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14. The bat was encased in a body mold with its head and legs protruding. A sling was looped around the bat's right leg just above the ankle and was attached to a strain gauge. Electric shocks were delivered to the bat's right thigh through two silver plates located above and below the thigh. The voltage was adjusted to the minimum level needed to elicit a consistent leg flexion. Conditioning sessions consisted of one 20-trial block (10 S+ and 10 S− trials, randomly ordered) at each frequency or delay. Trials were 30 s apart. In the frequency discrimination tests, each stimulus train consisted of 12 pairs of PCF_2-ECF_2 delivered at 80-dB sound pressure level (SPL). The PCF_2 and the ECF_2 each were 20 ms long, with 0.5-ms rise and fall times, and were separated by a 10-ms silent period. The PCF_2 was 500 Hz above the bat's resting frequency, which was measured at the start of training from a sample of 100 pulses. In the temporal discrimination tests, each stimulus train consisted of 24 pairs of PFM_1-EFM_2. We used EFM_2 rather than EFM_3 or EFM_4 because (i) H_2 (Fig. 1B) is the most intense, and (ii) the population of FM-FM neurons responding to PFM_1-EFM_2 is the largest. Both the PFM_1 and the EFM_2 were 3.5 ms long, with 0.5-ms rise and fall times, and were repeated at 20 pairs per second. The PFM_1 linearly swept from 31 to 21 kHz, and the EFM_2 linearly swept from 62 to 46 kHz. The PFM_1 and the EFM_2 were delivered at 80 and 75 dB SPL, respectively. For the 36-ms Δt, the presentation rate was decreased to 10 pairs per second.
15. Two to four weeks before the first muscimol application, the bat was given an intramuscular injection of a neuroleptic analgesic, Innovar-Vet [0.08 mg of Fentanyl per kilogram of body weight and Droperidol (4 mg/kg)], the dorsal surface of its skull was exposed, holes 200 by 200 μm^2 were made over the DSCF or FM-FM area, and a small well was placed over each hole.
16. Statistical analyses were based on the proportion of correct responses within selected time windows. These windows were chosen post hoc with the constraints that within a given experiment (i) all time windows were the same width and (ii) the same windows were used for every bat. Within each time window there was at least one 20-trial block at each Δf or Δt on each test day. We compared (i) baseline performance to performance after muscimol treatment (Tables 1 and 2); (ii) performance during each time window relative to chance (50%) (Fig. 3); and (iii) performance among the different Δf or Δt test blocks within each time window (not shown). There were no inconsistencies among these analyses.
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ACh Receptor–Rich Membrane Domains Organized in Fibroblasts by Recombinant 43-Kildalton Protein

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Neurotransmitter receptors are generally clustered in the postsynaptic membrane. The mechanism of clustering was analyzed with fibroblast cell lines that were stably transfected with the four subunits for fetal (α, β, γ, δ) or adult (α, β, ε, δ) type mouse muscle nicotinic acetylcholine receptors (AChRs). Immunofluorescent staining indicated that AChRs were dispersed on the surface of these cells. When transiently transfected with an expression construct encoding a 43-kilodalton protein that is normally concentrated under the postsynaptic membrane, AChRs expressed in these cells became aggregated in large cell-surface clusters, colocalized with the 43-kilodalton protein. This suggests that 43-kilodalton protein can induce AChR clustering and that cluster induction involves direct contact between AChR and 43-kilodalton protein.

A CRUCIAL STEP IN SYNAPSE FORMATION is the aggregation of neurotransmitter receptors in the postsynaptic membrane. In mammalian muscle and electric organ of the marine ray Torpedo, for example, the postsynaptic membranes contain about 10^4 AChR molecules per square micrometer (*1*), whereas nonsynaptic portions of the membrane are virtually devoid of AChRs. Although the precise colocalization of pre- and postsynaptic specializations at a synapse clearly involves interactions between nerve and muscle, muscle fibers are capable of aggregating AChRs even in the absence of nerves (*2*). A synapse-associated, intracellular, 43-kD peripheral membrane protein isolated from AChR-rich membranes of Torpedo electroplaque (*3*) has been indirectly implicated in this latter process. Exposure of electroplaque membranes to pH 11 releases this 43-kD protein and other peripheral proteins from the membrane and also causes an increase in lateral (*4*) and rotational (*5*) mobility of AChR molecules. The 43-kD protein is concentrated in AChR-rich membranes where it is present in equimolar amounts with AChR (*6*). Finally, chemical cross-linking experiments reveal a close association between 43-kD protein and the β-subunit of the AChR (*7*).

The availability of cDNA clones for the 43-kD protein (*8, 9*) permitted us to study directly its role in AChR cluster formation by coexpression with cDNAs for AChR subunits in fibroblast cell lines. Because the development of the neuromuscular junction is accompanied by a transition from fetal (α, β, γ, δ) to adult (α, β, ε, δ) AChR (*10, 11*), and by major changes in the morphology of the junctional AChR clusters (*12*), we determined the effects of 43-kD protein on both receptor types. The quail fibroblast line, QT-6 (*13*), which expresses no detectable endogenous AChR (*14*), was transfected with expression constructs for each of the four subunit cDNAs of the fetal or adult muscle AChR (*15*). Fibroblast clones selected on the basis of the cotransfected neomycin resistance gene were screened for cell-surface α-bungarotoxin binding sites and clones expressing fetal AChR (Q-F18) and adult AChR (Q-A33) were isolated. The functional integrity of AChR in the clones was determined by voltage-clamp studies on isolated membrane patches (*16*). Membrane patches excised from transfected cells showed currents elicited by ACh that were not blocked by 100 nM atropine (Fig. 1). The channels expressed in clone Q-F18 (*15*) showed a slope conductance of about 40 pS for inward current and a burst duration of about 5 to 10 ms. Q-A33 exhibited channels of larger conductance and briefer burst duration. Similar properties have been reported for fetal- and adult-type bovine AChRs

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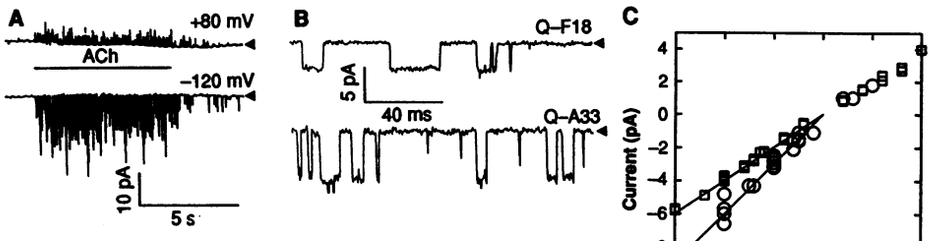

Fig. 1. Channels were activated by acetylcholine applied to outside-out patches from transfected fibroblasts. (**A**) Slow sweeps from a patch excised from a Q-F18 cell held at −120 or +80 mV. A perfusion pipette was positioned close to the patch and 1 μM ACh plus 100 nM atropine was applied during the time indicated by the bar. The channel activity during these records was too high to analyze; they are only shown to illustrate the nicotinic nature of the ACh-elicited currents. The baseline current (no channels open) is indicated by arrowheads. (**B**) Single-channel currents recorded in patches from Q-F18 or Q-A33 cells, held at −100 mV. Note the larger current and somewhat briefer duration of events in the patch from Q-A33 cells. (**C**) Single-channel current-voltage relations for four outside-out patches each from Q-F18 (squares) or Q-A33 (circles) cells. The lines drawn through the points have slopes of 40 pS (squares) and 60 pS (circles), respectively. The mean burst durations ± SD at a membrane potential of −100 mV were 7.5 ± 1.9 ms and 2.7 ± 2.2 ms, for the Q-F18 and Q-A33 lines, respectively (n = 4 patches). The conductances for inward current were 38 ± 0.5 pS and 56 ±5.0 pS, respectively.

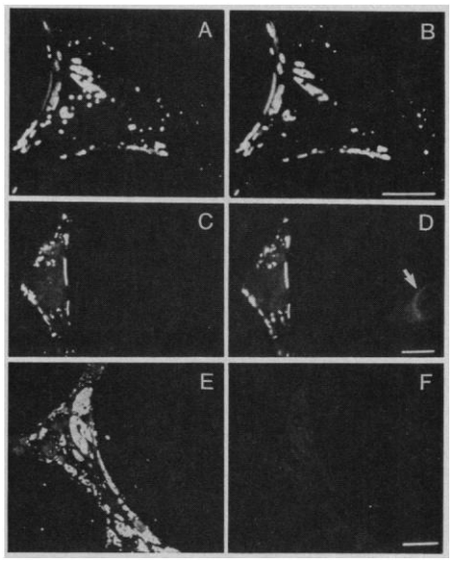

Fig. 3. Immunofluorescent colocalization of AChR and 43-kD protein in fibroblasts. Q-F18 (**A** and **B**), Q-A33 (**C** and **D**), or QT-6 (**E** and **F**) cells were transiently transfected with RSV 43-kD protein (*20*), doubly stained with antibodies to 43-kD protein and AChR with appropriate second antibodies (*24*) and viewed with Texas red (43-kD protein: A, C, E) or FITC (AChR: B, D, F) optics. Q-F18 and Q-A33 cells each display AChR clusters (B and D) that colocalize with 43-kD protein aggregates (A and C). Note that a cell expressing diffuse AChR (arrow in D) is negative for 43-kD protein expression (C). A 43-kD protein–transfected QT-6 (parent) cell displays 43-kD protein aggregates (E) in the absence of AChR (F). To control for fluorescent cross-bleed and second antibody specificity we processed parallel cover slip cultures and stained them in the absence of either the anti-AChR or anti–43-kD protein primary antibody. Such cells showed patches of either AChR or 43-kD protein staining similar to those shown above, but in the absence of the second channel fluorescent signal. For photomicrographs the digital image was normalized over the full range of video intensity levels (*24*). Bars, 10 μm.

expressed in *Xenopus* oocytes (*11*), for fetal-type endogenous receptors expressed by mouse myoblast-like cell lines (*17*), and for adult-type AChRs on dissociated mouse muscle fibers (*18*).

Immunofluorescent labeling of cell-surface AChR revealed a uniform amount of specific staining on cells expressing either receptor-type (Fig. 2A). In no case did we observe the large, spontaneously occurring clusters normally seen on cultured muscle cells (*19*). In contrast, after transient transfection of mouse 43-kD protein (*20*) into Q-F18 or Q-A33 cells, large brightly stained patches of cell-surface AChR formed (Fig. 2 B to D). AChR patches ranged in diameter from less than 1 μm to greater than 10 μm. The results suggest that 43-kD protein can induce clustering of both adult and fetal AChRs in fibroblast cells.

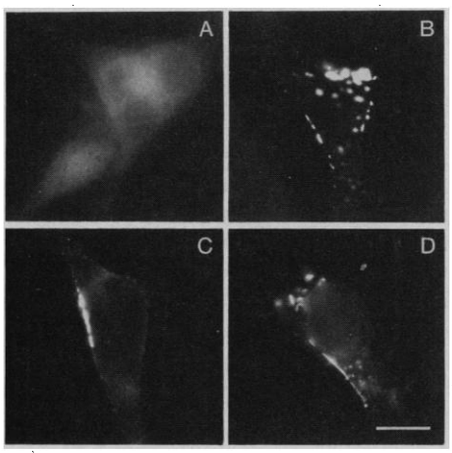

Fig. 2. Clustering of AChR on the surface of 43-kD protein–transfected fibroblasts. (**A**) Control Q-F18 cells (not transfected with 43-kD protein) stained by indirect immunofluorescence with α-bungarotoxin and anti–α-bungarotoxin (*23*) show a uniformly grainy distribution of staining across the cell surface. Similar results were obtained with Q-A33. (**B** and **C**) Q-F18 cells transiently transfected with 43-kD protein (*20*) show large, brightly stained surface clusters of AChR. (**D**) A Q-A33 cell transfected with 43-kD protein displays similar AChR clusters. Bar, 10 μm.

To determine whether the 43-kD protein interacts directly with the AChR, immunofluorescent double-labeling of 43-kD protein and AChR was performed after fixation and permeabilization of cells. In each case AChR clusters were colocalized with aggregates of intracellular 43-kD protein (Fig. 3). Because only a fraction of cells transiently expressed 43-kD protein in these experiments, some cells in each field were negative when stained with the antibody to 43-kD protein (anti–43-kD protein). These 43-kD protein–negative cells showed diffuse intracellular and cell-surface AChR staining (arrow in Fig. 3D) typical of Q-F18 and Q-A33 cells, and never showed condensed patches of cell-surface staining. Similarly, AChR aggregates were not observed on cells subjected to mock transfections, where Rous sarcoma virus (RSV)–*neo* was substituted for RSV–43-kD protein (*15*). Formation of AChR clusters was therefore correlated with 43-kD protein expression. Some fibroblast cell lines have been shown to express small amounts of endogenous 43-kD protein (*21*). Because reagents specific for quail 43-kD protein have not been characterized, we cannot exclude the possible presence of endogenous 43-kD protein in our cell lines. However, endogenous 43-kD protein, if present, was evidently inadequate in quantity or quality to induce clustering of mouse AChR. Thus, AChR clusters were only observed in cells expressing recombinant 43-kD protein and were always colocalized with 43-kD protein aggregates in those cells.

Finally, 43-kD protein alone was transfected into the parent cell line, QT-6. Anti–43-kD protein revealed patches of staining that were very similar to the AChR cluster–associated 43-kD protein patches on Q-F18 or Q-A33 AChR-expressing cell lines (Fig. 3, A, C, and E). Therefore, clusters of 43-kD protein were able to form independently of the interaction with AChR.

These observations suggest that this 43-kD intracellular peripheral membrane protein participates in organizing large high-density aggregates of fetal and adult AChR. It is possible, however, that the formation of AChRs clusters involves membrane and cytoskeletal proteins in addition to 43-kD protein. Indeed, several other proteins are concentrated at the neuromuscular junction (*22*). Our results suggest that if such proteins are required for AChR clustering, they

may be replaced by normal constituents of QT-6 fibroblasts. We propose that 43-kD protein serves as the key link between AChR and the cytoskeleton, and that aggregation of 43-kD protein drives AChR clustering rather than simply stabilizing AChR clusters formed by other means.

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15. Quail QT-6 fibroblasts were maintained, transfected, and stable AChR-expressing clones were isolated with four separate α, β, γ, and δ-subunit–expression constructs as described previously [P. Blount and J. P. Merlie, *Neuron* **3**, 349 (1989)]. The ε-subunit cDNA was isolated from an adult mouse muscle cDNA library; partial sequence analysis (P. Gardner, unpublished data) has shown it to be identical to the mouse ε gene coding sequence previously described [Buonanno *et al.*, *J. Biol. Chem.* **264**, 7611 (1989)]. Expression was driven by the RSV long terminal repeat promoter in each construct. Clones were selected by resistance to geneticin (300 μg/ml), conferred by cotransfection with an RSV-*neo* plasmid. Surviving colonies were chosen for further study on the basis of surface α-bungarotoxin binding capacity. The fibroblast clones described here had approximately 12,000 (Q-A33) and 20,000 (Q-F18) surface binding sites per cell. The surface binding capacity has been stable for 4 months of continuous passage.
16. Single-channel currents were recorded with standard methods [M. Covarrubias, C. Kopta, J. H. Steinbach, *J. Gen. Physiol.* **93**, 765 (1989)]. Outside-out patches were formed with a pipette solution containing 140 mM CsCl, 1 mM $MgCl_2$, 2 mM EGTA, and 20 mM Hepes (pH 7.35), osmolarity adjusted to 290 mosm with glucose. The bath contained 140 mM NaCl, 2 mM KCl, 0.5 mM $CaCl_2$, and 20 mM Hepes (pH 7.35), osmolarity adjusted to 320 mosm with glucose. The perfusion pipette (tip diameter about 1 μm) contained bath solution plus 1 μM acetylcholine chloride and 100 nM atropine sulfate. Parent QT-6 cells showed relatively few membrane channels; the dominant channel appeared to be a high-conductance calcium-activated potassium conductance. There were muscarinic responses, blockable by 100 nM atropine, which increased openings of this type of channel. The majority (more than 95%) of openings elicited by ACh application were of the conductance classes shown in Fig. 1. A few openings of lower conductance were seen [O. P. Hamill and B. Sakmann, *Nature* **294**, 462 (1981); R. Kullberg *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 2067 (1990)].
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20. The RSV–43-kD protein was constructed as follows. The Nar I–Hind III fragment of Pbα1 (*14*) was subcloned into the Sma I site of SK+ (Stratagene, San Diego) yielding SK-PBα. The full-length coding sequence for mouse 43-kD protein (*9*), devoid of 5′ and 3′ noncoding sequence, was synthesized by polymerase chain reaction and was substituted for the α-subunit coding (Sma I–Nco I) fragment of SK-PBα, thus resulting in pSK43-kD protein with a 3′ noncoding region derived from the mouse AChR α-subunit mRNA. The coding region was verified by sequencing. Cultures were transiently transfected via calcium phosphate precipitate as previously described (*14*) and were replated on ethanol-washed glass cover slips and incubated overnight.
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23. We added α-bungarotoxin (2 μg/ml final) to the culture medium for 1 hour to label cell-surface AChR. Cover slips were washed in warm serum-free medium, fixed for 20 min either in 4% paraformaldehyde in phosphate-buffered saline (PBS) or as described below (*24*), and washed three times in PBS and incubated for 1 hour at room temperature with affinity-purified rabbit antibody to α-bungarotoxin (anti–α-bungarotoxin) [J. P. Merlie and R. Sebbane, *J. Biol. Chem.* **256**, 3605 (1981)] (1:20). After they were washed three times over at least 30 min, cover slips were incubated for 1 hour with affinity-purified goat antibody to rabbit immunoglobulin G (anti–rabbit-IgG) (1:100, Boehringer), were washed again, and examined with epi-fluorescence.
24. On the day after plating onto cover slips, cells were rinsed in warm, serum-free medium and fixed in 1% paraformaldehyde, 100 mM L-lysine, 10 mM sodium *m*-periodate, and 0.1% saponin in PBS. After they were washed three times in PBS, cover slips were incubated for 1 hour at room temperature with a cocktail containing the rat monoclonal anti–α-subunit, MAb 210 [M. Ratnam *et al.*, *Biochemistry* **75**, 2621 (1986)] (1:500) together with the two mouse monoclonal anti–43-kD protein antibodies MAb 1234 and 1579 [S. C. Froehner, *J. Cell Biol.* **99**, 88 (1984)] (supernatants 1:4). Cover slips were washed three times over at least 30 min, incubated for 1 hour with affinity-purified fluorescein isothiocyanate (FITC) goat anti–rat-IgG (Boehringer) and biotin goat anti–mouse-IgG (Sigma) from which species cross-reactive antibodies had been adsorbed by the manufacturers. After they were washed again, cover slips were incubated for 1 hour with Streptavidin–Texas red (BRL), washed, mounted, and examined with a Bio-Rad MRC 500 laser scanning microscope (a gift of the Lucille P. Markey Charitable Trust).

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Protein Kinase C and Regulation of the Local Competence of *Xenopus* Ectoderm

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The limited competence of embryonic tissue to respond to an inductive signal has an essential, regulatory function in embryonic induction. The molecular basis for the competence of *Xenopus* ectoderm to differentiate into neural tissue was investigated. Dorsal mesoderm or 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) caused in vivo activation of protein kinase C (PKC) and neural differentiation mainly in dorsal ectoderm and to a lesser extent in ventral ectoderm. These data correlate with the observations that PKC preparations from dorsal and ventral ectoderm differ, the dorsal PKC preparation being more susceptible to activation by TPA and diolein than is the ventral PKC preparation. Monoclonal antibodies against the bovine PKC α plus β or γ isozymes immunostained dorsal and ventral ectoderm, respectively, which suggests different localizations of PKC isozymes. These results suggest that PKC participates in the establishment of embryonic competence.

AMPHIBIAN BLASTULA-STAGE ECTODERM can be induced to form mesoderm, whereas early gastrula-stage ectoderm cannot, but can be induced to neural tissue instead. This phenomenon is called embryonic competence; its molecular mechanisms remain obscure (*1*). Dorsal mesoderm, which induces overlying dorsal ectoderm to differentiate to neural tissue during normal gastrulation, is able to induce ventral ectoderm to differentiate to neural tissue (*2*), although it induces the expression of neural markers strongly in dorsal ectoderm and only weakly, if at all, in ventral ectoderm (*3*), indicating that dorsal and

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