A Role of Tyrosine Phosphorylation in the Formation of Acetylcholine Receptor Clusters Induced by Electric Fields in Cultured *Xenopus* Muscle Cells

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Abstract. During the development of the neuromuscular junction, acetylcholine receptors (AChRs) become clustered in the postsynaptic membrane in response to innervation. In vitro, several non-neuronal stimuli can also induce the formation of AChR clusters. DC electric field (E field) is one of them. When cultured *Xenopus* muscle cells are exposed to an E field of 5–10 V/cm, AChRs become clustered along the cathode-facing edge of the cells within 2 h. Recent studies have suggested the involvement of tyrosine kinase activation in the action of several AChR clustering stimuli, including nerve, polymer beads, and agrin. We thus examined the role of tyrosine phosphorylation in E field–induced AChR clustering. An antibody against phosphotyrosine (PY) was used to examine the localization of PY-containing proteins in E field–treated muscle cells. We found that anti-PY staining was colocalized with AChR clusters along the cathodal edge of the cells. In fact, cathodal PY staining could be detected before the first appearance of AChR clusters. When cultures were subjected to E fields in the presence of a tyrosine kinase inhibitor, tyrphostin RG-50864, cathodal AChR clustering was abolished with a half maximal inhibitory dosage of 50 μM. An inactive form of tyrphostin (RG-50862) had no effect on the field-induced clustering. These data suggest that the activation of tyrosine kinases is an essential step in E field–induced AChR clustering. Thus, the actions of several disparate stimuli for AChR clustering seem to converge to a common signal transduction mechanism based on tyrosine phosphorylation at the molecular level.

THE formation of acetylcholine receptor (AChR)[1] clusters in the postsynaptic membrane is the central event in the development of the neuromuscular junction (NMJ) (4, 10). Although this process is induced by innervation in vivo, a number of non-neuronal stimuli can substitute for the nerve in causing AChRs to cluster in vitro. These include bath application of soluble factors such as agrin (12), spontaneous interaction between the muscle cell and the culture substratum (4), treatment of muscle cells with polymer beads (1, 3, 26, 27), and electric fields (E fields) (16, 23, 33). The effect of the first three kinds of stimuli is presumably mediated by molecular interactions between the stimulus and the cell surface. For example, the agrin effect may be mediated by putative agrin receptors (22) and beads may act by local presentation of either endogenous, cell surface-bound or exogenous, bead-bound molecules (1, 3, 27). However, E field is unique among these stimuli in that it exerts its action at a distance, apparently without the mediation of a physical contact or a chemical intermediary. When cultured *Xenopus* muscle cells are exposed to a constant E field, AChR clusters are formed along the cathode-facing edge (16, 23, 33). In addition, ultrastructural and cytoskeletal specializations characteristic of the postsynaptic membrane also develop at these E field–induced clusters (16, 31). The simplicity and effectiveness of this stimulus make it an ideal tool to understand the mechanisms of postsynaptic development at the NMJ.

Several theories have been advanced to explain the mechanism of E field–induced AChR cluster formation. First, it was proposed that E field may aggregate AChRs by electromigration in the plane of the membrane that results from a combination of lateral electrophoresis and electro-osmosis (21, 28). However, recent studies by Stollberg and Fraser (34) have shown that E field is not the motive force, but rather the trigger for AChR clustering. Their data further suggest that the triggering mechanism is set up by the asymmetric accumulation of certain non-AChR molecules as opposed to the accumulation of AChRs themselves (34, 35). Another possibility is that the externally applied E field imposes an asymmetric change in membrane potential, resulting in a depolarization at the cathodal edge (5), and this may trigger AChR clustering by a voltage-dependent mechanism.

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1. *Abbreviations used in this paper*: AChR, acetylcholine receptor; E field, electric field; g.s., grey scale; NMJ, neuromuscular junction; PY, phosphotyrosine; R-BTX, tetramethylrhodamine-conjugated α-bungaroxin.

Through the use of pulsed voltage waveforms, Stollberg and Fraser (35) found that an E field too small to elicit voltage-dependent changes can still elicit AChR clustering along the cathode. Thus, changes in membrane potential are not necessary for the E field–induced AChR clustering. They concluded that E field triggers AChR clustering by a mechanism that involves the accumulation of certain non-AChR molecules along the cathodal edge of the muscle cell (35). The nature of this trigger is the subject of this investigation.

Despite the diverse nature of AChR clustering stimuli in vitro, it is reasonable to assume that their actions all converge on a common signal transduction pathway. Recent studies have suggested that tyrosine kinase activation is involved in AChR clustering induced by innervation (30), agrin (42), and beads (2, 3, 27). Thus, it is of interest to examine whether similar steps are also involved in E field–induced AChR clustering. In this study, we used immunocytochemical and pharmacological methods to explore the role of tyrosine kinase activation in this process. Here we report that E field also seems to act on the signal transduction pathway involving tyrosine phosphorylation as an early step.

Materials and Methods

Muscle Cultures and the Application of E Field

Muscle cells were isolated from *Xenopus* embryos according to a previously published method (25). In short, stage 20–22 embryos were freed from the surrounding jelly coat and vitelline membrane and the myotomal portion was dissected out and dissociated in calcium, magnesium-free Steinberg solution (60 mM NaCl, 0.7 mM KCl, 0.4 mM EDTA, 10 mM Hepes, pH 7.4). The dissociated cells were plated onto No. 1 coverglass circles (13-mm diam) and cultured in a medium composed of Steinberg solution (60 mM NaCl, 0.7 mM KCl, 0.4 mM Ca[NO₃]₂, 0.8 mM MgSO₄, and 10 mM Hepes, pH 7.4) supplemented with 10% L-15 medium, 1% FBS, and 0.1 mg/ml gentamycin. Cultures were maintained at 15° or at 22°C and were used in E field experiments within 3–4 d after plating.

The cultures were exposed to E fields in a chamber modified from the design of Harris et al. (13). In short, two strips of coverglass spacers were glued to the bottom of a 35-mm petri dish to form a groove with a dimension of 15 mm × 15 mm × 0.3 mm. The culture coverglass was placed within the groove and covered with a block made of Sylgard 184 silicone elastomer (Dow Corning, Midland, MI) that was fitted over the groove to give a uniform cross-sectional area around the culture coverglass. The chamber was connected to Ag-AgCl electrodes via 1% agar-saline bridges. Current was applied to the culture in complete medium with a constant voltage power supply and monitored with a digital ammeter. The field strength was adjusted with a potentiometer connected in series with the electrodes and the chamber. A field strength of 5–10 V/cm was used in this study.

Immunofluorescence

Before E-field exposure, the cultures were labeled with tetramethylrhodamine-conjugated α-bungarotoxin (R-BTX; Molecular Probes, Portland, OR) at a concentration of 0.3 μM for 30 min and washed. After E-field exposure, they were fixed with 95% ethanol at −20°C for 5 min and mounted for fluorescence microscopy. To study the localization of proteins that were phosphorylated on tyrosine, we labeled cultures with a mouse mAb (mAb 4G10; UBI, Lake Placid, NY) that was generated by using phosphotyramine as the immunogen. Before antibody labeling, cultures were blocked with a 75 mM Tris buffer containing 0.1% BSA for 1 h. The antibody was reconstituted to a concentration of 13 nM and the labeling was carried out for 1 h. After washing, cultures were labeled with a FITC-conjugated goat anti-mouse secondary antibody (Organon Technika, Durham, NC). The specimens were examined with a fluorescence microscope (E. Leitz Inc., Rockleigh, NJ) using a 63× (NA 1.4) objective and recorded on Kodak T-MAX film (Eastman Kodak Co., Rochester, NY).

Application of the Tyrosine Kinase Inhibitor

The tyrosine kinase inhibitors tyrphostin RG-50864 (active) and RG-50862 (inactive) were kindly provided by Rhone-Poulanc Rorer Central Research (Fort Washington, PA). They were dissolved in DMSO to make a 40 mM stock solution. This stock was divided into aliquots and stored at −80°C. Before each experiment, working solutions were prepared with culture medium by serial dilution. Cultures were preincubated in tyrphostin-containing medium for 2–3 h and then exposed to E field in the continued presence of the drug. To study whether the drug effect can be reversed, cultures were preincubated with tyrphostins for 2–3 h and then exposed to E field in the absence of the drug.

Quantitation of Asymmetric Phosphotyrosine and AChR Distribution

To quantify the effect of electric field in inducing the asymmetric distribution of both phosphotyrosine (PY)-containing proteins and AChRs, the fluorescence intensities in grey scale (g.s.) values along the cathodal and anodal edges of the cell were measured. Cells whose long axis was within 45° perpendicular to E field direction were selected for measurements. Images were acquired with a SIT camera (Cohu, San Diego, CA) and digitized with Image-1 hardware and software (Universal Imaging, West Chester, PA). The images were frame averaged and background subtracted before each measurement. In cultures that had clearly defined cathodal fluorescent patches, the measurements along the cathodal edge were taken at these patches. Three measurements were made on each patch, each encompassing a 2 × 3 pixel area. After each cathodal measurement, three measurements were also made along the anodal side that was exactly opposite to the cathodal fluorescent patch (in the direction of the E field). For cells that did not exhibit cathodal patches, e.g., in cultures exposed to the field for a short duration or after tyrphostin treatment, three arbitrary spots along the cathodal side and their counterparts along the anodal side were measured. The means of these measurements were calculated and used to determine the asymmetry index defined as:

A.I. = (cathodal g.s. − anodal g.s.)/(cathodal g.s. + anodal g.s.)

Results

Signaling of AChR Clustering by E Fields

When *Xenopus* muscle cells were exposed to a constant E field of 5–10 V/cm for 2 h, AChRs became clustered along the cathode-facing edge of the cell. In this study, only well-spread cells were examined as opposed to spherical myoballs used in previous studies (23, 35), because the flatness of the spread-out cells facilitated immunocytochemical observation conducted in this study. Examples of E field–induced cathodal clusters are shown in Fig. 1. AChR clusters were observed along the edge of the cell, including thin membrane lamellae or cell processes facing the cathode (Fig. 1). They were observed in nearly every muscle cell exposed to E field. In addition to the field-induced clusters, these muscle cells also exhibited spontaneously formed AChR clusters (hot spots) that bore no relationship to the orientation of the field as shown in Fig. 4 D.

Cathodal clusters could be observed in 10% of the cells after a 15-min field exposure (Fig. 2). The number of cells exhibiting these clusters increased as they were exposed to the field for longer duration until the response reached its maximum after 2 h of exposure. However, if the incubation was continued after a brief exposure to the field ("postfield relaxation"), a postfield increase in cathodal clustering was observed. As shown in Fig. 2, 45% of the cells exhibited cathodal clusters if they were first exposed to the field for 15 min and then allowed a 1 h and 45 min postfield relaxation.
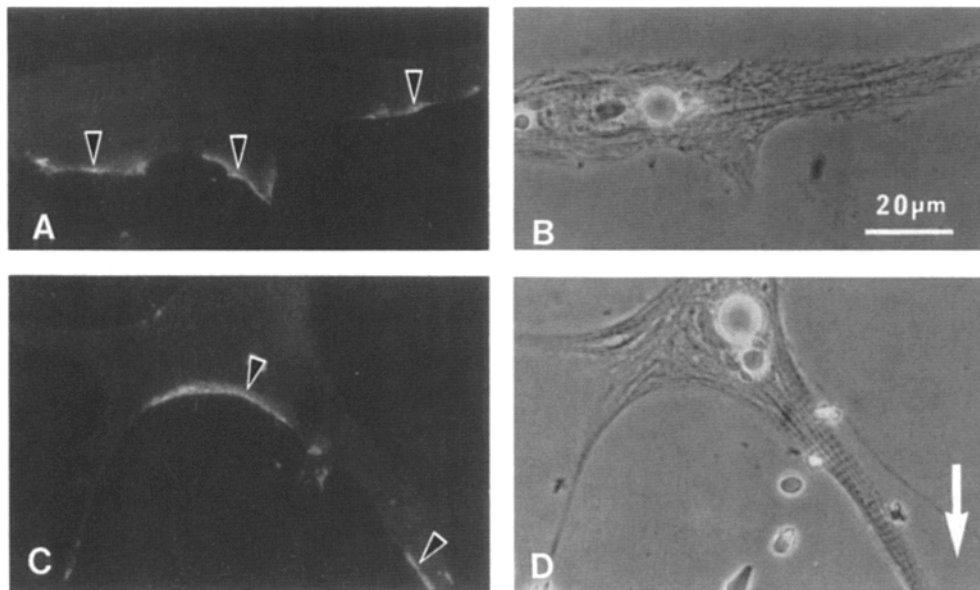
Figure 1. AChR clusters (*arrowheads*) along the cathode-facing edge of cultured *Xenopus* muscle cells after E-field treatment for 2 h at 6.6 V/cm. The fluorescence micrographs of R-BTX–labeled cells are shown on the left and their phase-contrast images are shown on the right. In this and subsequent micrographs, the white arrow indicates the direction of E field, pointing to the cathode.

A similar postfield increase in AChR clustering was observed after a 30 to 60 min E-field exposure (Fig. 2). This postfield increase in AChR clustering is consistent with previous results obtained from studies on spherical cells (33). It suggests that AChRs are not directly clustered by E field itself, but rather by a mechanism that is triggered by it. In these experiments, we prelabeled cells with R-BTX befor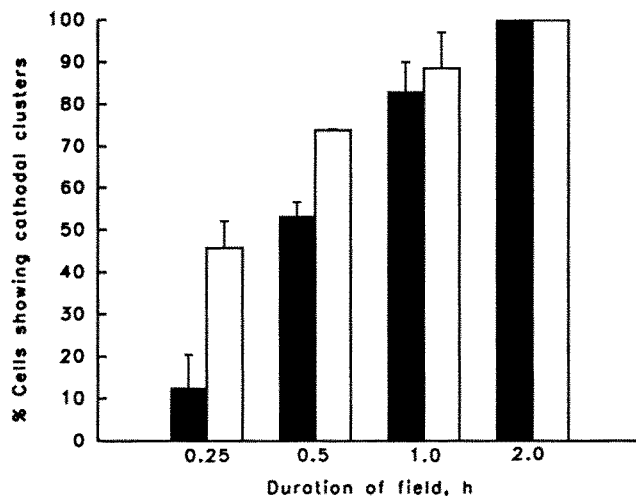e E-field application. Only the pool of AChRs preexisting on the cell surface was labeled. Thus, preexisting AChRs at the cell surface contributed extensively to the cathodal clustering induced by the field.

The precise timing of the onset of receptor clustering in response to E field allowed us to characterize the development of these clusters. As shown in Fig. 3 *A*, early cathodal clusters observable after 15–30 min in the field had a loose organization as compared to "mature" clusters that were seen in cells exposed to the field for 2 h as shown in Fig. 1. As shown in Fig. 3 *A*, they consisted of a cluster of small, punctate AChR aggregates scattered over a distance of 5–8 μm from the cathodal edge. As the field exposure was lengthened, a consolidation of these punctate aggregates into a narrow, linear pattern along the immediate cathodal edge was seen (Fig. 3 *B*). This process continued until solid bright clusters along the cathodal edge were formed in 2 h (Fig. 3 *C*).

Colocalization of Phosphotyrosine with E Field–Induced Clusters

To understand the role of tyrosine phosphorylation in the action of E field, immunofluorescence studies with anti-PY mAb 4G10 were conducted to study the distribution of PY-positive proteins in relation to AChR clusters. In addition to E field–induced clusters, we also examined the localization of PY at the NMJ and at aneural AChR clusters (hot spots) in vitro. As shown in Fig. 4, *A–C*, NMJs in culture were associated with strong PY staining. The precise colocalization of PY staining (Fig. 4 *B*) and AChR clusters (Fig. 4 *A*) suggests that PY was concentrated at the postsynaptic membrane. In addition, PY staining was also clearly concentrated at most, but not all AChR hot spots (Fig. 4, *D* and *E*). The similarity in PY and R-BTX staining patterns suggests that at least part of the PY-positive proteins at these sites may be the AChR itself, since it is known that several subunits of the AChR can be phosphorylated on their tyrosine residues (41).
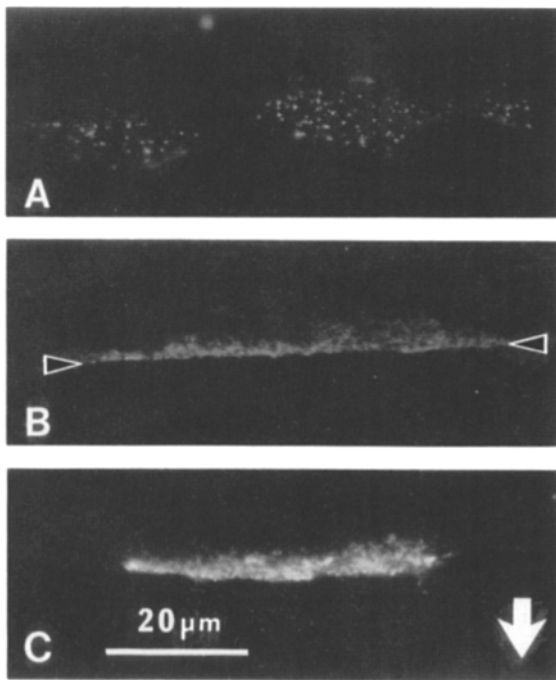
Figure 2. Continuation of AChR clustering after E-field termination. The cells were exposed to the field for the duration indicated on the horizontal axis. After E-field termination, cultures were either fixed and examined right away (■) or allowed a period of postfield relaxation up to a total of 2 hr before fixation (□). Clustering of AChRs along the cathodal edge continued after the termination of the field. Results from two separate experiments were pooled. A mean of 120 cells per treatment were scored. The error bars show standard error of the mean (SEM). For the 0.5 h "Field + Postfield" bar (□), the SEM is 0.23%. For the 2 h bars, the SEM is 0.

Figure 3. E field–induced AChR clusters at three stages of formation. Early cathodal clusters (*A*, 15–30 min) were punctate and spread over a distance of 5–8 μm from the cathodal edge. Intermediate (*B*, 1 h) and mature (*C*, 2 h) cathodal clusters were composed of linear, consolidated AChR patches along the immediate cathodal edge (between arrowheads in *B*).

Figure 4. Colocalization of PY with AChR clusters. (*Left*) A NMJ in culture. PY (*B*), visualized by staining with mAb 4G10 and FITC secondary antibody, was colocalized with AChR clusters (*A*) at the postsynaptic membrane. Phase-contrast image of this NMJ is shown in *C*. (*Right*) Most, but not all, AChR hot spots (*D*) were also associated with PY staining (*E*). In this cell, clusters 2 and 3 were PY-positive, whereas cluster 1 was not.

However, the absence of PY concentration at some aneural clusters (e.g., Fig. 4 *D, 1*) indicates that tyrosine phosphorylation of AChRs may not be necessary for the maintenance of these AChR clusters. The specificity of the PY staining was ascertained by immunoabsorption studies. After absorbing the PY antibody with 40 mM *o*-phospho-L-tyrosine, the staining was totally abolished (*2*). In addition to being concentrated at AChR clusters, PY staining was also seen as diffuse, punctate structures in the muscle cell that were not stained by R-BTX (Fig. 4 *E*). This staining appeared to be specific since it was also abolished by preabsorbing mAb 4G10 with *o*-phospho-L-tyrosine.

To examine the role of tyrosine phosphorylation in the action of E field, we exposed muscle cells to E field and then stained them with the anti-PY antibody. As shown in Fig. 5, *A* to *B*, mature cathodal clusters were clearly associated with PY staining. There was generally a high degree of congruency between the PY labeling and AChR clusters and all cathodal clusters examined were positive in the antibody labeling. Like AChRs, PY concentration also displayed a highly asymmetric distribution along the cathodal edge. The ability to time the onset of the field-induced cluster formation afforded us the opportunity to examine the temporal relationship between AChR and PY accumulation along the cathodal edge. As shown in Fig. 5, *C* and *D*, muscle cells exposed to a brief field (15–30 min) often exhibited clear cathodally associated PY staining in the absence of visible AChR clusters. Even after 1-h exposure, R-BTX staining still lagged behind PY staining in brightness (Fig. 5, *E* and *F*).

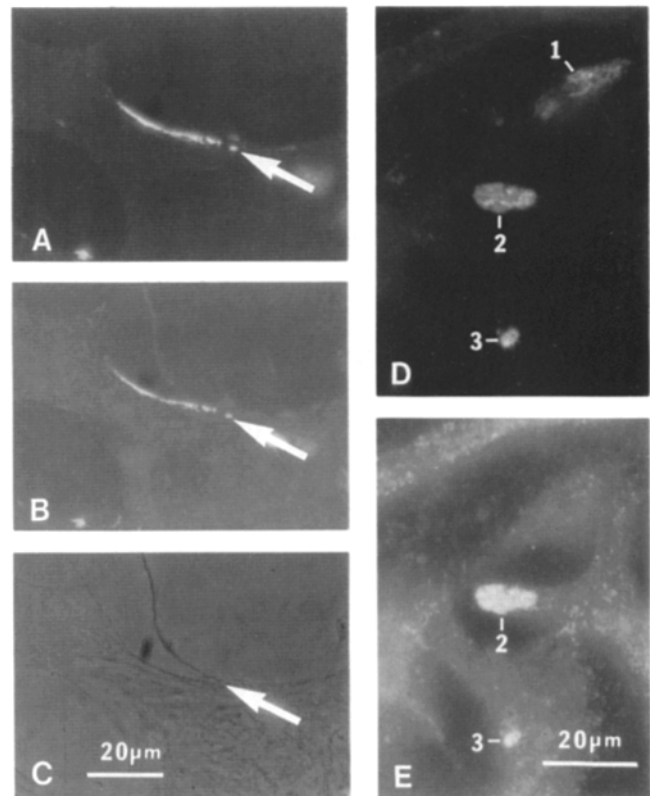We quantified the time course of AChR clustering and PY accumulation in Fig. 6. First, the percentage of cells that exhibited either cathodal PY labeling or AChR clustering were scored. As shown in Fig. 6 *A*, muscle cells exposed to E field developed cathodal PY clusters earlier than AChR clusters. To quantify the polarized localization of PY and AChR accumulation along the cathodal edge, we determined the FITC and rhodamine fluorescence levels along the cathodal and anodal edges and calculated an asymmetry index from these values (see Materials and Methods). This index has a theoretical range from −1 to +1. Any positive value indicates an asymmetric distribution along the cathodal edge. This analysis was carried out at the level of individual cathodal clusters. A cathodal cluster, showing either PY or AChR labeling, was defined by the appearance of a discrete patch of fluorescence along the cathodal edge of the cell. For example, four cathodal clusters were scored in Figs. 5, *A* and *B*, whereas one patch was scored in Fig. 3 *C*. However, microclusters (<1-μm diam) that appeared to belong to a larger group were not individually analyzed. For example, the cell in Fig. 3 *A* was considered to have only three discrete clusters and the cell in Fig. 5 *D* showed only one cluster. As shown in Fig. 6 *B*, the onset of the asymmetric PY distribution preceded that of AChRs by at least 15 min. These results thus suggest the possibility that, at least during the early stages of cluster formation, the PY-positive proteins along the cathodal edge may
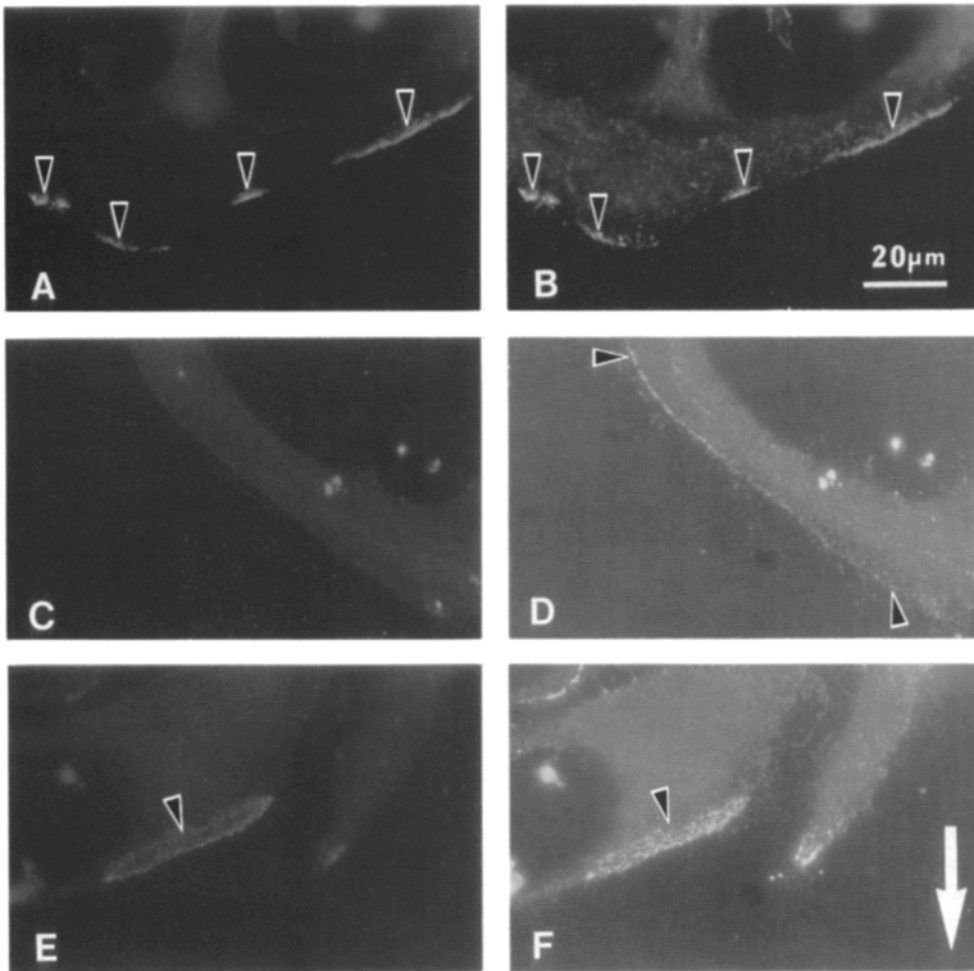
Figure 5. Colocalization of PY with E-field-induced AChR clusters. (*Left*) R-BTX images; (*right*) mAb 4G10 images. (*A* and *B*) Mature cathodal clusters were always associated with PY. (*C* and *D*) In cells exposed to E field for a shorter period of time (15–30 min), cathodal PY patches (*arrowheads* in *D*) were often unaccompanied by AChR clusters. (*E* and *F*) At intermediate cathodal clusters (1 h in the field), PY labeling was often stronger than R-BTX labeling (*arrowheads*).

not be AChRs and a possible causal relationship between tyrosine phosphorylation and AChR clustering.

Effect of a Tyrosine Kinase Inhibitor on E Field–Induced AChR Clustering

To study further the role of tyrosine kinase activation in AChR clustering induced by E field, we studied the effect of a tyrosine kinase inhibitor, tyrphostin RG-50864, on this process. This inhibitor has been shown to block specifically tyrosine kinase activation in a number of cellular responses elicited by peptide growth factors (15). The cultures were first treated with this compound for 2 to 3 h and then exposed to E field for 2 h. At a concentration of 60–80 μM, RG-50864 dramatically inhibited the clustering of AChRs along the cathodal edge. An example of this inhibition is shown in Fig. 7, *A* and *B*. This effect was quantified in Fig. 8. We measured the R-BTX fluorescence intensity along the cathodal edge and along the anodal edge and calculated and asymmetry index from the means of these measurements. This graph shows that RG-50864 was effective at a concentration above 25 μM and half inhibition was achieved at a dose of 50 μM. Cluster formation was completely inhibited at a concentration above 80 μM. The cells remained viable after the tyrphostin treatment for the duration of these experiments as shown by the persistence of hot spots not induced by E field. Furthermore, the reversibility of tyrphostin inhibition was

studied by first treating cultures with 80 μM tyrphostin for 3 h and then allowing the cultures to recover in normal medium for 2 h before E-field exposure. This resulted in a recovery of the cathodal AChR clustering to 80% of the control level (data not shown). Thus, the inhibitory effect of tyrphostin is not due to a non-specific, deleterious influence on the viability of the cells.

To further examine the specificity of this inhibition, we studied the effect of another compound in the tyrphostin family, RG-50862, on AChR clustering. This analog is inactive in blocking receptor tyrosine kinases and does not inhibit bead-induced AChR clustering (3, 27). As shown in Figure 7, *C* and *D*, this compound was also ineffective in blocking E field–induced AChR clustering at all concentrations (up to 100 μM) tested.

Discussion

E field offers a unique tool for studying the cellular mechanisms of AChR clustering in cultured muscle cells. Although it is different from other stimuli in its lack of physical contact or molecular interaction between the stimulus and the cell surface, it is one of the most effective means of eliciting AChR clustering. In cultured *Xenopus* muscle cells, nearly all cells exhibit cathodal clusters after a 2-h E-field treatment at a strength of 5–10 V/cm. With this stimulus, the onset of
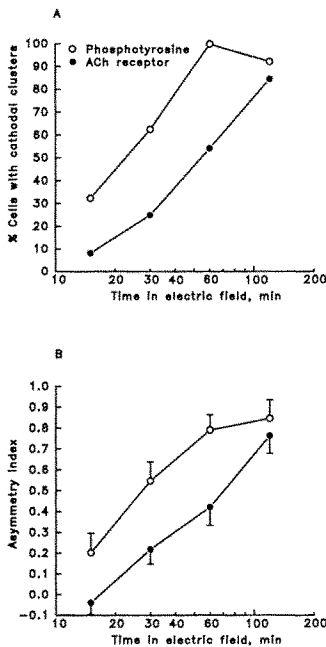
Figure 6. Time course of the appearance of PY and AChRs along the cathodal edge of the cells. (○) PY; (●) AChR. (A) Percentage of cells that exhibited cathodal PY or AChR clusters. (B) Development of the asymmetry in PY and AChR accumulation along the cathodal edge in comparison to the anodal edge. The asymmetry index, as defined in the Materials and Methods, has a theoretical range from −1 to +1. A positive value indicates an increased accumulation along the cathodal edge. A mean of 30 cells were scored at each time point. The error bars in B denote SEM.

This study suggests local activation of tyrosine kinases may underlie the mechanism of AChR clustering induced by E field. We presented two lines of evidence to support this premise. First, we used an anti-PY antibody to examine the localization of proteins that were phosphorylated on tyrosine residues. These antibodies are generated by using PY or its analogs as immunogens. They are very useful tools for demonstrating the concentration of PY-containing proteins at membrane specializations, such as focal contacts, cell–cell contacts, and AChR clusters (11, 19, 30). This study has shown a colocalization of PY-containing proteins with developing AChR clusters induced by E field and the appearance of these proteins along the cathodal edge actually precedes the concentration of AChRs. It may be argued that this earlier cathodal accumulation of PY proteins relative to AChRs is due to a superior sensitivity in the detection of signals from the anti-PY antibody followed by the FITC-secondary antibody over the R-BTX labeling of AChRs. To address this question, we compared the labeling of AChR clusters with R-BTX with that produced by labeling them with a mAb against the cytoplasmic domain of AChR (mAb 88B, kindly provided by Dr. S. C. Froehner, University of North Carolina, Chapel Hill, NC) followed by FITC-conjugated secondary antibody. The latter method is nearly identical to the labeling of PY used in this study. Our results showed that no amplification of the AChR signal over what was obtained with R-BTX labeling was gained by this antibody-labeling procedure (2). This suggests that the early PY accumulation before AChR clustering reflects a true physiological event.

the clustering process can be temporally controlled and the location of the clusters can be predicted. Previous studies by Stollberg and Fraser (34) have shown that the accumulation of AChRs by electromigration is neither necessary nor sufficient for clustering induced by E field. Our present study shows that the clustering process initiated by a brief field exposure still continues after the termination of the field. Thus, E field itself is not the motive force for moving AChRs, but it locally triggers the clustering mechanism along the cathodal edge of the cell. Through the use of DC or AC pulsed waveforms, Stollberg and Fraser (35) also showed that the field effect can not be mediated by changes in membrane potential, even though these changes can be significant enough to effect asymmetric cellular responses, such as increases in intracellular calcium level. They concluded that an accumulation of certain non-AChR molecules along the cathodal edge of the cell may trigger the clustering process.

These results are consistent with our recent finding that the accumulation of PY-positive proteins is an early event during the formation of AChR clusters induced by beads in cultured Xenopus muscle cells (2). In this study, we also showed PY colocalization with nerve-induced AChR clusters and with aneural AChR hot spots in vitro. Previous studies have shown PY colocalization with AChRs at the NMJ in vivo (30) and with agrin-induced AChR clusters in vitro (42). Thus, the localization of PY-positive proteins seems to be a common denominator of the cellular response to this diverse array of AChR clustering stimuli.
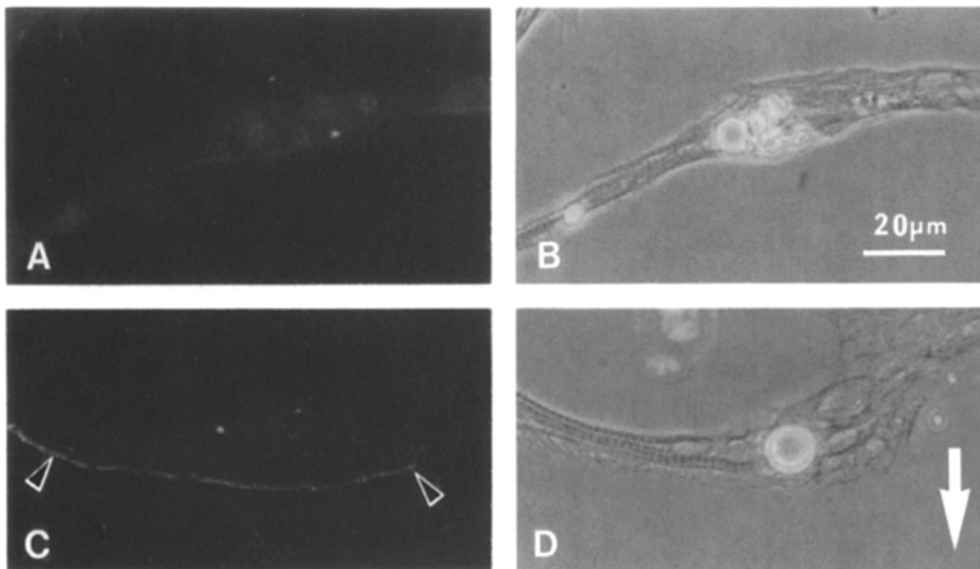

Figure 7. Effect of tyrphostins on E field–induced AChR clustering. (A and B) Tyrphostin RG-50864 at 100 μM inhibited the formation of AChR clusters along the cathodal edge as shown in this example. The culture was treated with tyrphostin for 3 h and then exposed to E field for 2 h in the continuous presence of this drug. (C and D) In contrast, an inactive from of tyrphostin, RG-50862, left the E field–induced cathodal AChR clustering (arrowheads) intact.
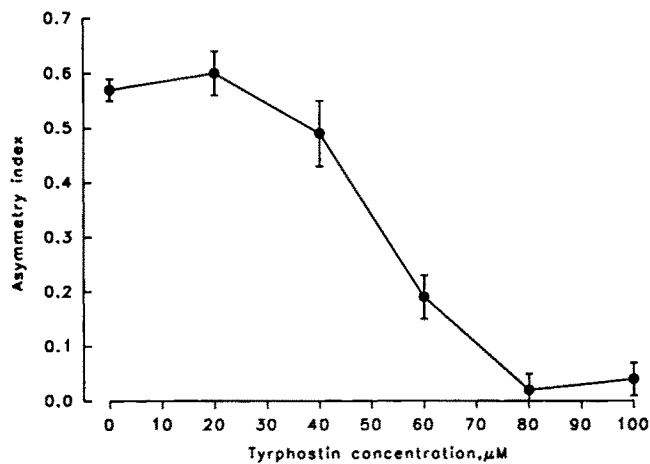
Figure 8. Quantitation of the inhibition of E field–induced AChR clustering by tyrphostin RG-50864. The asymmetry index is an indicator for the extent of cathodal AChR clustering as defined in Materials and Methods. 20 cells were scored to calculate this index at each drug concentration. The error bars show SEM.

In addition to immunocytochemistry, we showed that a member of the tyrphostin family of tyrosine kinase inhibitors abolished the effect of E field in clustering AChRs. Tyrphostins have been shown to inhibit tyrosine kinases with high specificity (7, 15, 18, 43). Previously, we showed that the same tyrphostin (RG-50864) used in this study also blocked AChR clustering (3, 27) and PY accumulation (2) induced by beads in Xenopus muscle cells. The tyrphostin concentrations that inhibited E field–induced AChR clustering are comparable to those used in previous studies (2, 3, 18, 27).

There are two kinds of membrane-associated tyrosine kinases: receptor-associated kinases that are integral membrane proteins, such as receptors for peptide growth factors, and non-receptor kinases that are peripherally linked with the internal surface of the plasma membrane, such as the src family proteins (20). Since the latter is on the inside of the membrane, they are unlikely to be influenced by E field due to the electrical insulation property of the lipid bilayer. However, receptor-associated tyrosine kinases have extracellular domains that can serve as targets for E field. Peptide growth factors, such as EGF, basic FGF (bFGF), PDGF, and insulin-like growth factor-1 (IGF-1), are physiological ligands for these receptors (40). It is believed that the ligand-receptor interaction results in dimerization of these receptors and thus brings the cytoplasmic tyrosine kinase domains of both receptors into close proximity with each other (32, 40). This enables the dimerized receptors to transphosphorylate each other on tyrosine residues and to activate a signal transduction cascade. Our recent biochemical studies have shown that bFGF receptors are present in cultured Xenopus muscle cells (27) (L. P. Baker, unpublished results). We have shown that the activation of bFGF receptors resulting from bead-mediated presentation of bFGF may be involved in the formation of AChR clusters in these muscle cells (3, 27). It is conceivable that these receptors or other transmembrane tyrosine kinases can serve as targets for E field. Their activation may be mediated by an E field–induced oligomerization process as schematically shown in Fig. 9 (A and B). We envision that these kinase molecules can accumulate along the cathodal edge of the cell by electromigration due to lateral

electrophoresis or electro-osmosis similar to the processes previously proposed for the accumulation of AChRs. This accumulation decreases the intermolecular distance to a range that allows transphosphorylation of the receptors to take place and thus activates the signal transduction pathway for AChR clustering along this edge of the cell (Fig. 9, C and D). Two main features of this model, that the triggering process involves the accumulation of a non-AChR molecule and is independent of changes in membrane potential, are consistent with the results of Stollberg and Fraser (35).

The events downstream from the initial signaling at the membrane level are unknown. Previous studies on signal transduction pathways activated by peptide growth factors have shown that receptor tyrosine kinases, after they are transphosphorylated, become active in phosphorylating other substrate proteins to initiate a cascade of events that results in cellular activation (32, 40). The substrates for E field–induced tyrosine phosphorylation are unknown. Previously we showed that E field–induced AChR clusters are associated with a cytoskeletal specialization (31). It is conceivable that local tyrosine kinase activation elicited by E field can result in an assembly of the cytoskeletal specialization. Such a mechanism is suggested by recent evidence that several cytoskeletal proteins, such as paxillin and tensin, are substrates for tyrosine kinases (6, 37, 38). Both proteins are concentrated at focal contacts (6, 38) in non-muscle cells and paxillin is also concentrated at the NMJ in muscle (39). In addition to these cytoskeletal proteins, AChRs themselves are
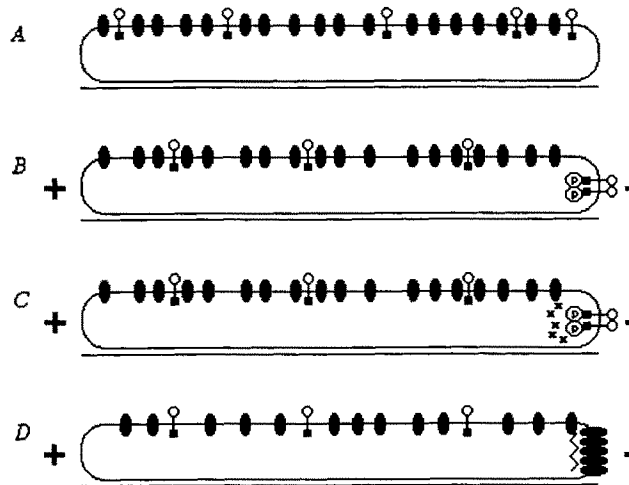

Figure 9. A model depicting the role of tyrosine phosphorylation in E field–induced AChR clustering. (A) In the resting state, AChRs and transmembrane tyrosine kinases are both present on the cell surface. (B) When the cell is exposed to E field, kinase molecules become aggregated at the cathode-facing edge, presumably by an electromigration process. This causes oligomerization of kinase molecules, leading to transphosphorylation. (C) The transphosphorylation of kinase molecules results in the local activation of a signal transduction cascade at the cathodal edge of the cell. This may involve tyrosine phosphorylation of substrate proteins and generation of second messengers. (D) This local signaling event triggers the assembly of a cytoplasmic apparatus, e.g., a cytoskeletal specialization, that effects clustering of AChRs via a diffusion-mediated trapping mechanism. (Y) Transmembrane tyrosine kinase; (■) tyrosine kinase domain; (●) AChR; (x) 2nd messengers; (⟨⟩) trapping mechanism.

also substrates for tyrosine kinases (41). A recent study has shown that tyrosine phosphorylation of the β subunit of the AChR in cultured chick myotubes may be involved in agrin-induced AChR clustering (42). Our preliminary biochemical studies have shown that certain subunits of AChRs are indeed tyrosine phosphorylated in *Xenopus* muscle cells (Peng, H. B., unpublished results). However, our immunofluorescent studies have suggested the cathodal accumulation of PY-containing proteins before the appearance of AChR clusters. This suggests that the initiation of AChR clustering may involve tyrosine phosphorylation of certain non-AChR molecules. Our current study and the one conducted by Stollberg and Fraser (33) have shown that the clustering process draws AChRs from the surface pool that is preexistent in the membrane. Previous measurements have shown that lateral diffusion of AChRs in the membrane of *Xenopus* muscle cells is fast enough that a diffusion-mediated trapping mechanism can effectively cluster the receptors (8, 29, 35). In view of our current results, E field–induced tyrosine phosphorylation may be fundamental to the establishment of this trap as illustrated in Fig. 9.

The present study, together with several other recently published works, has suggested a unifying signal transduction mechanism based on tyrosine kinase activation for understanding the induction of AChR clustering. The potency of the E field effect makes it a useful tool for understanding the nature of the tyrosine kinases and substrates involved in AChR clustering. In addition, E field is also a powerful activator of a range of other cellular processes, such as cell orientation, outgrowth, and locomotion (5, 9, 13, 14, 17, 24, 36). The concept of tyrosine phosphorylation developed in this work may also offer an explanation for these other fascinating effects produced by this stimulus.

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