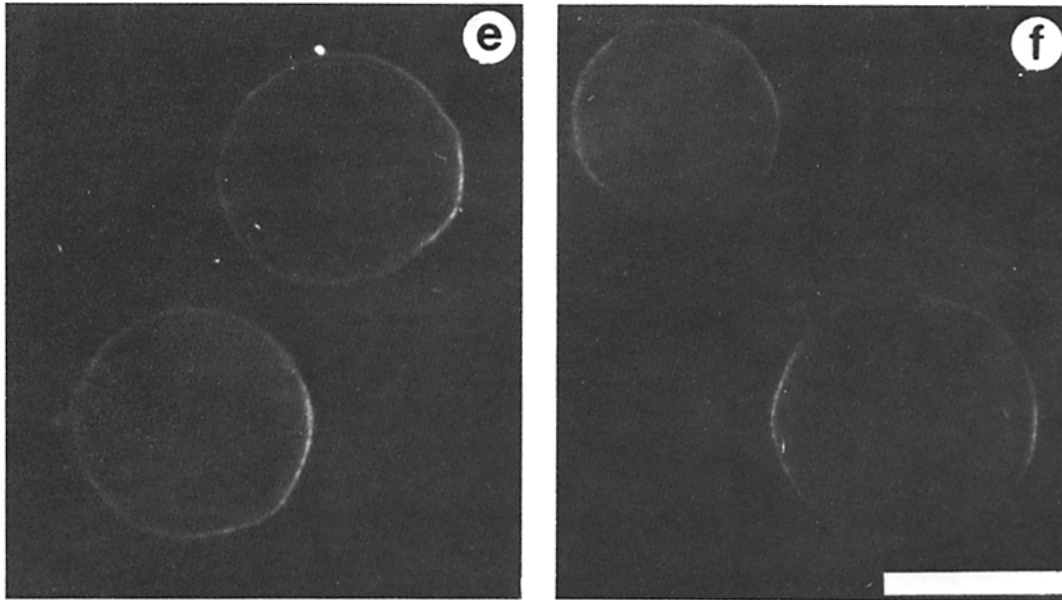
within the characteristic diffusion time ($\approx$20 min), no increase in asymmetry can be achieved regardless of the duration of further exposure to the same field, since the distribution of the receptor is already in the state of equilibrium. Fig. 5 showed that a field of 1 V/cm induced insignificant asymmetry even when applied for 4.5 h, while a 10 V/cm field induced definite asymmetry in 10 min. This is consistent with the above predictions. Moreover, the low-field experiments (Table II) indicated that the threshold field needed to induce observable accumulation is between 1.0 and 1.5 V/cm, very close to the threshold observed in the short-term (1.5 h) experiment shown in Fig. 4. In other words, the equilibrium state between the electrophoretic and diffusional transport of most of the Con A receptors has indeed been reached within 1.5 h. The consistency of these results gives strong support of the idea that the movements of Con A receptors observed are electrophoretic and diffusional in nature.

Properties of the Con A Receptors

The accumulation of the Con A receptors occurred on the side of these cells facing the negative pole of the field. It indicates that the majority of these molecules are either positively charged or less negatively charged than other mobile membrane components. The origin of this positivity is unknown; the basic amino groups on the glycoproteins are one possibility. It is unlikely that most of the Con A receptors are neutral in their charge and that the observed accumulation of the Con A receptors is due to exclusion by other redistributed charged membrane components, since for an effective exclusion to occur, nearly all other membrane components have to be negatively charged. Studies of the composition of cell membranes indicate that only a small minority of the membrane lipids are charged at physiological pH (2, 4, 8).

The Con A receptors in the embryonic muscle cell membrane probably consist of a heterogeneous population of glycoproteins with different charge properties and/or varying degrees of mobility in the plane of the membrane. Three observations in the present report suggest this heterogeneity of the Con A receptors: First, we have never observed complete segregation of the Con A receptors. Some stain was always seen on the side of the cell facing the positive pole of the field even when extensive accumulation has occurred on the other side. This indicates the existence of subpopulations of receptors which are relatively more negative in charge or essentially immobile. Second, the data on the relaxation of asymmetry depicted in Fig. 8 indicate that the characteristic relaxation time increased when more receptors had been accumulated. This is consistent with the expectation that less mobile receptors accumulated more slowly by the field also diffused more slowly with longer relaxation time, resulting

in an increase in average relaxation time. Finally, "double accumulation" of the receptors shown in Fig. 13 C and F indicates that there exist subpopulations of the receptors that are different in their capability in forming aggregates and/or susceptibility to receptor anchoring by submembrane cytoskeletal organization (15, 16). Evidence of the heterogeneity in the Con A receptor mobility was also reported for cultured rat myoblast cells (22).

Comparison with Previous Report

Both the electrophoretic mobility and diffusion coefficient reported here are higher than those in the previous preliminary report (21). We attribute the differences to the following: (a) Estimate of electrophoretic mobility from the relation between asymmetry cell counts vs. duration, as was done previously, is less adequate as discussed in the above. In the previous study, we did not find the complete recovery of distribution after the relaxation, hence the diffusion coefficient was inadequately estimated from the partial relaxation data. (b) The use of high-resolution epi-illumination optics and the FITC selective filter combination in the present study greatly facilitates the resolution of weak asymmetry. Presumably, the receptor mobility reported here was that of the most mobile population of the Con A receptors. The dark-field transmission fluorescence optics used in the previous report resolved only more extensive accumulation. The mobility estimated previously may represent an average mobility for a more heterogeneous population of receptors. The low-field study in the present report also indicated a higher threshold field (1.0–1.5 V/cm) for inducing detectable accumulation of the receptors. This is probably due to inadequate estimate of the culture chamber cross section (hence field strength) and/or insufficient cell counting in the previous report.

Finally, we have used pure Steinberg's saline to substitute for the culture medium before the start of all of the experiments, except in the long-duration studies (Table II). This modification in the procedure simplified the interpretation of the observed results, since it eliminated the possibility of interaction between the supplemented serum proteins and the cell surface receptors. This interaction may result in changes of receptor mobility that could account for some of the differences in the mobilities observed in the two reports.

Alternate Mechanisms

The two types of redistribution of the Con A receptors described in the present report are consistent with the idea of electrophoretic and diffusional movements in the plane of cell membrane. We have not, however, excluded all other possible mechanisms by which an external electric field could produce redistribution in surface receptors. As discussed in the previous report (21), the action of the electric field is unlikely to be intracellular, since the intracellular field strength is too small to be effective in redistributing cytoplasmic components. It is also unlikely that the observed redistribution is due to oriented locomotion of the cell (10) or to the directed flow process (3) in the membrane. We have shown in Fig. 7 that the cell hardly moved during a 3-h period of field application when extensive accumulation of the Con A receptors occurred. Moreover, cell locomotion or directed flow process in the membrane presumably requires a supply of metabolic energy, while the movements we observed were independent of cell metabolism.

In conclusion, postfield labeling of FITC-Con A has enabled us to study both the electrophoretic mobility and the diffusion coefficient of nonliganded Con A receptors in the plane of the cell membrane. The results suggest that the Con A receptors are heterogeneous in their mobility and that the most mobile population of the Con A receptors have mobilities higher than those reported for Con A-receptor complexes in other culture cells (12, 22, 25). Extensive accumulation of the receptors by an electric field resulted in the formation of immobile aggregates. Cell scoring over a population of cells was required to study the movements of nonliganded receptors in the present report. In situ electrophoresis, however, may be equally applicable in the quantitative measurements of the mobilities of receptor-ligand complexes in the membrane of single cells.

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