The Number of Na⁺ Channels in Cultured Chick Muscle Is Increased by ARIA, an Acetylcholine Receptor–inducing Activity

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ARIA is a glycoprotein purified from chick brain on the basis of its ACh receptor–inducing activity (ARIA). In this study we present evidence that ARIA increases the number of voltage-gated sodium channels in chick muscle as well as the number of ACh receptors (AChRs). Exposure of chick myotubes to ARIA increased by twofold the number of ³H-saxitoxin binding, an effect that is comparable to the increase of AChRs assayed by ¹²⁵I- α -bungarotoxin (¹²⁵I- α -BTX) binding. We also documented effects of ARIA on myoblasts: the number of ¹²⁵I- α -BTX binding sites in the mononucleated muscle cells was increased by 1.5-fold, and the peak TTX-sensitive inward currents increased by the same amount. No change was detected in the voltage dependence of channel activation, in mean channel current, or in mean channel open time.

Thus, the Na⁺ channel is the first molecule, other than AChR subunits, whose expression has been shown to be induced by ARIA. Since sodium channels are concentrated at motor end plates, our results provide circumstantial evidence that ARIA may regulate several genes expressed at developing neuromuscular junctions. Moreover, the finding that ARIA's effects extend to mononucleated myoblasts suggests that this protein may be important during the earliest stages of muscle formation and innervation.

[Key words: neuromuscular junction, sodium channel, ACh receptor, neurotrophic factor, muscle, myoblasts]

During formation of the neuromuscular junction, several proteins become concentrated in the synaptic cleft, the postsynaptic membrane, and the postsynaptic cytoplasm. For example, the 20S form of AChE, s-laminin, and neural cell adhesion molecules (N-CAMs) accumulate in the cleft (Massoulié and Bon, 1982; Covault and Sanes, 1985; Hunter et al., 1989); ACh receptors (AChRs) and voltage-gated sodium channels accumulate in the postsynaptic membrane (Fertuck and Salpeter, 1976; Beam et al., 1984); and the 43 kDa protein, an 87 kDa protein, a form β -spectrin, and a dystrophin-related protein accumulate in the cytoplasm (Froehner et al., 1981; Bloch and Morrow, 1989; Carr et al., 1989; Ohlendieck et al., 1991). The remarkable concen-

tration of these proteins implies an external organizing influence, which most likely comes from the motoneuron itself.

The inductive influence of motoneurons has been studied in most detail in regard to the accumulation of AChRs. The density of AChRs at developing end plates begins to increase within hours of growth cone–muscle contact (Role et al., 1985). The accumulation of AChRs is primarily due to two processes: (1) aggregation of receptors in the plane of the membrane; and (2) increased local insertion of newly synthesized receptors. The upregulation of AChRs at the junction occurs in the face of an overall downregulation of AChRs in extrasynaptic regions, a decrease that is driven by muscle activity. The effect of putative trophic factors at the end plate must overcome this negative influence of activity.

In the search for neuronal factors that regulate the expression of synapse-specific molecules, we have purified a protein from chick brain based on its ability to increase the synthesis of AChRs in cultured chick muscle cells. This molecule, named ARIA for its ACh receptor–inducing activity, is a 42 kDa glycoprotein, and we suggest that it plays a role in synaptogenesis. Information about the spectrum of action of highly purified ARIA preparations would provide insight into its possible biological role at developing synapses. It is already known that ARIA does not regulate the synthesis of all junctional proteins. No effect has been detected on cellular or secreted forms of AChE (Usdin and Fischbach, 1986) or on the cytoplasmic 43 kDa protein that is thought to play a role in AChR immobilization (Falls et al., 1988).

In this study we have investigated the effect of ARIA on voltage-gated Na⁺ channels, which, like AChRs, are integral membrane proteins concentrated in the postsynaptic membrane. Early evidence that Na⁺ channels accumulate at the neuromuscular junction came from loose-patch microelectrode measurements. TTX-sensitive inward currents recorded in the immediate vicinity of rodent or snake end plates are much greater than currents recorded 100–200 μ m away (Beam et al., 1984; Betz et al., 1984; Caldwell et al., 1986). Electron microscopic immunogold localization of Na⁺ channels at rat end plates showed that they are packed at high density (ca. 5000/ μ m²) in the depths of secondary synaptic folds (Flucher and Daniels, 1989; Boudier et al., 1992), while AChRs are restricted to the upper third of the postsynaptic folds (Fertuck and Salpeter, 1976; Flucher and Daniels, 1989). Thus, in this species, Na⁺ channels and AChRs are in the same region but not exactly in the same membrane domain. Na⁺ channels also colocalize with AChRs in innervated chick myotubes *in vitro*. Binding sites, detected with fluorescent scorpion toxin, were concentrated at neurite-associated receptor patches (NARPs) on myo-

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tubes that were cocultured with embryonic spinal cord neurons (Angelides, 1986). No association between Na^+ channels and AChRs was observed in myotubes grown in the absence of neurons. An effect of neural tube explants on the number of functional Na^+ channels has been found in *Xenopus* myocytes (DeCino and Kidokoro, 1985).

We have used a combination of saxitoxin (STX) binding and electrophysiologic measurements in chick myotubes and myoblasts to show that ARIA increases the number of functional Na^+ channels. The effect of ARIA on myoblasts is particularly noteworthy because most myogenic cells are mononucleated at the time the motor nerves enter muscle masses in chick limb buds.

Materials and Methods

Muscle culture. Pectoral muscles dissected from 11 d chick embryos (E11) were incubated in a $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free phosphate-buffered saline for 30 min. The tissue was then mechanically disrupted by repeated passage through a fire-polished Pasteur pipette. Mononucleated cells were plated in Eagle's minimum essential medium (MEM) supplemented with horse serum (10% v/v), glutamine (2 mM), penicillin (50 U/ml), streptomycin (50 $\mu\text{g}/\text{ml}$), and transferrin (40 $\mu\text{g}/\text{ml}$). Cells were plated on either collagen-coated 35 mm culture dishes, 24-well plastic dishes, or collagen-coated coverslips at the densities described in the text.

ARIA purification and treatment. ARIA was partially purified as described in Falls et al. (1990). Briefly, adult chicken brains were dilypdated, acid extracted, and then desalted on a Vydac C18 column. The material was then carried through several chromatographic steps: a CM sepharose column equilibrated in 25 mM 2-[N-morpholino]ethanesulfonic acid (MES; pH 6) eluted with a gradient of NaCl, a Vydac C4 reverse-phase column equilibrated in 0.1% trifluoroacetic acid (TFA) eluted with a gradient of isopropyl alcohol (C4/TFA), and the same C4 column equilibrated in 0.13% heptafluorobutyric acid (HFBA) eluted with a gradient of isopropyl alcohol (C4/HFBA). For treatments, C4/HFBA material was dried by vacuum centrifugation, redissolved in culture medium to a final C4/HFBA protein concentration of approximately 10 $\mu\text{g}/\text{ml}$, and applied to cultures.

AChR binding assay. The total number of surface AChRs and the rate of incorporation of AChRs into the surface membrane were measured as described in Usdin and Fischbach (1986). For measuring the rate of incorporation of receptors, all exposed receptors were blocked with unlabeled α -bungarotoxin (α -BTX) (10^{-7} M for 1 hr at 37°C). The cells were then washed by immersing the plates in 2 liters of Ca^{2+} -free Hank's balanced salt solution containing 2% BSA (HBSS). Fresh medium containing 5 nM ^{125}I - α -BTX was added and plates were returned to the incubator for five hr. Then cells were washed in HBSS, solubilized with a solution of 1 N NaOH, 0.5 mg/ml deoxycholate, and then counted in a γ -counter. Nonspecific binding of ^{125}I - α -BTX was measured in the presence of 1.2 μM unlabeled α -BTX. The total number of surface receptors was measured essentially in the same way but without blocking existing AChRs.

AChR autoradiography. Mononucleated cells were plated on collagen-coated slide chambers (Nunc Inc.) as described above. Four hours after plating, cells were treated with ARIA. After 48 hr of treatment all exposed receptors were blocked with unlabeled α -BTX (10^{-7} M for 1 hr at 37°C) and the receptors incorporated into the membrane were labeled with ^{125}I - α -BTX as described above. Slides were washed first with HBSS and then with PBS, and fixed in 4% paraformaldehyde in PBS for 10 min and in 100% methanol (-20°C) for four min. Slides were coated with Kodak NTB-2 emulsion and exposed for two days at 4°C in a desiccator box. Slides were then randomly screened and all mononucleated muscle cells found in the screened area were photographed using an image analysis system (Image-1/AT, Universal Imaging Corp.). Images of fibroblasts were also acquired. The number of grains per cell as well as the length of the cell's longer axis were quantified.

Saxitoxin binding assay. Cultured myotubes grown on 35-mm-diameter Petri dishes (10^6 cells/dish) were treated with ARIA for 48 hr starting four days after plating. Binding of radioactively labeled saxitoxin (^3H -STX; specific activity, 12.2 Ci/mmol; radiochemical purity, 75%, kindly provided by Dr. Catterall, University of Washington, Seattle) was performed essentially as in Sherman et al. (1983). Briefly,

incubations were carried out in an Na^+ -free medium (K^+ -substituted medium, KSM: 135 mM KCl, 5.5 mM glucose, 0.8 mM MgSO_4 , 1.8 mM CaCl_2 , and 50 mM HEPES adjusted to pH 7.4 with Tris base). Cells were washed three times with 2 ml of KSM (37°C), incubated with 750 μl of 10 nM ^3H -STX for 30 min at 37°C, washed very rapidly (10–15 sec) with 10 ml of cold KSM (4°C), and dissolved by incubation with 750 μl of an NaOH/deoxycholate solution for 10 min. After harvesting, dishes were washed with 750 μl of an HCl solution for an additional 10 min in order to further remaining counts further and to neutralize the solution. Lysates were counted in a scintillation counter. Nonspecific binding for ^3H -STX was measured in the presence of 2 μM tetrodotoxin (TTX) in the incubation medium. The assay was carried out in duplicates.

Electrophysiological recordings. Cell-attached patch-clamp recordings of myotubes and whole-cell patch-clamp recordings of myoblasts were performed essentially as described in Hamill et al. (1981). Sylgard-coated electrodes were filled with extracellular solution for cell-attached recordings (140 mM NaCl, 5 mM KCl, 3 mM CaCl_2 , 1 mM MgSO_4 , 10 mM HEPES, 11 mM glucose, pH 7.3) or intracellular solution for whole-cell recordings (140 mM CsF, 5 mM EGTA, 10 mM NaCl, 10 mM HEPES, pH 7.4). Polished electrodes had resistance of 2–4 M Ω and seals with 20–50 G Ω resistance were obtained by applying negative pressure in the pipette. An axopatch 1B amplifier connected to a pCLAMP system (Axon Instruments) was used for data acquisition and analysis. Currents were low-pass filtered at 3 kHz with an 8-pole Bessel filter (902LPF, Frequency Devices Inc., Haverhill, MA).

For cell-attached recordings of myotubes, patches were held at 50 mV negative with respect to the resting potential to remove channel inactivation. Voltage-gated sodium currents were elicited by applying depolarizing steps to the patch from the holding potential to more positive potentials (from -10 up to $+60$ mV relative to the resting potential in 10 mV steps). Peak currents were measured from the average of 16 traces for each voltage. Single-channel characteristics were measured from events identified visually from records and measured on-screen.

For whole-cell recording of myoblasts, cells were held at -100 mV to remove channel inactivation. Several depolarizing steps (from -60 up to $+30$ mV at 10 mV intervals) were applied to elicit voltage-gated Na^+ currents and peak sodium current (I_{Na}) was measured from the average of 10 records.

Results

ARIA increases the number of STX binding sites in myotubes

The number of surface membrane Na^+ channels in multinucleated myotubes was assayed by measuring the specific binding of ^3H -STX. Initial experiments, using a range of ^3H -STX concentrations, showed that the K_D was approximately 1.5 nM, similar to values previously reported in studies of chick myotubes maintained in culture (Baumgold et al., 1983). Maximal binding was obtained at 5–10 nM. At these concentrations, specific binding of ^3H -STX binding was 50% of the total. The amount of toxin bound specifically at saturation was 28 ± 12 fmol/dish (mean $\pm$ SEM, $N = 3$), which is also similar to previously reported values. The estimated number of ^3H -STX binding sites was less than the number of ^{125}I - α -BTX sites (113 ± 32 fmol/dish) measured in sister cultures.

In three experiments the number of specific ^3H -STX sites was increased in cultures exposed to ARIA for 48 hr compared to controls (Fig. 1). The mean ^3H -STX ratio (treated/control) was 2.0 ± 0.3 . In each experiment the total number of ^{125}I - α -BTX binding sites was assayed in sister cultures treated with ARIA for the same period of time (Fig. 1). As expected, ARIA produced a clear increase in α -BTX binding sites. It is noteworthy that the cultures with the greatest α -BTX binding ratio also had the greatest STX.

We attempted to demonstrate that the increase in myotube ^3H -STX binding was accompanied by an increase in the number of functional Na^+ channels by measuring the maximum rate of rise of the action potential ($\dot{V}_{\text{max}}$) using two intracellular micro-

Figure 1. ARIA increases the number of ³H-STX binding sites in proportion to the increase in ¹²⁵I- α -BTX binding sites in cultured chick myotubes. Specific ³H-STX and ¹²⁵I- α -BTX binding sites were measured in cells treated with ARIA for 48 hr in three independent experiments. Each bar represents the mean specific counts of two treated dishes compared to the mean specific counts of two control dishes.

electrodes under current-clamp conditions. However, $\bar{V}_{\max}$ varied widely between myotubes, perhaps because of differences in input resistance and capacitance (many myotubes branch *in vitro*), and this variation obscured a possible effect of ARIA on I_{Na} . We also examined spherical myoballs grown on a nonadhesive surface (Fischbach and Lass, 1978) under whole-cell, volt-

age-clamp conditions, but these experiments were abandoned when we found that ARIA did not reliably increase the total number or the rate of insertion of ¹²⁵I- α -BTX into myoball membranes.

ARIA does not alter the voltage dependence of I_{Na} , single-channel current, or mean open time detected in myotubes

The properties of single channels were examined with patch-clamp microelectrodes in the cell-attached configuration. Membrane patches were hyperpolarized by applying a holding potential of +50 mV to the pipette. Intracellular recordings in preliminary experiments showed that the myotube resting membrane potential was not affected by ARIA treatment. Resting potential was at least -40 mV, so we assume that the patch potential was at least -90 mV. Depolarizing pulses ranging between 40 mV and 110 mV were applied, which we assume shifted the trans-patch potential from -50 mV to +20 mV. Figure 2*A* illustrates results from membrane patches in a control and an ARIA-treated myotube, selected because their currents were similar in size. Maximal inward currents were evoked when the membrane patch was depolarized by 80 mV with respect to the holding potential. No transient inward current was observed when 500 nM TTX was included in the micropipette (not shown). The magnitude of I_{Na} varied widely between patches (Fig. 2*B*). However, normalized V - I curves showed that ARIA did not alter the voltage dependence of activation (Fig. 2*C*).

Under the conditions described above no outward currents were observed. Single-channel openings carrying outward currents were observed when the membrane was depolarized further. The size of the single-channel currents suggested that they

Figure 2. ARIA does not alter the voltage dependence of I_{Na} recorded in myotube cell-attached membrane patches. *A*, Examples of voltage-gated inward currents from a control and an ARIA-treated cell matched for similar peak I_{Na} . Membrane patches were hyperpolarized by applying a holding potential of +50 mV to the pipette, and the currents were elicited by applying negative pulses (-40, -60, -80, and -100 mV) that depolarized the membrane patch. Each trace is the average of 16 sweeps. Current kinetics did not differ consistently between control and treated cells. *B*, Examples of V - I_{Na} curves from patches in control (●) and ARIA-treated (○) cells show that the magnitude of I_{Na} varied widely between patches. *C*, Normalized V - I_{Na} curves from control (●, $n=9$) and ARIA-treated (○, $n=9$) cells. For each patch, peak inward currents were normalized to the maximum peak current (error bars represent SEM). *RP*, resting potential.

Figure 3. ARIA does not alter myotube Na^+ channel current or channel mean open time. The histograms depict single-channel open time (solid bars) and single-channel currents (hatched bars) in control and ARIA-treated cells. Channel open times and currents were measured following a 70 mV depolarizing step from the presumed holding potential of -90 mV (control, 119 openings in 15 patches; ARIA, 154 openings in 19 patches).

are due to activation of Ca^{2+} -activated K^+ channels. In many patches a small inward tail current was evident on repolarization to the holding potential. We have not investigated this current, which was also present in myoblasts (see Fig. 7) in detail, but preliminary experiments showed that it is not blocked by 500 nM TTX or by 6 mM CoCl_2 . It may, therefore, be carried by Cl^- ions (see Hume and Thomas, 1989).

Single Na^+ channel open times and channel currents were measured following depolarization by 70 mV from the holding potential (20 mV positive to the initial resting potential). Neither parameter was affected by ARIA (Fig. 3). Thus, we have no evidence that ARIA alters the gating properties of Na^+ channels.

Peak inward currents, measured in 39 patches from ARIA-treated myotubes and 39 control patches, ranged between 0 pA to more than 20 pA (Fig. 4). The current was less than 6 pA in most patches. The mean peak current was slightly greater in patches from treated cells (3.95 ± 0.77 pA; mean $\pm$ SEM) than in those from control (3.26 ± 0.47 pA). However, neither the difference in the means nor in the overall distribution was statistically significant (*t* test, Mann-Whitney, and χ^2). Moreover, the percentage of patches with zero channels was similar in treated and control cells (11% and 13%, respectively). Our failure to demonstrate an effect of ARIA on patch current is most likely a sampling problem: the clamped patches are small, and the distribution of Na^+ channels may not be uniform.

ARIA increases the synthesis and total number of AChRs in myoblasts

Myoblasts provide a means of avoiding the sampling problem encountered with small membrane patches because the entire surface membrane can be voltage clamped. However, the effect of ARIA on myoblasts has not been previously studied, so we first assayed the total number of AChRs and their rate of incorporation into the surface membrane, which provides a measure of receptor synthesis. Cells were plated at low density (300 cell/mm²) to minimize fusion, and 4 hr later ARIA was added to the medium. The rate of incorporation of new AChRs (appearance of ^{125}I - α -BTX sites after blocking exposed sites with unlabeled α -BTX; see Materials and Methods) was measured at

Figure 4. ARIA does not increase the peak I_{Na} in myotube cell-attached membrane patches. *A*, Examples of inward currents from three control and three ARIA-treated cell membrane patches chosen to compare equivalent peak currents. Patches were hyperpolarized by applying a holding potential of $+50$ mV to the pipette. Voltage-gated currents were then elicited by the application of a 70 mV depolarizing step. *Bottom traces* in each column show the largest responses recorded. *B*, The histogram depicts the distribution of peak inward current measured in control ($N = 39$) and ARIA-treated ($N = 39$) cells.

various times thereafter. No effect was observed after 12 hr of treatment. However, after 24 hr the rate of insertion of AChRs was 1.5-fold higher than in control. The rate of insertion was about twofold greater after exposure to ARIA for 48 and 72 hr. The total number of AChRs, rather than the rate of incorporation, is most relevant for comparison with whole-cell I_{Na} . ARIA increased total AChR number over approximately the same time course. After 48 hr, the amount of ^{125}I - α -BTX bound was 1.5 times control.

Autoradiography was employed to determine if ARIA affected all myoblasts or only a subpopulation. Mononucleated cells were plated and treated with ARIA for 48 hr. Within a few

CONTROL

ARIA

40μM

Figure 5. Autoradiography of newly inserted AChRs in control and ARIA-treated myoblasts. Myogenic mononucleated cells were treated with ARIA for 48 hr beginning 4 hr after plating. AChRs inserted during a 5 hr period were labeled with ^{125}I - α -BTX after blocking preexisting receptors with cold toxin. The myoblasts have been photographically aligned. Cells were selected to match in size. The bottom panel shows that some ARIA-treated cells are not labeled.

hours after plating, myoblasts assume a spindle shape that can be distinguished from the more polygonal fibroblasts. Examples of control and treated myoblasts that illustrate the range of ^{125}I - α -BTX binding and cell size are shown in Figure 5. In this experiment, all AChRs were blocked with unlabeled α -BTX five hr before addition of ^{125}I - α -BTX, so the grains reflect newly synthesized receptors.

Histograms of grains per cell in control and ARIA-treated myoblasts are shown in Figure 6. ARIA produced a marked shift in the entire distribution. The mean number of grains per cell in ARIA-treated cultures (62.1 ± 3.4 grains; $n = 121$) was 1.9-fold greater than the mean in controls (32.3 ± 2.4 grains; $n = 117$; $p < 10^{-6}$). This ratio is very similar to the ratio of specific counts observed in the incorporation rate binding assay. Under the same conditions, fibroblasts exhibit little or no binding (3.2 ± 0.02 grains/cell; $n = 32$) in both ARIA-treated and control cultures. Cell size (length) was not affected by ARIA (control, $84.34 \pm 2.39 \mu\text{m}$; ARIA, $85.82 \pm 2.66 \mu\text{m}$). Moreover, the number of grains per cell was not correlated with cell size

in either control or treated cells (data not shown). We conclude that chick myoblasts synthesize AChRs, and that ARIA increases the rate of this synthesis.

ARIA increases I_{Na} in myoblasts

Whole-cell recordings were obtained from myoblasts after 40–48 hr of ARIA treatment and compared to age-matched controls. The mononucleated cells were held at -100 mV to remove channel inactivation. Depolarizing pulses evoked transient inward currents that were completely blocked by TTX (Fig. 7A). As observed in myotubes, the V - I_{Na} relation in myoblasts was similar in control and ARIA-treated myoblasts (Fig. 7B). Note that the inward tail current first observed in myotube patches was prominent in myoblasts (Fig. 7A). This current is not blocked by TTX.

The mean peak I_{Na} was significantly higher in ARIA-treated myoblasts ($146.5 \pm 17.9 \text{ pA}$, $n = 23$) than in controls ($96.5 \pm 16.6 \text{ pA}$, $n = 21$; $p < 0.05$ by t test). ARIA induced a shift in the distribution of peak I_{Na} (Fig. 8) similar to the shift observed

Figure 6. ARIA increases the rate of synthesis of AChRs in myoblasts. Histograms of silver grain counts per myoblast (control, $n = 117$; ARIA, $n = 121$) labeled with ^{125}I - α -BTX as described in the Figure 5 caption. The mean number of grains per cell in ARIA-treated cultures (62.1 ± 3.4) was greater than the mean in controls (32.3 ± 2.4) ($p < 10^{-6}$). In the same cultures, fibroblasts exhibited few or no grains (3.2 ± 0.02 per cell; $n = 32$ cells).

in AChRs (see Fig. 6): 33% of the control myoblasts exhibited peak I_{Na} of less than 50 pA, whereas only 9% of the ARIA-treated cells were in this category. The ratio of mean peak I_{Na} values (1.5) was within the range of ratios (treated/control) of ^3H -STX binding in myotube cultures (1.3–2.5). As observed with AChRs, peak I_{Na} did not correlate with cell size, estimated in this case by whole-cell capacitance (data now shown).

The increase in myoblast I_{Na} was not unexpected considering the increase in ^3H -STX binding observed in myotubes. Unfortunately, we could not measure specific ^3H -STX binding to myoblasts grown under conditions used for the electrophysiological recordings. The density of sites at this stage of development is apparently too low to detect specific binding above background levels.

Discussion

We conclude that ARIA increases the number of functional Na^+ channels in embryonic chick muscle membranes. This is based on estimates of ^3H -STX binding to multinucleated myotubes and on voltage-clamp measurements of I_{Na} in mononucleated myoblasts. As discussed above, both measurements could not be made on the same cell population. The observed increases in myotube ^3H -STX binding and in myoblast I_{Na} are significant effects of ARIA, independent of our interpretation. While they might be due to different cellular responses to ARIA, the simplest interpretation is that the mechanism is the same.

In chick muscle, the properties of AChR channels and Na^+ channels do not change with development (Schuetze, 1980; Frelin et al., 1981). In mammalian muscle, on the other hand, the

Figure 7. ARIA does not alter the voltage dependence of I_{Na} in myoblasts. *A*, Voltage-gated inward currents in myoblasts recorded in the whole-cell configuration. Cells were held at -100 mV and depolarized to -50 , -30 , -10 , and $+10$ mV. CsCl was included in the pipette solution to block outward currents. The top trace shows currents from a representative control cell, the middle trace shows currents from an ARIA-treated cell, and the bottom trace shows currents from a control cell in the presence of $0.5 \mu\text{M}$ TTX. *B*, Normalized V - I_{Na} curves from control ($\bullet$, $N = 11$) and ARIA-treated ($\circ$, $N = 11$) cells.

properties of AChRs and Na^+ channels do change as the muscle and the postsynaptic membrane mature. Low-conductance, long-duration embryonic AChR channels are replaced by high-conductance, short-duration channels during the first 2 weeks after birth (Brenner and Sakmann, 1978; Fischbach and Schuetze, 1980). The change in Na^+ channel properties from relatively TTX-insensitive, low-conductance embryonic channels to TTX-

Figure 8. ARIA increases peak whole-cell I_{Na} in myoblasts. Histograms of peak I_{Na} measured in the whole-cell patch-clamp configuration in control ($n = 21$) and ARIA-treated ($n = 23$) myoblasts. Cells were held at -100 mV and currents elicited by depolarizing steps. Peak currents were obtained from $V-I_{Na}$ curves for each cell. The mean peak I_{Na} was significantly higher in ARIA-treated myoblasts (146.5 ± 17.9 pA) than in controls (96.5 ± 16.6 pA; $p < 0.05$ by t test).

sensitive, high-conductance channels also occurs postnatally (Frelin et al., 1983; Sherman et al., 1983; Weiss and Horn, 1986). The switch from embryonic to adult AChR channels is correlated with the appearance of a new subunit (ϵ) that replaces the γ -subunit to form $\alpha\beta\delta\epsilon$ receptors (Mishina et al., 1986; Gu and Hall, 1988). ARIA produces a large increase in the level of ϵ mRNA in mouse muscle (Martinou et al., 1991), suggesting that ARIA may play a role in receptor "maturation" as well as in the initial accumulation of receptors at developing junctions. The change in mammalian Na⁺ channel properties is also due to the expression of a new gene product (Kallen et al., 1990). It would be of interest to determine if ARIA promotes the appearance of adult-type Na⁺ channel genes in mammalian muscle. This might be expected to occur since the expression of Na⁺ channels in rat muscle becomes nerve-dependent beginning approximately on postnatal day 10 (Sherman and Catterall, 1982; Bambrick and Gordon, 1988).

AChR and Na⁺ channel genes can be regulated by the same agents. The expression of both genes in cultured muscle is regulated by levels of intracellular Ca²⁺ and cAMP. Agents that decrease intracellular Ca²⁺ (e.g., TTX, verapamil, or ryanodine at high concentration) increase both receptor and channel mRNA and protein (Birnbach et al., 1980; Sherman et al., 1985; Klarsfeld et al., 1989). The same has been shown to result from

treatment with forskolin, which increases intracellular cAMP levels (Sherman et al., 1985; Harris et al., 1988). Conversely, downregulation is evident following treatments that increase intracellular Ca²⁺ levels, such as exposure to a calcium ionophore or to low doses of ryanodine (Birnbach et al., 1980; Sherman et al., 1985). ARIA can be added to the list of factors that coregulate AChRs and Na⁺ channels. Changes in cAMP and/or Ca²⁺ are probably not involved in the chain of events triggered by ARIA. ARIA does not increase intracellular cAMP levels (Johnson et al., 1990) and our experiments suggest that ARIA does not act by an overall change in intracellular Ca²⁺. ARIA's effect on AChR synthesis adds to that of saturating concentrations of TTX, verapamil, calcium ionophore, high (10^{-4} M) or low (10^{-6} M) concentrations of ryanodine, or a combination of these drugs (G. Corfas and G. D. Fischbach, unpublished observations). We have recently found that ARIA's action on chick and mammalian myotubes involves the activation of a transmembrane receptor tyrosine kinase in muscle cells (Corfas et al., 1993). This may shed light on molecular mechanisms of coordinated regulation of AChRs and Na⁺ channels.

It has been known for some time that mononucleated muscle cells express AChRs (Fambrough and Rash, 1971; Smilowitz and Fischbach, 1978) and Na⁺ channels (Weiss and Horn, 1986), but ARIA regulation of these proteins in myoblasts has not been previously reported. Histograms of autoradiographic grains generated by ¹²⁵I- α -BTX and of peak I_{Na} suggest that ARIA affected most if not all myoblasts, rather than a small subpopulation. In both cases there is a shift in the distribution to higher values following ARIA treatment, and the lowest values became the least frequently observed. On the other hand, earlier work suggested that not all muscle nuclei that are incorporated into myotubes respond to ARIA (Harris et al., 1989). In this study, *in situ* hybridization showed that AChR α -subunit expression was not increased above background levels in a substantial fraction of the nuclei. Our present data suggest that heterogeneity among myotube nuclei is not characteristic of the mononucleated precursor cells; it may arise from interactions among nuclei once they are incorporated in a common cytoplasm.

The effect of ARIA on myoblast AChRs and Na⁺ channels is important considering that the myogenic regions of chick limb buds contain few if any multinucleated myotubes when the first motor axons arrive on E5 (Hilfer et al., 1973). In the experiments reported here, we assayed myoblasts dissociated from E11 pectoral muscle. We cannot comment on E5 myoblasts. However, we should note that myotubes formed by E5 myoblasts do respond to ARIA at least in terms of AChR levels (Corfas and Fischbach, unpublished observations). Therefore, primary as well as secondary myotubes respond to ARIA.

The fact that ARIA increases the number of muscle membrane Na⁺ channels as well as the number of ACh receptors, and that it increased the expression of ϵ subunit mRNA (in mouse myotubes), adds to the evidence that this protein acts as a trophic factor at developing neuromuscular junctions. More direct evidence depends on the *in vivo* assay of reagents that block the action of ARIA specifically.

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