Accumulation in Fetal Muscle and Localization to the Neuromuscular Junction of cAMP-dependent Protein Kinase A Regulatory and Catalytic Subunits RIα and Cα

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Abstract. Using probes specific for cAMP-dependent protein kinase, we have analyzed by in situ hybridization the patterns of expression of regulatory and catalytic subunits in mouse embryos and in adult muscle. RIα transcripts are distributed in muscle fibers exactly as acetylcholinesterase, showing that this RNA is localized at the neuromuscular junction. The transcript levels increase upon denervation of the muscle, but the RNA remains localized, indicating a regulation pattern similar to that of the ε subunit of nicotinic acetylcholine receptor. RIα transcripts have accumulated in the muscle by day 12 of mouse embryogenesis, and localization is established by day 14, at about the time of formation of junctions. This localization is maintained throughout development and in the adult. Immunocytochemical analysis has demonstrated that RIα protein is also localized. In addition, RIα recruits Cα protein to the junction, providing at this site the potential for local responsiveness to cAMP. PKA could be implicated in the establishment and/or maintenance of the unique pattern of gene expression occurring at the junction, or in the modulation of synaptic activity via protein phosphorylation.

Embryonic skeletal muscle shows a high level of Cα transcripts and protein throughout the fiber; the transcripts are already present by day 12 of embryogenesis, and their elevated level is maintained only through fetal life. In the adult, the Cα hybridization signal of muscle is weak and homogeneous.

THE cAMP-dependent protein kinase (PKA)[1] is composed of regulatory and catalytic subunits. The subunits associate in tetrameric complexes of two regulatory and two catalytic subunits, the holoenzyme, which is cytoplasmic and inactive. Intracellular synthesis of cAMP results in the binding of this second messenger to the regulatory subunits and liberation of monomeric catalytic subunits, endowed with serine-threonine kinase activity, which can phosphorylate target proteins in the cytoplasm and the nucleus (Taylor et al., 1990; McKnight, 1991).

The regulatory subunits belong to two different families, RI and RII, each of which has distinct properties concerning binding of cAMP derivatives and the state of phosphorylation (Scott, 1993, and references therein). Each family has two different members, α and β, resulting in a total of four genes. The catalytic subunits, Cα, β, and γ are encoded by three genes. Relatively little is known concerning the cellular distributions of the gene products of these different family members. RIβ and RIIβ, as well as Cβ, are primarily expressed in the nervous system. RIIβ is also found in adipose tissue, bone marrow, glands, and reproductive organs. The α forms are considered to be ubiquitous and constitutive (Cadd and McKnight, 1989). In contrast, Cγ is testis specific (Beebe et al., 1990).

Several studies have implicated cAMP-dependent protein kinase in control of differentiation of cells in culture: PKA as an inhibitor of myoblast differentiation (Li et al., 1992; Winter et al., 1993), and RIα as a specific extinguisher (Tse1) of cAMP- and glucocorticoid-responsive, liver-specific genes in hepatoma cells (Boshart et al., 1991; Jones et al., 1991). In addition, a series of recent papers has implicated PKA in major developmental decisions in *Drosophila* (Lepage et al., 1995; Jiang and Struhl, 1995; Li et al., 1995; Pan and Rubin, 1995). It is therefore tempting to speculate that this kinase could be important during the

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1. *Abbreviations used in this paper*: AChE, acetylcholinesterase; Cy3, indocarbocyanine; nAChR, nicotinic acetylcholine receptor; NMJ, neuromuscular junction; PKA, cAMP-dependent protein kinase; PKI, heat-stable inhibitor of PKA; RT, room temperature.

course of development and of cell differentiation in mammals as well.

We have examined the expression of the various PKA subunits in different mouse tissues and during the course of development. Since most tissues are composed of a mixture of cell types and much of the available information comes from use of tissue extracts, we have chosen in situ hybridization and immunocytochemistry as a means to achieve characterization at the cellular level.

These analyses revealed a nonuniform localization of RIα mRNA and protein, respectively, in skeletal muscle. This localization was shown to correspond to the neuromuscular junction (NMJ) by demonstration of the same localization of a known NMJ marker and of RIα. While this work was in progress, Morita et al. (1995) described the localization of RIα transcripts to NMJs in adult rat tongue. We also examined RIα expression on sections of mouse embryos: the establishment of localized expression of transcripts coincides with or may even precede the formation of junctional complexes. To determine the relationship between the maintenance of NMJ localization and electrical activity, the effect of denervation upon RIα expression was investigated. The observation of localized expression of RIα provides an intriguing example of NMJ localization for a molecule known to be implicated in the regulation of gene expression. Hence, PKA could be involved in the molecular mechanism responsible for localized gene expression.

In parallel, in situ hybridization analysis of expression of the other PKA subunits was carried out. This revealed that muscle presents a curious expression pattern, not only of RIα, but of Cα as well. This catalytic subunit shows high expression throughout all skeletal muscles of the early fetus, but not the adult.

Materials and Methods

Probes

cDNA clones of the PKA subunits Cα, Cβ, RIα, RIIα, RIβ, and RIIβ were generous gifts from G.S. McKnight (University of Washington, Seattle, WA) (and are detailed in Cadd and McKnight, 1989). Linearized plasmids were transcribed with either T7 or SP6 RNA polymerase in the presence of ^{35}S-UTP-αS (Amersham International, Buckinghamshire, UK) or digoxigenin-11-UTP (Boehringer Mannheim GmbH, Mannheim, Germany). After digestion of the template with RQ1 DNase (Promega, Madison, WI), radiolabeled probes were separated from unincorporated nucleotides by gel filtration chromatography on Sephadex G-50 (Pharmacia Biotech, St. Albans, UK) in 10 mM Tris-HCl, pH 7.5, 5 mM EDTA, 0.1% SDS, and 10 mM DTT. All probes used in these experiments had a specific activity of ~10^9 cpm/μg. Digoxigenin-labeled probes were ethanol precipitated after DNase digestion and used without further purification. The RIIβ probe (1,200 nucleotides) was hydrolyzed 15 min at 60°C in a mixture of 80 mM $NaHCO_3$ and 120 mM Na_2CO_3. The other probes (of 290–410 nucleotides) were used without hydrolysis. The specificity of each of the probes was verified by Northern and Southern blot analysis (data not shown).

Tissue Preparation

Paraffin-embedded sagittal sections (7 μm) of 12-, 14-, or 16-d-old mouse embryos were obtained from Novagen (Madison, WI). Paraffin-embedded tissue sections (3-μm) of 18-d-old mouse fetuses were prepared at the Hôpital Beaujon (Clichy, France).

Cryosections of Innervated and Denervated Muscle Tissue. 2-mo-old mice were anesthetized with ketamine (Rhône Mérieux, Lyon, France) and xylazine (Bayer, Leverkusen, Germany), and their right legs were denervated by removing a 2–3-mm section of the sciatic nerve high in the thigh. It was verified 3 d later that the animals lacked the toe extension reflex in the operated leg, and the animals were then killed. The tibialis anterior muscle of each leg was dissected and frozen immediately in isopentane at −70°C. 5-μm cryosections were made at −20°C with a 5030 Microtome (Bright Instrument Co Ltd., Huntingdon, UK).

Diaphragm. 2-wk-old mice were killed by cervical dislocation, and their diaphragms were dissected with some ribs attached (to keep the muscle flat) and immediately fixed in freshly prepared 4% paraformaldehyde-PBS.

In Situ Hybridization

Paraffin sections were pretreated and hybridized with radiolabeled probes according to Sassoon and Rosenthal (1993). Exposure time ranged from 3 d to 1 mo. After development of the emulsion, slides were stained with toluidine blue; the stain was neutralized for photography with a blue filter. On Northern blots, the RIα probe shows a slight cross-hybridization with RIIα transcripts (data not shown). For in situ hybridization and as will be described below, RIα signal is not uniform on the muscles, being concentrated as "stripes" corresponding to the NMJ. With the RIIα probe, the appearence of weak but discernable stripes on muscles was observed only upon long exposure (>3 wk). When hybridization with labeled RIIα probe was carried out in the presence of a 20-fold excess of cold RIα, cross-hybridization was essentially eliminated.

Cryosections. 5-μm longitudinal cryosections of innervated and denervated tibialis anterior muscle were fixed with 4% paraformaldehyde as described by Watkins (1989) and hybridized to ^{35}S-labeled probes according to the method of Sassoon and Rosenthal (1993). After RNase treatment, the sections were contact exposed to Hyperfilm β-Max x-ray film (Amersham) for 30 h at 4°C. The slides were then coated with Hypercoat LM-1 emulsion (Amersham) and exposed at 4°C for 7 to 37 d. Whole-mount in situ hybridization of digoxigenin-labeled RIα probe was performed according to Wilkinson and Nieto (1993). Proteinase K treatment (10 μg/ml) of fixed diaphragms was in 50 mM Tris-HCl, pH 7.0, 5 mM EDTA, 0.1% Tween 20 for 1 h at room temperature (RT). It should be noted that in all cases, the hybridization conditions were more stringent than those used by Cadd and McKnight (1989) for the same probes. These authors demonstrated specificity of the probes by incubation with a 500-fold molar excess of unlabeled probe.

Acetylcholinesterase (AChE) Histochemistry

The method of Koelle and Friedenwald (1949) was used.

Densitometry

Semiquantitative evaluation of RIα transcript distribution in control and denervated tibialis anterior muscle was obtained from Hyperfilm β-Max contact exposure autoradiograms. The images of the autoradiograms were recorded by a Tri-CCD (Hitachi) camera linked to a Polyvar-2 microscope (Reichert-Jung, Vienna, Austria), and the measure of number and area of spots was carried out using a P.C. Compac Pontium with the program Sapphire from Quantel (Newbury, UK).

Microphotography of Radioactive In Situ Hybridizations

The methods used are outlined in Fig. 1 and explained in the legend.

Immunocytochemistry

Polyclonal antibodies raised against Cα or the first 17 amino acids of human RIα, as well as Cα and RIα proteins used in blocking experiments, were a gift from Susan Taylor (UCSD, La Jolla, CA). The mAb against mouse RIα was purchased from Transduction Laboratories (Lexington, KY). Secondary antibodies coupled to FITC, indocarbocyanine (Cy3), or Texas red were obtained from Caltag (San Francisco, CA) and Amersham. TRITC- and FITC-α-bungarotoxins, used to label NMJ through binding to the nicotinic acetylcholine receptor (nAChR), were purchased from Molecular Probes, Inc. (Eugene, OR).

4-μm cryosections of mouse tibialis anterior muscle (adult or 16.5-d fetal) or of intercostal muscle of a 16.5-d-old fetus were air-dried at RT for 2–3 h, pre-incubated for 1 h at RT in PBS containing 10% of appropriate nonimmune serum, and then incubated overnight at 4°C with monoclonal (12.5–25 ng/μl) anti-RIα or polyclonal (1:200–1:500) anti-Cα or anti-RIα

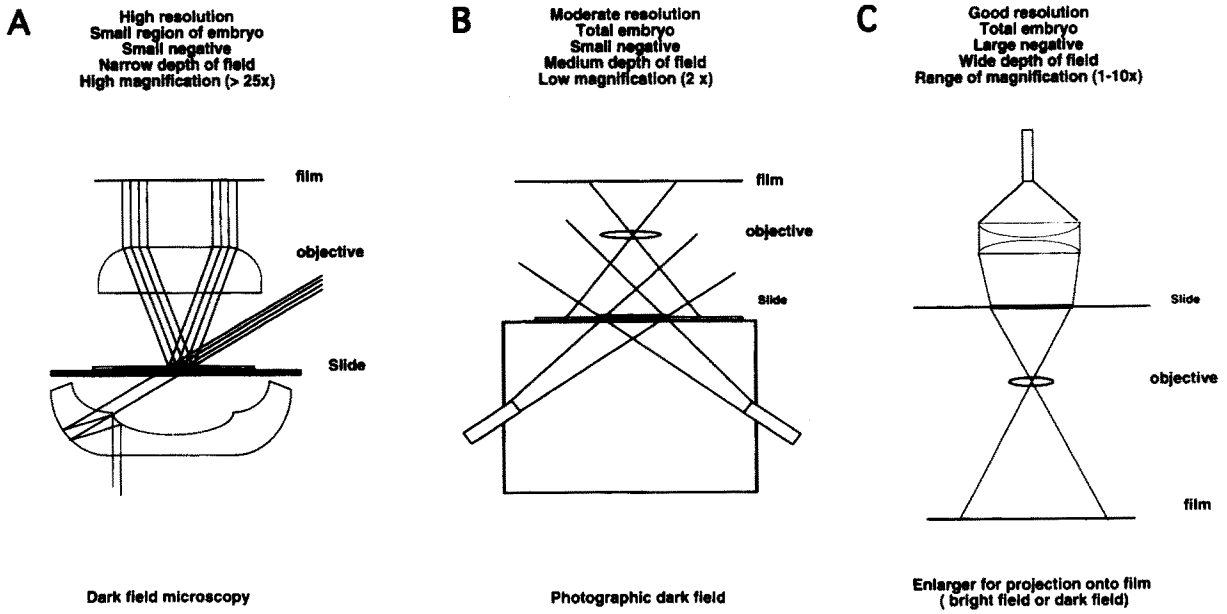


Figure 1. Photographic procedures used or developed here for mouse embryo sections. The characteristics of each method are indicated above the corresponding scheme. (*A*) Classical dark-field microscopy, indicating positions of film (24 × 36–mm), objective, and the microscope slide. The major drawbacks are the necessity of using relatively high magnification, and the sometimes confusing image obtained because of the equivalent reflective properties of silver grains, specks of dust, and chromatic proteins such as hemoglobin. (*B*) Photographic dark-field based on direct use of a camera placed above a black-lined box and two light sources (optical fibers) whose beams cross at 45° at the level of the microscope slide. (*C*) A system for obtaining bright field, and in a second-step simulated dark field, using a photographic enlarger in place of a microscope. The microscope slide replaces the photographic negative on the negative carriage. The enlarger can be equipped with a filter to neutralize a histological stain. (However, the neutralization is incomplete for heavily stained preparations. This system is optimal when the signal is strong or moderate, and the slide has not been stained; otherwise, weak signals can be lost.) The image at the desired magnification is exposed directly onto 10 × 12.5–cm film. The first film can then be used to produce a contact negative to obtain "simulated dark field." With this system, confusion among silver grains, chromatic proteins, and dust specks is diminished. Moreover, the degree of magnification is flexible.

antibodies diluted in PBS containing BSA (10 or 100 mg/ml). The sections were rinsed (five times for 10 min at RT in PBS) and incubated with secondary antibody (1:200) and α-bungarotoxin (2 ng/μl) for 1–3 h at RT, rinsed as described above, and mounted with Immu-mount (Shandon, Pittsburgh, PA). The specificity of the antibody staining was verified by preincubation (2 h at RT) of the diluted antibody with 13 μg/μl of the corresponding protein.

The sections were examined with a photomicroscope (Carl Zeiss, Inc., Thornwood, NY). Confocal microscopy of samples labeled with two fluorochromes was performed with a Confocal Laser Scanning Microscope (Leica Instruments, Nussloch, Germany), which uses an argon-krypton laser operating in multiline mode. Tissue preparations labeled with both Texas red (or Cy3) and fluorescein conjugates were sequentially analyzed at 567- and 488-nm wavelengths with filters that transmit light very selectively and optimally. A long-path filter RG590 was used to detect Texas red (or Cy3) emission, while a narrow-band filter centered on 535 nm was used for FITC signal detection. For each neuromuscular junction, six to eight optical sections taken at intervals of 0.5 μm were generally recorded.

Results

RIα and Cα Transcripts in Fetal Intercostal Muscle

In situ hybridization using probes for the different subunits of the PKA family revealed that skeletal muscle presents an unexpected and striking signal with probes for both the Cα catalytic and RIα regulatory subunits. The illustration of Fig. 2 shows a section of ribs and intercostal muscles from an 18-d-old mouse fetus. Hybridization with a Cα probe revealed an intense signal throughout the muscle tissue (Fig. 2 *A*), while that with RIα showed a nonuniform distribution of signal (Fig. 2 *B*). The strong signal for Cα observed with muscle tissue contrasts with the weak signal for cartilage of the ribs, and was observed for all skeletal muscles of mouse fetuses (see below). Fig. 2 *B* demonstrates the unequal distribution of grains obtained with the RIα probe: a strong signal is limited to stripes across the muscle tissue. Such a distribution is reminiscent of that of NMJs along the middle of muscle fibers. A suitable system to test whether RIα is indeed localized at the NMJs is offered by a well-characterized muscle, the diaphragm.

Localization of RIα Transcripts to the Neuromuscular Junction

The diaphragm is a flat circular muscle, which contains neuromuscular junctions limited to the form of an arc. Junctions can be identified by histochemical staining of proteins known to be associated with them, such as AChE (illustrated by the half diaphragm on the right of Fig. 3). On the left (Fig. 3) is shown the result of whole-mount in situ hybridization of a diaphragm from a 2-wk-old mouse with a digoxigenin-labeled RIα probe. The staining patterns of the two diaphragm halves are identical, demonstrating that RIα transcripts are indeed localized to the NMJ. Intercostal muscles also show a characteristic pattern of NMJs running in a line halfway between and parallel to the ribs (see Piette et al., 1993); RIα transcripts were

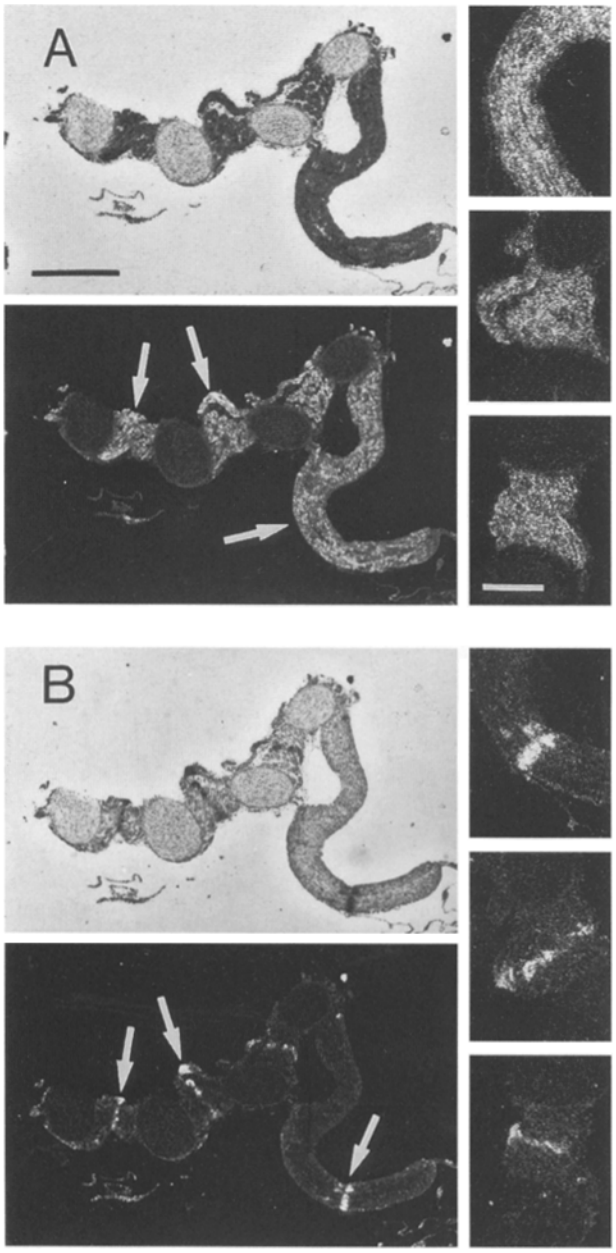


Figure 2. Hybridization of Cα and RIα probes with fetal muscle. Sections of 18-d mouse fetal ribs and intercostal muscles, hybridized with radioactive Cα (*A*) or RIα (*B*) probes (6-d exposure). The images of *A* and *B* are positive (*upper*) and negative (*lower*) images prepared according to method C of Fig. 1. The small images to the right were obtained by standard dark-field microscopy (Fig. 1, method A) of the regions indicated by arrows in *A* and *B*, permitting comparison of the different photographic methods. Bars, 0.5 mm on the whole section and 0.2 mm on the small image.

similarly localized in the 2-wk-old intercostal muscles (not shown).

NMJ localization of nAChR subunit mRNAs has been established (Merlie and Sanes, 1985; Fontaine et al., 1988). The pentameric receptor is composed of $\alpha_2\beta\gamma\delta$ subunits, with γ being replaced by ε at birth (Mishina et al., 1986; Witzemann et al., 1987). The corresponding transcripts are first present throughout the muscle fiber during the early

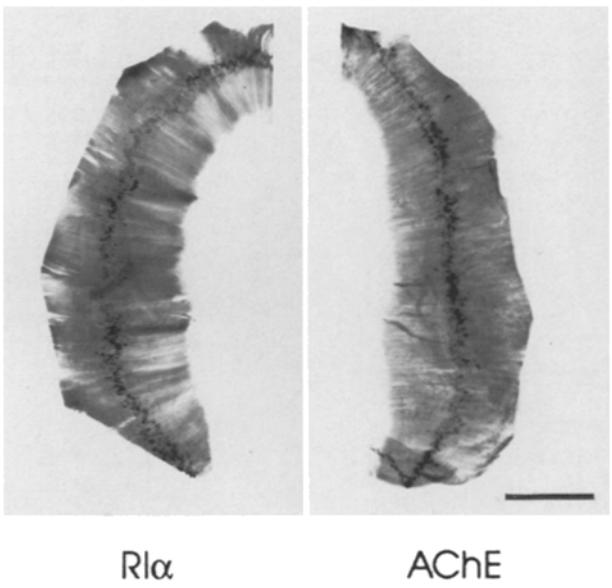


Figure 3. Distribution of AChE and of RIα transcripts on a 2-wk-old mouse diaphragm. A diaphragm stained for AChE is represented on the right panel; the left panel shows the hybridization obtained with a digoxigenin-labeled RIα probe. Bar, 2 mm.

stages of development (Fontaine and Changeux, 1989), and begin to be localized at ~ day 13.5 in the mouse embryo, coincident with the formation of neuromuscular synapses (Piette et al., 1993). Moreover, upon denervation, injury, or blockage of nerve impulses to adult muscle, three types of changes concerning the nAChR subunits are observed. The mRNAs for α, β, and δ are delocalized, and their amount is increased (Goldman et al., 1988); the γ subunit is reexpressed in a delocalized fashion; the mRNA for the ε subunit remains localized, and its expression is increased (Witzemann et al., 1991; Kues et al., 1995; Brenner et al., 1990). These results imply that electrical activity is critical for the maintenance of localization of the corresponding messenger RNAs and for the correct expression of the adult receptor components (see reviews by Hall and Sanes, 1993; Duclert and Changeux, 1995; Chu et al., 1995).

RIα Transcripts Remain Localized and Are Augmented upon Denervation

To determine whether the parameters associated with nerve stimulation are implicated in regulation of the RIα expression pattern, the effect of denervation was examined. The sciatic nerve was cut in the right leg of 2-mo-old mice 3 d before death. The tibialis anterior muscle was then dissected away from both sides, frozen and sectioned, and subjected either to in situ hybridization with radioactive RIα probe or to histochemical staining for AchE (Fig. 4). At the microscopic level, the enzyme activity and RIα mRNA show the same distribution in this muscle. The right panel of the figure shows the in situ hybridization with RIα of the denervated muscle: the signal remains localized, and it has become more intense. Although the sections shown were subjected to long exposure, the result was already apparent after 15 d.

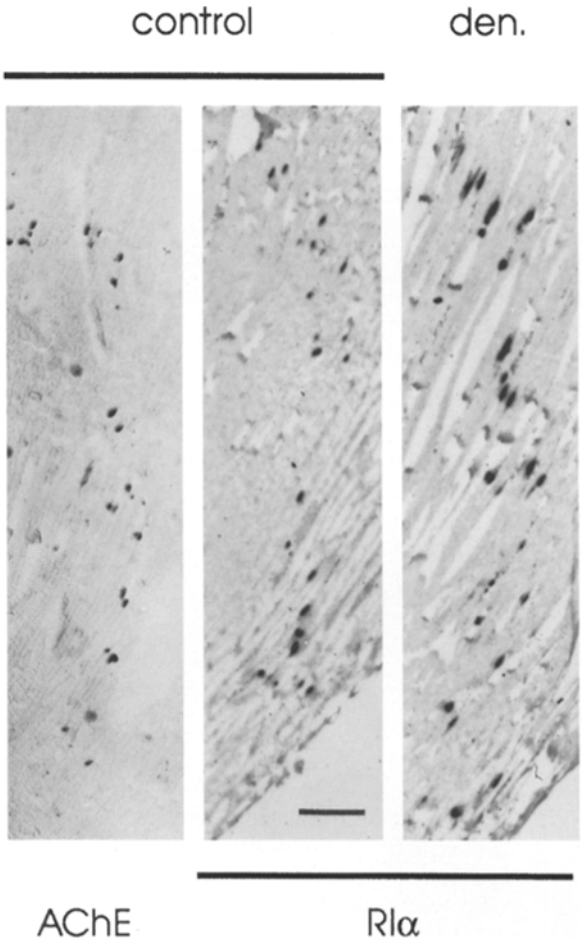


Figure 4. Distribution of AChE and RIα transcripts in normal and denervated adult muscle. Microphotographs (Fig. 1, method C) of sections of 2-mo-old control or denervated tibialis anterior mouse muscle, stained for AChE or hybridized with RIα probe (competed with unlabeled RIIα), and exposed for 37 d. *den*, denervated. Bar, 0.2 mm.

Fig. 5 shows x-ray film images of a series of control or denervated muscle sections, hybridized with the Cα, RIα, and RIIα probes. No change in signal strength or distribution with the Cα and RIIα probes is observed upon denervation, and the signal obtained with both is uniform and diffuse. The very intense signal observed with the Cα probe on 18-d fetal intercostal muscle is not found in the 2-mo-old leg muscle, even upon very long exposure (not shown). Consequently, at some time after birth, the level of mRNA for the Cα subunit is strongly reduced. The arc-like distribution of presumed NMJs is outlined by the RIα probe, and after denervation, the number of spots and the intensity of the RIα signal is enhanced.

Evaluation of the strength of the RIα signal on sections of normal and denervated muscle was carried out using measurements of the number and the area of labeled spots. The average labeled surface per section was four times greater on the denervated muscle (Table I). Thus, RIα transcript accumulation responds to denervation as does the ϵ subunit of the nAChR, remaining localized and increasing in amount. It cannot be excluded, however, that increased expression from extrajunctional nuclei occurs, but that the signal remains too weak to be detected by in situ hybridization. It should be noted that Morita et al. (1995) observed a similar increase in junctional RIα transcripts after denervation of rat tongue with a maximum at 2–7 d. These results imply that regulation of RIα gene expression, like those of the nACh receptors, is achieved by mechanisms that are regulated by electrical activity and/or the physiological status of the muscle.

Expression of RIα and Cα Transcripts during Mouse Development

We examined sections of mouse embryos of 12 d, before the establishment of neuromuscular synapses, of 14 d, when these connections have just been established, and of 16 d, when the fetus is well formed. Below, we emphasize only the very intense labeling obtained on skeletal muscle with the RIα and Cα probes. It is important to note that both gene products are known to be ubiquitous in their distribution. Indeed, weaker signals were observed with both probes on essentially all tissues. In particular, RIα signal is observed throughout the muscle tissue, but at a modest level compared to the intense localized signal.

The sagittal section of an entire 12-d embryo shown at the top of Fig. 6 has been hybridized with a radioactive Cα probe. The section is a little to one side of the midline and misses most of the spinal cord. The dark areas of radioactive label reveal the presence of Cα transcripts in developing muscle tissue, visible between the dorsal ribs and vertebral regions (*pale round areas*) and at the back of the neck and the throat region. Higher magnifications of the region along the back (*bright field*) and the neck (*simulated dark field*) are shown on the left, hybridized with Cα (*top and middle*), and RIα (*bottom*). The Cα probe marks all muscles, which are discretely outlined by the hybridization signal. Along the back and irrespective of the known rostro-caudal progression in maturation of the muscle, all muscles are intensely marked by the Cα probe, indicating that these transcripts are a very early marker of skeletal muscle. The RIα signal is diffuse in the throat region, but the neck muscles are well outlined. For the larger neck muscles, the grains do not trace a clear outline of the muscles as they do with the Cα probe: they could be concentrated in the center of the muscle mass, where the NMJs will presumably form.

The 14-d fetus sagittal section of Fig. 6 shows a quantitative and qualitative evolution of muscle tissue, clearly outlined by the Cα probe on the bright-field image. Tongue muscle is well formed, as are the throat muscles and those all along the back, hip, and abdomen. All show intense labeling with the Cα probe. This is confirmed by the higher magnification of the back, neck, and throat regions shown on the right side of Fig. 6, and as before, hybridized respectively with Cα (*top and middle*) and RIα (*bottom*). The back region shows that the muscles are intensely marked while cartilage is not. The RIα signal on the back of the neck and throat region is weaker than at 12 d and has become discrete; it probably delineates the positions of the NMJs in these muscles.

Fig. 7 shows sagittal sections of a 16-d fetus hybridized with the Cα (*left*) and RIα (*right*) radioactive probes. The images shown are bright field (*upper*) and simulated dark

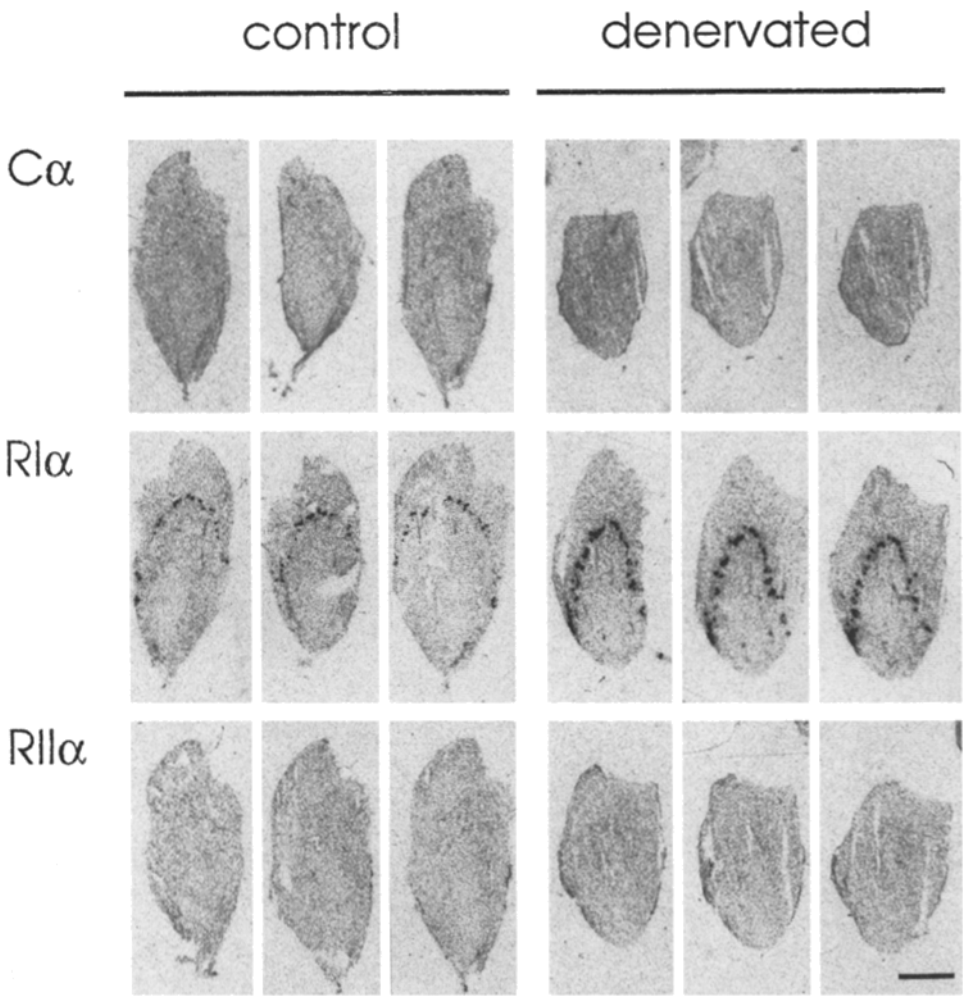


Figure 5. Hybridization of normal or denervated adult muscle with Cα, RIα, and RIIα probes. Contact exposure photographs of sections of tibialis anterior muscle from denervated or control leg, hybridized with radioactive probes for Cα, or with RIα or RIIα competed with a 20-fold excess of the appropriate unlabeled RNA and exposed for 30 h. Bar, 2 mm.

field (lower, with an inset showing part of the same section in true dark field). Cα hybridization of all skeletal muscle remains remarkably intense. The comparison of true and simulated dark field images reveals that the latter has a higher degree of resolution. For example, the salivary gland in the throat shows the same intensity as muscle in the true dark field, while simulated dark field reveals that the silver grain deposits are more intense on muscle. The Cα signal has become even more intense on the brown adipose tissue of the back than at 14 d. Several groups of muscles have become very apparent because of the strong Cα signal: the hip, lower abdominal, diaphragm, and intercostal muscles, as well as a layer of muscle underlying the skin throughout the body surface. The neck, throat, and even eye muscles have become well developed. Only skeletal muscle shows an intense Cα signal: in the heart, discernable labeling is present, whereas none is visible on smooth muscles surrounding the gut. However, the strong and uniform Cα signal in muscle is not accompanied by a comparable signal for a regulatory subunit (see below).

Table I. Effect of Denervation upon RIα Transcript Accumulation in Tibialis Anterior NMJs

	Labeled area per section (pixels)	Fold increase
Innervated	546 ± 76	
Denervated	2,024 ± 316	3.7

RIα hybridization signals of the 16-d fetus (Fig. 7) probably delineate the NMJs of the muscles that were visualized with the Cα probe. The diaphragm reveals two patches of RIα signal corresponding to the oval distribution of NMJs already established for this muscle. In addition, the junctions running along the center of the ventral intercostal muscles are nicely labeled. Compared to the strong and uniform Cα signal, the RIα signal outside of the stripes remains weak, even after long exposure. While at 12 d the RIα signal is sufficiently strong and uniform to highlight muscle compared to neighboring tissues, this is not true at later times (see Fig. 6).

RIIα, RIβ, RIIβ, and Cβ Transcripts in the 16-d Fetus

Figs. 8 and 9 complement the preceding images: they show the results of hybridization of the 16-d fetus with the PKA subunits RIIα, RIβ, RIIβ, and Cβ. Fig. 8 shows parallel sections of the head region hybridized with RIα and RIIα probes. RIα (*top*) marks the eye and throat muscles, as well as the trigeminal ganglion, and to a lesser extent, the brain. The RIIα signal is strong on the lens, the trigeminal ganglion, and the skin of the muzzle, but not on skeletal

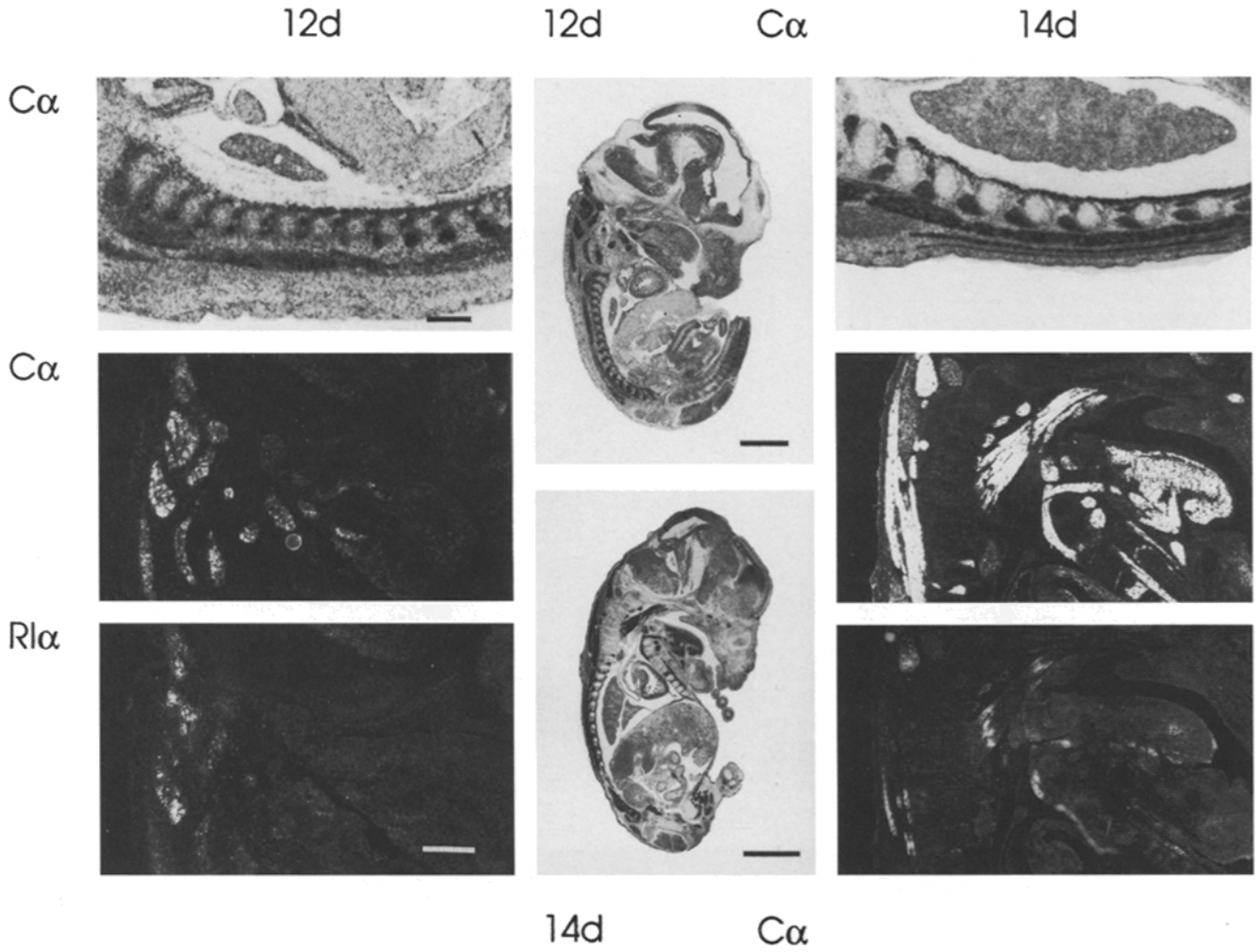


Figure 6. Hybridization of 12- and 14-d mouse embryos with Cα and RIα probes. The central part of the figure shows sagittal sections of entire 12- and 14-d mouse embryos with Cα probe. Magnified images of hybridization with Cα and RIα probes are shown on the left (*12 d*) and on the right (*14 d*). (*Top*) Bright field of the back (*rostral to the left*) hybridized with Cα. (*Middle and bottom*) Simulated dark field of the back of the neck hybridized with Cα and RIα, respectively. Bars, 1 mm for the 12-d embryo, 2 mm for the 14-d, 1 mm for the enlargements of the back, and 0.5 mm for those of the neck region. For both Cα and RIα probes, exposures were for 16 d (12 d embryo) or 27 d (14 d embryo).

muscle. In addition, the signal is discernible for most other tissues.

Fig. 9 shows the region of the back of the neck hybridized with Cβ, RIβ, and RIIβ. The expression patterns of these subunits in the nervous system have been detailed by Cadd and McKnight (1989). Both Cβ and RIβ mark the spinal cord, as previously described. RIIβ shows intense hybridization on brown fat tissue. None of the three probes showed significant hybridization with muscle (data not shown).

Localization of RIα Protein to the NMJ

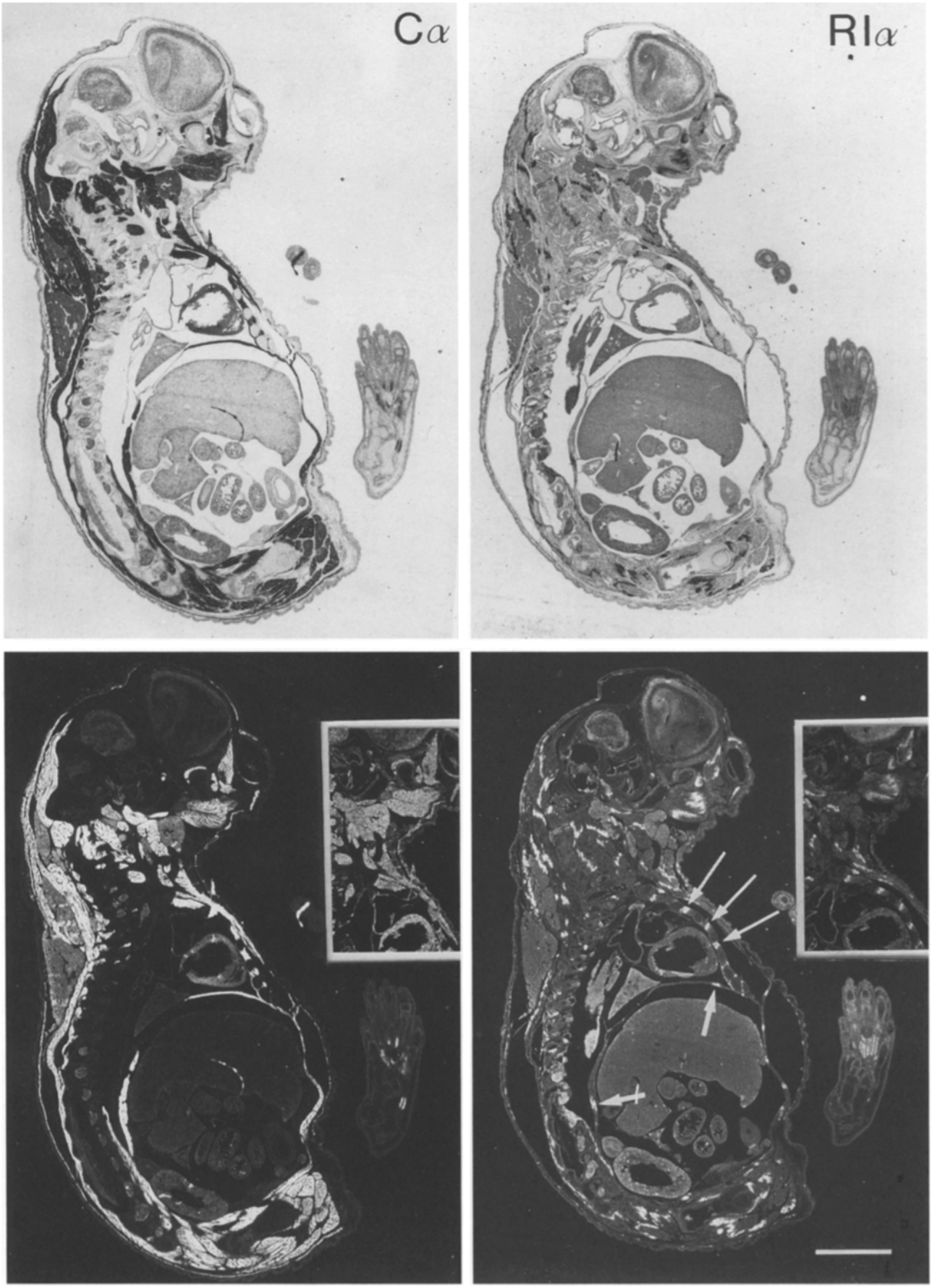
Immunocytochemistry has been used to demonstrate that RIα protein localization corresponds to that of the transcript. Indirect immunofluorescence of unfixed cryostat sections of tibialis anterior muscle using two different RIα-specific antibodies revealed localized staining (Fig. 10). When sections were simultaneously stained with fluorochrome-labeled α-bungarotoxin, which binds specifically to the α subunit of nAChR, colocalization was evident. NMJ-specific staining with RIα-mAb is essentially abolished by preincubation of the antibody with an excess of RIα (Fig. 10).

RIα Recruits Cα to the NMJ

A polyclonal antibody was used for immunocytochemical detection of Cα protein in adult tibialis anterior muscle. As was observed for RIα, sites of α-bungarotoxin staining always show strong Cα staining (Fig. 10). Preincubation of the antibody with an excess of Cα protein essentially abolished or caused significant reduction in staining. Even though no enrichment of Cα transcripts occurs at the NMJ, the localized RIα recruits Cα protein and achieves a local enrichment of the catalytic subunit.

RIα and Cα Protein in Fetal and Adult Skeletal Muscle

Intercostal and tibialis anterior muscles from a 16.5-d fetus were immunostained for Cα. In line with the transcript ac-


Cα
RIα

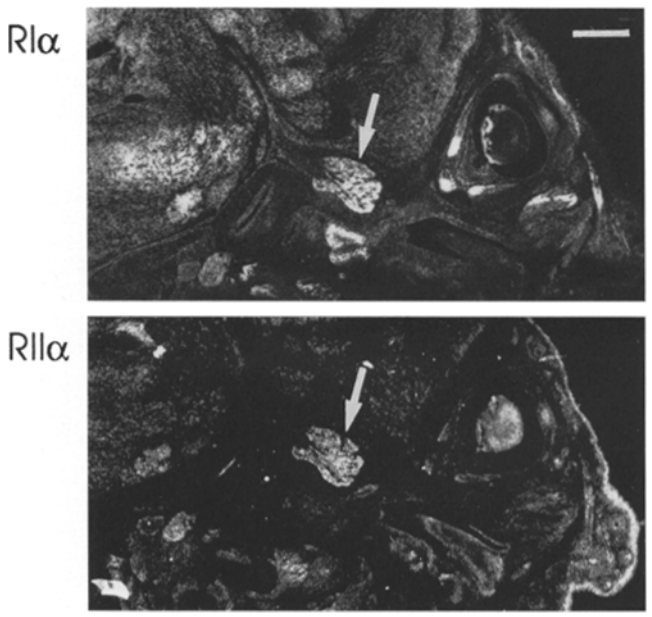


Figure 8. Hybridization of 16-d mouse fetus with cross-competed RIα and RIIα probes. Sagittal sections of the head region of a 16-d mouse fetus, hybridized as indicated, both exposed for 21 d. The arrow points to the trigeminal ganglion. These preparations were not stained with toluidine blue, thus contrast is provided only by silver grains and tissue refringence. Bar, 0.5 mm.

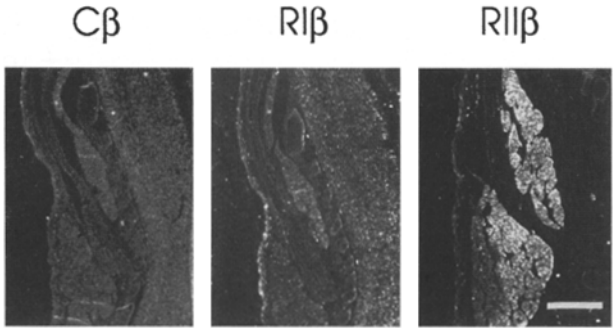


Figure 9. Hybridization of 16-d mouse fetus with Cβ, RIβ, and RIIβ probes. The back of the neck region was hybridized with Cβ or RIβ (3 d exposure) or RIIβ (9 d). The bar indicates 0.5 mm.

cumulation (see Figs. 2 and 5), a strong signal was observed (Fig. 11, *A* and *C*). In comparison, 1-mo-old tibialis anterior muscle showed a strong reduction of the Cα signal (Fig. 11 *E*).

To investigate whether RIα staining was also stronger in fetal compared to adult muscle, parallel sections were immunostained for RIα, revealing the existence of a strong signal on the fetal tissue only (Fig. 11, *B*, *D*, and *F*). Thus, even though the RIα in situ hybridization signal of extrajunctional regions was weaker than that of Cα in fetal muscle, posttranscriptional mechanisms or protein stability (Uhler et al., 1986) could intervene to achieve a balance of regulatory and catalytic subunits in the fetal tissue.

A final point concerns the delay in the maturation of limb compared with trunk muscle (Sassoon, 1993). By this criterion, the tibialis anterior is ~2 d behind the intercostal muscles of the 16.5-d fetus. The Cα and RIα signals of the intercostal muscles are less intense than those of the leg, implying a gradual decline in the amount of these PKA proteins as muscle development advances (Fig. 11).

Discussion

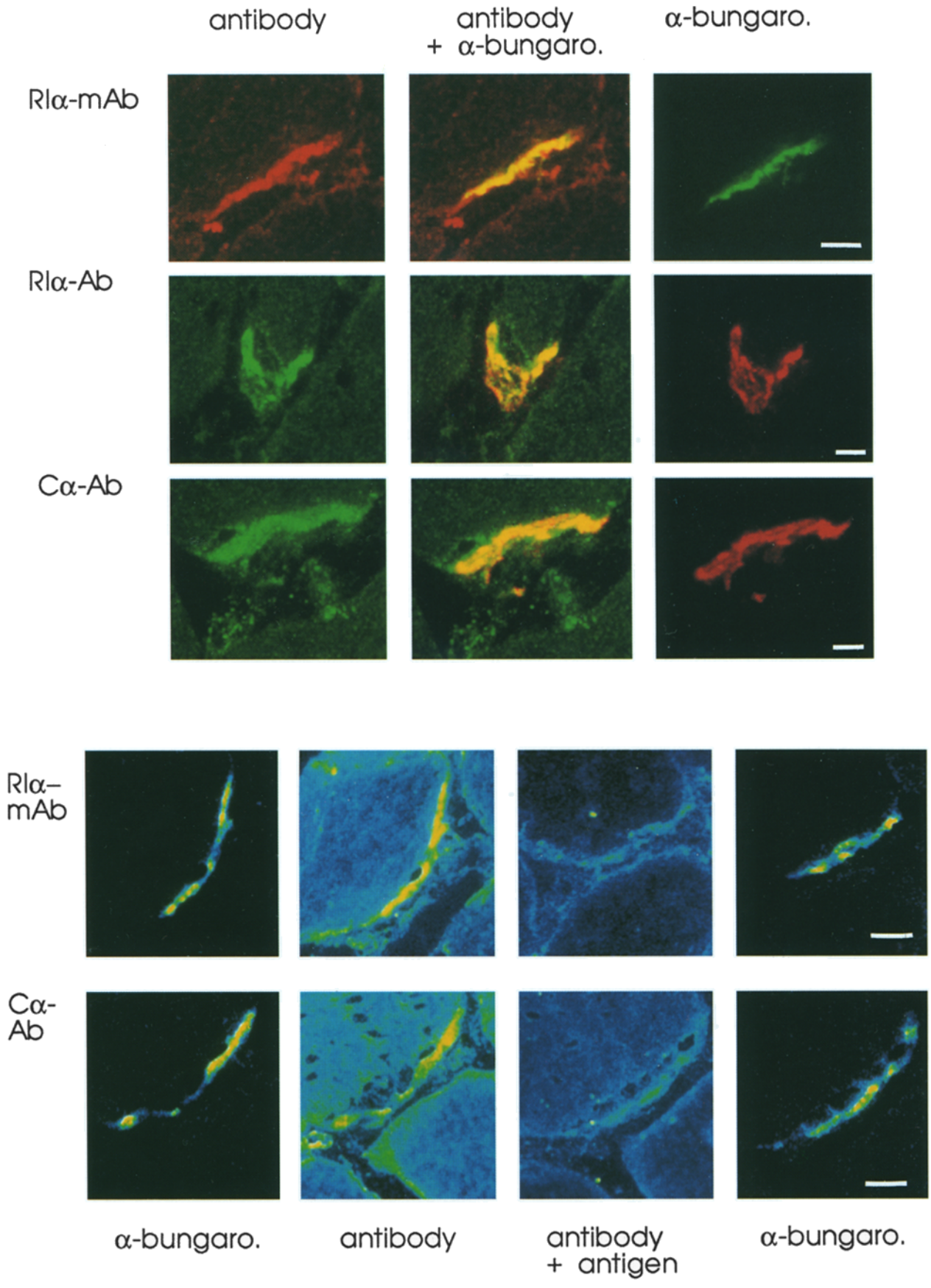
The results presented here imply the existence in skeletal muscle of unsuspected roles of PKA. The neuromuscular junction is highly enriched in RIα and Cα. In addition, high levels of Cα transcripts are uniformly distributed throughout embryonic and fetal skeletal muscle, but only low levels are present in adult muscle. Immunostaining revealed that not only Cα, but also RIα protein, is uniformly distributed and abundant in early fetal but not adult muscle.

The Localization of RIα to the NMJ

In situ hybridization and immunocytochemical analyses have revealed that RIα transcripts and protein are localized to the NMJ. In addition, RIα protein recruits Cα to the junction. Nearly perfect coincidence of the staining patterns of RIα or Cα and of α-bungarotoxin makes it likely that some mechanism exists to restrict the movement of RIα at the junctions. Although A-kinase–anchoring proteins have been described for the regulatory subunits of the RII family (Scott and McCartney, 1994), including in skeletal muscle (McCartney et al., 1995), available information implicates RIα predominantely as a soluble cytoplasmic protein.

Localized transcription has been deduced to be the mechanism underlying localization to the NMJ of the nAChRs: intronic probes reveal transcripts in the subjunctional nuclei (Fontaine and Changeux, 1989), and transgenes whose expression is directed by the nAChR promoters show expression localized to the postsynaptic nuclei (Klarsfeld et al., 1991; Sanes et al., 1991; Simon et al., 1992). In addition, manipulations that lead to delocalization of the nAchR mRNA cause extrasynaptic nuclei to express the transgene (Simon et al., 1992; Gundersen et al., 1993). For the α, β, γ, and δ subunits, generalized expression throughout the muscle fiber precedes the restriction to localized expression. In addition, generalized reexpression throughout this syncitium follows denervation, even though expression remains highest at the NMJ (Goldman and Staple, 1989; Simon et al., 1992), and this delocalized reexpression can be prevented by electrical stimulation (Goldman et al., 1988; Witzemann et al., 1991). These observations have led to the notion that localization is a con-

Figure 7. Hybridization of 16-d mouse fetus with Cα and RIα probes. Sagittal sections of a 16-d mouse fetus hybridized with Cα (left, 27 d of exposure) or RIα (right, 9 d exposure), and photographed with bright field (*top*) or simulated dark field (*bottom*, with an inset of true dark field). The thin arrows point to intercostal muscles, and the thick arrows designate two zones of RIα signal on the diaphragm. The section hybridized with RIα was heavily stained with toluidine blue: the filter failed to neutralize the stain on some tissues (e.g., liver, brain, salivary gland). Bar, 2 mm.


antibody
antibody + α-bungaro.
α-bungaro.
Rlα-mAb
Rlα-Ab
Cα-Ab
Rlα–mAb
Cα-Ab
α-bungaro.
antibody
antibody + antigen
α-bungaro.

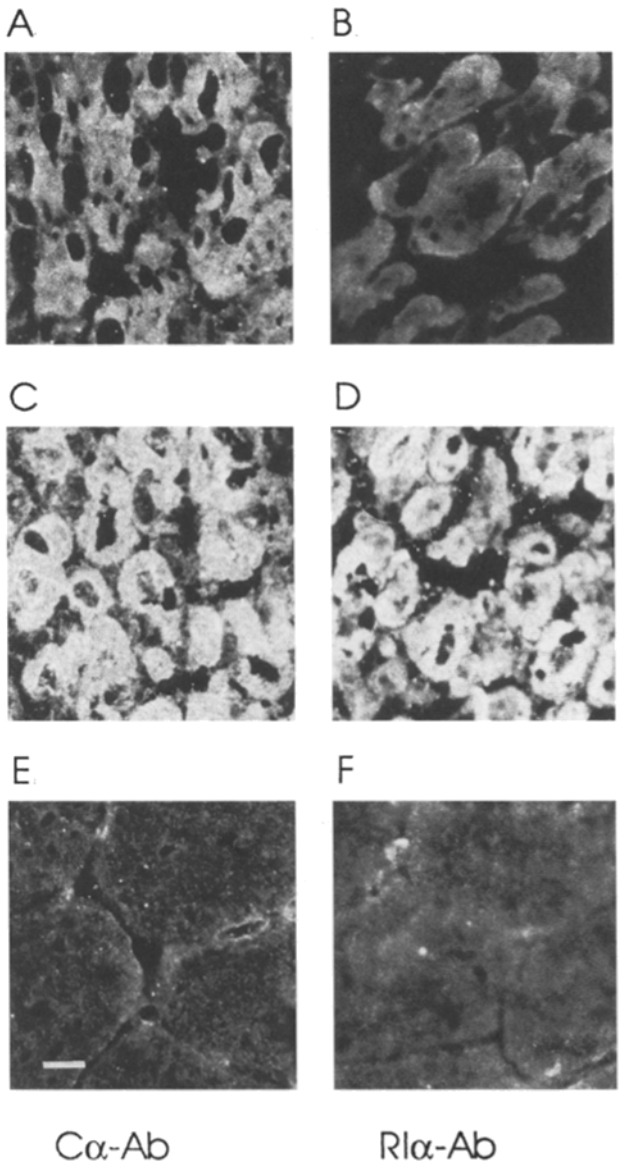


Figure 11. Immunostaining of fetal or adult skeletal muscle with anti-Cα and anti-RIα antibodies. 4-μm cryosections of 16.5-d old-intercostal (*A* and *B*) or tibialis anterior (*C* and *D*) or of 1-mo-old tibialis anterior (*E* and *F*) muscle were stained by indirect immunofluorescence with anti-Cα (*A*, *C*, and *E*) or anti-RIα (*B*, *D*, and *F*) polyclonal antibodies and a secondary antibody coupled to Cy3. Bar, 10 μm.

sequence, at least in part, of extrajunctional repression of gene expression.

The time course of localization of RIα transcripts is consistent with a role of this molecule in the establishment and/or maintenance of neuromuscular junctions. Earlier studies have demonstrated that the junctions are formed in the diaphragm at days 13–14 during mouse development, and localized expression of nAChR can already be demonstrated by day 14 (Piette et al., 1993). RIα mRNA localization may occur earlier than NMJ formation; the in situ hybridization signal is already visible in the neck muscles in 12-d embryos, and localized expression is clearly established by day 14.

The final pattern that emerges of RIα transcript expression at the NMJ is different from that of the other gene products described so far. The transcripts are present early, already detectable at embryonic day 12, localized soon thereafter, and this localization is retained during adult life, and even after denervation. The early expression and localization is reminiscent of the behavior of most of the nAChR subunits (α, β, γ, and δ), while maintenance of localization and enhanced accumulation after denervation is similar to the expression pattern of the nAChR subunit ε, which, however, is first expressed after birth (Brenner et al., 1990). More detailed studies will be required to establish whether localized transcription or transport of the transcripts is implicated in the localization of RIα mRNA. Since RNA diffusion seems to be limited in the syncitial myotube (Pavlath et al., 1989; Hall and Ralston, 1989), the simplest model in view of the precedent of the nAChRs would be localized transcription.

Overexpression of Cα in Fetal Muscle and Its Down-regulation after Birth

The current model of PKA activity assumes that more or less equimolar amounts of regulatory and catalytic subunits coexist within the cell. cAMP induces the release of catalytic subunits that can either phosphorylate cytoplasmic proteins or move into the nucleus and phosphorylate target transcription factors (Lalli and Sassone-Corsi, 1994). Nuclear catalytic subunits are captured by still another protein, the PKI (heat-stable inhibitor of PKA; Van Patten et al., 1992), which upon binding to C, exposes a hidden nuclear export signal that results in escorting of the C-PKI complex back into the cytoplasm, permitting the reformation of holoenzyme owing to the greater affinity of R than PKI for C (Taylor et al., 1990; Wen et al., 1995).

We describe here that Cα transcripts were abundant in embryonic and fetal muscle at all the stages examined. None of the R subunits showed a comparable signal in the

Figure 10. Confocal microscope images of NMJs of adult tibialis anterior muscle double-stained with α-bungarotoxin and anti-RIα or anti-Cα antibodies before (*above*) and after (*below*) adsorption of the antibody with purified RIα or Cα protein. *RIα-mAb*, anti-RIα *mAb*; *RIα-Ab* and *Cα-Ab*, polyclonal anti-RIα and anti-Cα, respectively. The fluorochrome conjugates to the secondary antibody were Cy3 for RIα-mAb, FITC for RIα-Ab, and FITC (*above*) or Texas red (*below*) for Cα-Ab. Staining of NMJs with α-bungarotoxin was obtained with the complementary FITC- or TRITC-coupled toxin. (*Upper panels*) The photos on the left and the right represent the superposition of the images of all individual optical sections; in the center, the two are superimposed. (*Lower panels*) The photos show mean values of the intensities of individual optical sections; the signal intensities were converted into pseudocolors to enhance contrast. Weak signal is shown as blue, intermediate is green, and intense signal is yellow. Note that for Cα-mAb, sequential tissue sections of a single NMJ are shown. The longer bars correspond to 10 μm, the shorter ones to 5 μm.

muscle except RIα, for which strong and relatively uniform expression was observed in neck and back muscles at 12 d, and thereafter, the abundant transcripts were restricted to NMJs. These results implied that Cα subunits that are not counterbalanced by regulatory subunits could exist in fetal muscle. However, immunocytochemical staining of the tibialis anterior and intercostal muscles from a 16.5-d fetus revealed strong and uniform staining of both subunits in the leg muscle, whereas the intercostal muscles showed reduced but still intense staining for Cα, as well as significant reduction in RIα staining. In view of the documented delay in maturation of limb compared to trunk muscles, we interpret the result obtained with the tibialis anterior to represent the situation preceeding junctional localization of RIα, and the staining patterns of the intercostal muscles to correspond to the expression profile after localization of the RIα mRNA. The stronger immunostaining for both subunits in the more immature fetal muscle implies a gradual decline to the weak levels observed in 1-mo-old muscle. Consequently, strong expression of both regulatory and catalytic subunits of PKA can be considered to be characteristic of immature muscle.

PKA family members may exist at different concentrations and in distinct intracellular compartments. In adult muscle, RIIα is probably bound to a muscle-specific A-kinase–anchoring protein at the sarcolema membrane (McCartney et al., 1995); low levels of other regulatory and catalytic subunits are probably present throughout muscle cells, with a high level of accumulation of both RIα and Cα proteins being found at the NMJ. The distinct compartments could display differential sensitivity to cAMP, depending on both subunit composition and distance from the site of cAMP production.

The possible role of PKA in expression and/or activity of members of the myogenic gene family, Myf-5, MyoD, myogenin, and MRF4, is controversial. While these factors are involved in the regulation of expression of the nAChR genes (Piette et al., 1990; Simon and Burden, 1993; Dürr et al., 1994), recent studies show that the myogenic factors may not be involved in the preferential expression of the nAChRs at the NMJ (Duclert et al., 1993; Tang et al., 1994). In addition, PKA has been thought to play an as yet unidentified but important role at the NMJ, since one of the presumably important molecules localized at the NMJ, CGRP (calcitonin gene–related peptide), stimulates adenylate cyclase (Fontaine et al., 1987). PKA-mediated phosphorylation of the γ and δ subunits of nAChR (Huganir and Greengard, 1983) contributes to the modulation of the sensitivity of this receptor to acetylcholine (Huganir et al., 1986; Mulle et al., 1988). Current models for gene regulation at the NMJ implicate PKC, PKA, or both (Duclert and Changeux, 1995; Chu et al., 1995).

In apparent contradiction, it has been reported that an active PKA system and/or the presence of free catalytic subunits is antagonistic to myogenesis of cells in culture via an inhibitory effect on transcriptional activity of myogenic factors. This effect does not seem to be mediated in a direct fashion by phosphorylation of the potential PKA sites of these factors (Li et al., 1992; Winter et al., 1993). These findings may be consistent if PKA is indeed present in muscle in distinct compartments with different properties and at changing concentrations. Moreover, new gene products involved in the regulation of muscle cell differentiation and maturation probably await identification before contradictions concerning kinases can be reconciled. For example, a new receptor tyrosine kinase localized at the NMJ has recently been discovered (Valenzuela et al., 1995), and its role remains to be specified. In addition, the receptor tyrosine kinases erbB3 and erbB4 that bind neuregulin have also been localized to the junction (Jo et al., 1995; Altiok et al., 1995; Zhu et al., 1995; Moscoso et al., 1995).

A totally different role that PKA could play in myogenesis concerns nuclear import of the myogenic factors. Vandromme et al. (1994) and Gauthier-Rouvière et al. (1995) have reported that PKA is required for nuclear transport of transcription factors that possess a nuclear localization signal (Vandromme et al., 1995), including MyoD and serum response factor. Although the serum response factor contains potential PKA recognition sites, mutation of the relevant serine does not modify the requirement for active PKA to achieve nuclear transport. It is proposed that the role of PKA in nuclear transport is indirect, acting, for example, to phosphorylate a nuclear localization signal–binding protein. During muscle development, Cα could insure nuclear transport of the myogenic factors, permitting them to accumulate in the nucleus and trigger the expression of muscle-specific genes.

Speculations Concerning the Role of PKA in Muscle

We propose that the role of RIα and Cα localized to the NMJ is to antagonize, in a cAMP-dependent manner, the repression exerted by electrical activity on the expression of nAChR and myogenic factor genes. Several lines of evidence have implicated protein kinases in expression of the nAChR genes and in control of the calcium pumps involved in nervous transmission and muscle contraction (Walke et al., 1994). In addition, cultured myotubes subjected to electrical stimulation, considered to mimic the effects of innervation, show modifications in expression of members of the MyoD family as well as receptor subunits, and these changes can be reversed by the addition of cAMP (Chahine et al., 1993). Consequently, a tightly localized cAMP responsive system could well be involved in the maintenance of the junctional pattern of gene expression (see Scott and McCartney, 1994, for a discussion of the probable importance of maintaining the labile second messenger cAMP close to its target). Since protein phosphorylation is involved in neuronal plasticity in the central nervous system (Schulman, 1995), regulation of protein kinase activity at the NMJ could also contribute to the modulation of the activity of this synapse.

Determination of the role of the high level of RIα and Cα in early fetal muscle awaits further studies. In particular, it will be critical to determine whether their appearance precedes myogenic differentiation, in which case, PKA could be involved in developmental decisions, or whether their expression occurs only later, implying a role in muscle maturation and/or physiology. In line with the first possibility, PKA has been demonstrated to play a key role in *Drosophila* and zebrafish embryos in the maintenance of repression of genes induced by hedgehog: abolition or inhibition of PKA leads to ectopic expression of

target genes under the inducing influence of the hedgehog product (Li et al., 1995; Jiang and Struhl, 1995; Pan and Rubin, 1995; Lepage et al., 1995; Hammerschmidt et al., 1996). In addition, cAMP can interfere with sonic hedgehog induction of sclerotome markers (Fan et al., 1995). These findings, combined with our observations of elevated expression of PKA subunits in embryonic muscle, imply a role for PKA in the developmental induction of myotome and sclerotome, and warrant investigation of PKA subunit expression in other tissues in the early mouse embryo.

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